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M.R. Ahuja
S. Mohan Jain *Editors*

Genetic Diversity and Erosion in Plants

Indicators and Prevention

Volume 1

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Volume 7

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Preface

The term erosion implies a gradual loss of something important that will eventually undermine the health or stability of dependent individuals or communities. As applied to genetic diversity, erosion is the loss of genetic diversity within a species. It can happen fairly quickly, as with a catastrophic event, or change in land use that removes large numbers of individuals and their habitat. Similarly, it can also occur more gradually and go unnoticed for a long time. Genetic erosion represents the loss of entire populations genetically differentiated from others, or the loss or change in frequency of specific alleles within a population, or the species as a whole, or the loss of allelic combinations in plants, trees, and animals.

Until the 1940s, the centers of origin of crop species and woody plants were considered limitless sources of genetic variability. After World War II, agriculture in developing countries suffered great changes. The expanded use of improved varieties resulted in the reduction of traditional varieties, a process called genetic erosion. The expansion of the agricultural frontiers also contributed to the risk of loss of the wild relatives of crop species. Some 10,000 different plant species have been used by humans for food and fodder production since the dawn of agriculture 10,000 years ago.

Yet today just 150 crops feed most humans on the planet, and just 12 crops provide 80 % of food energy, while wheat, rice, maize, and potato alone provide 60 % of stable food. Reduction of agricultural biodiversity means fewer options for ensuring more diverse nutrition, enhancing food production, raising incomes, coping with environmental constraints, and sustainably managing ecosystems. Recognizing, safeguarding, and using the potential and diversity of nature are critical for food security and sustainable agriculture. Biodiversity conservation targets three interdependent levels: ecosystems, species, and genes. Genetic erosion can represent the loss of entire populations genetically differentiated from others, the loss or change in frequency of specific alleles (i.e., different forms of a gene) within populations or over the species as a whole, or the loss of allelic combinations. Genetically eroded populations may be less competitive with introduced, new, invasive species. Genetic diversity is important to a species' fitness, long-term viability, and ability to adapt to changing environmental conditions. Genetic erosion can

be addressed at several levels in the spectrum of management activities. This book deals with a broad spectrum of topics on genetic erosion and biodiversity in crop plants, and trees.

We believe that this book will be useful to botanists, geneticists, molecular biologists, environmentalists, policy makers, conservationists, and NGOs working for the protection conservation of species in a changing environment.

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Chapter 1
Genetic Erosion: Context Is Key

Deborah Rogers and Patrick McGuire

Abstract Genetic erosion is a useful concept for conservationists, collection curators, natural lands managers, and practitioners of restoration and revegetation. However, there is variation in how the term has been used and how faithfully it follows from the genetic concepts upon which it was based. Genetic erosion is the loss of genetic diversity—often magnified or accelerated by human activities. It can result from habitat loss and fragmentation, but it also can result from a narrow genetic base in the original populations or collections or by practices that reduce genetic diversity. Just as loss of diversity is relative (to some baseline condition), so too is the biological significance of that loss, the management implications, and the human-applied value. Thus we emphasize the context in this chapter's treatment of genetic erosion. Although few species-specific guidelines are available, practitioners can minimize the risk of genetic erosion by being familiar with the biology of the affected species (including breeding system, mode of reproduction, and pattern of genetic diversity). Narrowly based genetic collections should be avoided, providers of plant materials for revegetation projects should offer information on their collection methods, and nursery managers should endeavor to minimize diversity losses at all stages of nursery culture.

Keywords Genetic diversity · Reforestation · Restoration · Revegetation · Source materials · Inbreeding · Natural areas · Conservation

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1.1 Introduction

1.1.1 Genetic Erosion—What Is It?

Genetic erosion is the loss of genetic diversity—often magnified or accelerated by human activities. The first well-publicized use of the term genetic erosion was in reference to the loss of the primitive races and varieties of cultivated plants, as they were gradually replaced in agriculture with newer and more productive crop varieties. This trend of changing agricultural techniques and land use and widespread transfer of improved cultivars led to the disappearance of traditional cultivars.

The term is now more generally applied to loss of genetic diversity, including the loss of diversity in native plant species. But just as the term 'climate change' is more commonly understood to represent an *accelerated* change in climate patterns and reflecting human influences rather than simply natural cycles, genetic erosion is more often used in the context of human-driven or-related losses in genetic diversity that are faster in rate or larger in scale than would be expected under natural processes alone. Here, we focus on the anthropogenically related loss of genetic diversity in plant populations.

1.1.2 How Is Genetic Erosion Measured?

Efforts to quantify genetic erosion vary according to how genetic diversity is being measured. Implicit in the concept of genetic erosion is that there is a baseline against which erosion can be measured (Brown 2008). At a population genetic level, where diversity is measured as the number and frequency of alleles (i.e., different forms of a gene), erosion is expressed as a reduction in allele number and frequency. Many phenotypic and molecular genetic markers have been deployed to measure and monitor this level of genetic diversity, as exemplified by several chapters in the volume. At higher orders of biological organization, measures of genetic erosion still have population genetic diversity as an implicit foundation. At a species level, genetic erosion can mean reduced population sizes, loss of populations, and reduced range. At a landscape or ecosystem level, genetic erosion can mean a simplification of trophic levels in the system, reduction in numbers of species represented, reduced density of one or more represented species, and reduced diversity of represented species. At a farmscape level, genetic erosion has been quantified by the simplification of the agroecosystem moving from diverse commodities, variable habitats, and differing scales toward the monoculture extreme. At the level of national agricultural production, genetic erosion can be marked by a reduction in the numbers of different commodities produced, a reduction in the number and diversity of variants of any specific commodity, and by the replacement of older, presumably more genetically diverse commodity varieties by new, more uniform, often imported, commodity varieties (e.g., replacement of landraces by modern cultivars).

There is a genetic resources conservation perspective of genetic erosion as well. A key conservation objective is to capture and conserve samples of genetic diversity representative of the genetic diversity available in the targeted population, species, or environment for maintenance in either ex situ conditions (genebanks, gardens, arboreta, zoos, and animal parks) or in situ conditions (genetic reserves, natural areas, ecosystems, and on-farm situations). All conservation methods incur risks of genetic erosion that are inherent in such necessary practices as sampling, regeneration and propagation, culling, storage, and record keeping.

In general, genetic erosion at all these levels is loss of genetic diversity within a species. It can represent the loss of entire populations genetically differentiated from others, the loss or change in frequency of specific alleles within populations or over the species as a whole, or the loss of allele combinations. The ultimate loss of genetic diversity is the extinction of a species and on a national or global scale this loss of biological diversity has been measured traditionally by frequency of species extinctions.

1.1.3 How Does Genetic Erosion Take Place?

In many instances, the genetic diversity of a species or population may be severely degraded without an immediate loss in census number. For example, there are a few tree species in Canada or the US that are so diminished in presence that they have been federally listed as 'endangered' or 'threatened'. However, there is serious concern about genetic erosion in forest tree species, as expressed at a 1995 international workshop on the status of temperate North American forest genetic resources (Rogers and Ledig 1996) and most recently in the State of the World's Forest Genetic Resources report which noted that half the forest species reported by countries are threatened by genetic erosion in forest ecosystems (FAO 2014).

Genetic diversity is lost in much the same manner as species become extinct. Habitat loss and habitat fragmentation can reduce the size of plant populations. If the habitat and not just the plants are removed (such as in land conversion), and there is no subsequent regeneration from seedbanks or previously collected seeds, then loss of genetic diversity can occur immediately, assuming that there is some diversity in the removed plants that is not contained elsewhere. The link between habitat fragmentation and loss of genetic diversity has been well established, both theoretically and empirically, particularly in forest tree species (e.g., Templeton et al. 1990; Ledig 1992).

Even if genetic diversity is not lost immediately, it is often reduced gradually as populations become smaller (e.g., Lacy 1987). When organisms reproduce, the progeny or offspring that result do not necessarily contain all of the genetic diversity from the parental generation. The genes from some potential parent individuals may not be represented in the progeny because of random factors such as phenological and developmental differences, distance from other plants or inability to find a mate, environmental factors that affect reproduction, random mortality, and random abortion of embryos, among other factors. At each generation,

reproduction represents a sampling of the genetic diversity that was available in the parental generation—a sample that is affected by random processes. For example, rare alleles may be present in only a few individuals, increasing the probability that at some point, by chance, they are not passed to the next generation and will be lost forever from the population unless reintroduced (see discussion of 'genetic drift' below).

In addition to habitat loss and fragmentation, other less obvious influences can also cause genetic erosion. For example, there is the potential to inadvertently reduce genetic diversity through propagation activities associated with restoration, rehabilitation, or reforestation, particularly in large-scale projects or in captive breeding programs and reintroduction scenarios. For plant-focused such projects, the genetically appropriate decision is often framed as 'planting local'—which is a proxy for planting or seeding with a genetic source that is adapted to the target habitat (see Sect. 1.4 below for further discussion). However, using genetically appropriate planting materials is not only a matter of using the correct source, but also of how the source was sampled. That is, 'genetically appropriate' planting material should be appropriate in both the nature and amount of genetic diversity relative to the scale of the project. As noted by Kitzmiller (1990), the ceiling on genetic diversity is established by the seed collected. But that level of genetic diversity can be seriously eroded by subsequent events.

Some management practices may contribute to loss of genetic diversity. The perennial herb Mead's milkweed [*Asclepias meadii* Torr. (Asclepiadaceae)] is a species federally listed as threatened, occurring primarily in prairie hay meadows in Kansas and Missouri, with a few small populations in Iowa and Illinois. The species can reproduce both sexually (and is self-incompatible) and asexually (through rhizomes). Over much of its remaining habitat, annual mowing has been common practice for over a century. However, some remaining habitat has been fire-managed since the mid-1950s, fire being a natural disturbance, historically, in these tallgrass prairie ecosystems. A comparison of genetic diversity between populations under the two different management methods provided evidence of much lower genetic diversity in the mowed versus burned sites. As mowing usually removed the milkweed pods, preventing seed dispersal and sexual reproduction, this led to increased rhizomatous growth (Tecic et al. 1998). Thus, genetic diversity was quickly lowered per unit area because of fewer and larger genetically distinct individuals. However, over time, genetic diversity in the mowed area would likely continue to decline, as selection removed additional clones, and no new clones (from sexual reproduction) were recruited. Thus the continuing trend would be one of fewer, larger clones and less genetic diversity.

Management plans for species conservation or habitat conservation may represent the best recommendations for maintaining genetic diversity in the targeted organisms that science can provide. Yet, there may still be inadvertent obstacles to preventing genetic erosion. In a managed area, there may be more than one target species with a management plan and the plans may be in conflict or the habitat management plan may conflict with the management plan for a target species within it.

Populations that are less genetically diverse may be more susceptible to pathogens (e.g., for plants: Schmid 1994) or other environmental stresses. Without genetic diversity, there is no adaptation and no evolution. Natural selection acts on genetic diversity; the more fit individuals survive and reproduce. Loss of genetic diversity reduces the ability of the population to adapt over time, reduces evolutionary potential, and lowers reproductive fitness. In fact, one of the basic tenets of evolutionary biology is that the rate of evolutionary change is proportional to the amount of genetic variability in a species (Futuyma 1979).

1.1.4 Is Reduction in Genetic Diversity Ever Useful?

Although reductions in genetic diversity are generally considered detrimental, there may be exceptions—such as reduction in the genetic load. For inbreeding species, typically found in plants, these detrimental alleles are rather quickly removed from the population because they are quickly exposed even when recessive, and the resulting individuals carrying them usually don't last long, or contribute much, if any, to future generations. For largely outcrossing species of plants and animals, the process takes longer because recessive alleles are more likely to be paired with more favorable dominant alleles and it takes longer for their exposure to selection. The 'uncloaking' and expression of these deleterious alleles is probably the explanation for much of the inbreeding depression observed when plant species experience higher-than-natural levels of inbreeding. The level of inbreeding depression depends on the nature of the deleterious mutations, the breeding system of the species, and the size of the populations (e.g., Charlesworth and Charlesworth 1987; Lynch and Gabriel 1990). The efficacy of purging deleterious alleles is related to population size, the dominance level of the mutation (e.g., mildly or highly recessive), and the type of purging process (i.e., drift or selection, or their interaction) (Glémin 2003; these population genetic features are discussed further below).

Other than the obvious example of the benefit of losing deleterious alleles, one other context in which loss of genetic diversity may not be necessarily disadvantageous is that of exotic invasive plant species. When exotic plant species are introduced to a new environment, they often experience what is called a 'genetic bottleneck' as the introduced plants just represent a sample—perhaps a very small sample—of the entire range of genetic diversity of the species. Although in theory the loss of genetic diversity could serve the invasion potential of plant invaders, this remains largely unconfirmed by conclusive experimental evidence. Some studies have revealed low levels of genetic diversity within populations of some invasive species including *Phragmites australis* (Cav.) Trin. ex Steudel (Poaceae) (Pellegrin and Hauber 1999), *Bromus tectorum* L. (Poaceae) (Bartlett et al. 2002), and *Setaria viridis* (L.) Beauv. (Poaceae) (Wang et al. 1995), although this is not a consistent feature among invasive species or populations (e.g., Pappert et al. 2000).

One direct and elegant example of loss of genetic diversity increasing invasive potential is the invasive Argentine ant [*Linepithema humile* (Mayr) Shattuck (Formicidae)]. A genetic study revealed that the Argentine ant has substantially less genetic variation in its introduced populations—even though they occur over a wide geographic area—than in its native range, and that the loss of diversity is associated directly with a behavioral change that allows the introduced ants to have widespread ecological success (Tsutsui et al. 2000).

In contrast, one genetic study of the common reed (*Phragmites australis*) provided an example of genetic erosion within native plant populations by invasion of exotic genotypes of the same species. More specifically, comparisons among historical and extant samples of native populations of common reed in the US showed that certain historical haplotypes (i.e., the genetic diversity was measured with alleles of closely linked loci in chloroplast DNA) seem to have disappeared and that one haplotype is now very widespread and invasive in the US, probably a more recent introduction to the US and possibly of Eurasian origin (Saltonstall 2002). In this case, the species is also clonal—a trait that could be beneficial to the invasive haplotype. So although the direct advantage of the narrow genetic base of the invader was not demonstrated in this study, the haplotype is highly successful and apparently outcompeting conspecific locals despite little genetic diversity.

1.1.5 Who Is Paying Attention to Genetic Erosion?

Genetic erosion was a topic of discussion in the international agricultural community in the mid-1900s and received prominence with the twin catastrophic outbreaks in 1970 of southern corn-leaf blight in the US and of coffee rust in Brazil. These events illuminated the consequences of genetic erosion, stimulated international discussions, and provided a major focus at the United Nations Conference on Human Environment in Stockholm in 1972 (UNEP 1972). The lesson was that "*genetic uniformity is the basis of vulnerability to epidemics and, more generally, to biotic and abiotic stresses*" (Scarascia-Mugnozza and Perrino 2002). Concerns about genetic erosion were motivation for the initiation of a global network of genebanks to conserve agriculturally important genetic resources. From the initial agricultural focus, there was increasing concern for and attention to genetic erosion at all levels, reinforcing the assertion made for plant genetic resources by Brown and Brubaker (2002) that: "*Genetic erosion, or the steady loss of genetic diversity in on-farm agriculture, is perhaps the key 'pressure' on the sustainable management of domesticated plant genetic resources.*"

Genetic diversity, which underlies species diversity and is lost with species extinctions, has been often recognized in its own right as comprising one of three levels of biological diversity critical for conservation (for example, McNeely et al. 1990; Jensen et al. 1993; FAO 1999). Conservation of genetic diversity has been codified as a goal in several international strategies and instruments, such as the Convention on Biological Diversity (CBD 1992), the Global Strategy for the

Management of Farm Animal Genetic Resources (FAO 1999), the International Treaty for Plant Genetic Resources for Food and Agriculture (ITPGRFA 2004), and the Interlaken Declaration on animal genetic resources for food and agriculture (FAO 2007a). There is urgency associated with the current rate of genetic diversity loss. In fact, the term 'sixth extinction' has been coined to convey the serious scale of the problem, and to equate it in magnitude to the previous five mass extinctions that are known from the geological record. Species currently are being lost at a rate that far exceeds the origin of new species and, unlike the previous mass extinctions, this is primarily the result of human activities (Frankham et al. 2004). Similarly, the seriousness of recent and ongoing losses of genetic diversity—in particular, locally adapted gene complexes—has been recognized with the term 'secret extinctions' (Ledig 1991). As suggested by this term, however, it is difficult to sense the urgency of taking measures to mitigate genetic losses, as such losses are often cryptic.

At the international level, impacts of genetic erosion on biodiversity in general have been a focus by the Secretariat of the Convention on Biological Diversity. At the start of the century, the CBD's Global Biodiversity Outlook 1 recognized declining genetic and species diversity and analyzed and recommended actions to address that decline (CBD 2001). The most recent revision and update of the CBD's Strategic Plan adopted the Aichi Biodiversity Targets which include recognition of pressures on biodiversity and taking steps to alleviate them (CBD 2010). Initially focused on plant genetic resources, the mandate of the UN FAO Commission on Plant Genetic Resources for Food and Agriculture was broadened and its name became the Commission on Genetic Resources for Food and Agriculture. The inaugural state of the world report on animal genetic resources and the second state of the world report on plant genetic resources both emphasize the threats and mechanisms of genetic erosion and advocate documenting and monitoring at national levels the progress of genetic erosion (FAO 2007b, 2010). In each case, global plans of action have been established and national progress at adopting the facets of these plans is facilitated and monitored by the UN FAO for the Commission (FAO 2007a, 2011).

Against this backdrop of a range of levels at which to measure genetic erosion and a range of impacts from populations to national and international levels, one can opine that the term genetic erosion may have become too vague to be useful. For example, currently the UN FAO urges its member nations to report periodically on the extent of genetic erosion through several mechanisms, such as the country reports expected from each member nation as the foundation for state-of-the-world reports on plant, animal, and forestry genetic resources. In addition, the global plans of action for plant and animal genetic resources each have priority actions relevant to reporting the status of genetic erosion. However, the types of responses to such efforts to collect information vary greatly as does the resulting value of the responses. On the one hand, presenting quantifiable data, studies have been done documenting displacement of breeds and local varieties, genetic marker-based assessments of genetic diversity in collections, in wild populations, or in breeding populations have been reported, and national surveys of varietal

diversity in specific crops have been conducted. Such information is extremely limited given the global scale of these reporting efforts. On the other hand, in spite of great efforts by international, regional, and national organizations, responses are sometimes almost statements of faith: "*If introduced, modern cultivars are being grown, there must have been a concomitant loss of local cultivars or landraces.*" Or: "*If climate change impacts increase in severity, there will necessarily be increased erosion of genetic diversity.*" The weakness is the absence of a specific context for specific questions about genetic erosion. Accordingly, we will focus in this chapter on one specific context for genetic erosion: that of natural, wild populations and plant restoration and revegetation.

1.2 Genetic Erosion—Dynamics of Genetic Diversity

Genetic diversity is always changing—over space and over time. Spatially, it sometimes reflects patterns in the environment (i.e., abiotic conditions such as elevation, soil moisture gradients, or climatic patterns, or biotic conditions such as predator, pollinator, or microbial interactions), suggesting adaptation of organisms to their conditions. But whether the genetic diversity is adaptive or not, it is constantly in motion over the landscape, moving by migration and through pollen and seeds and other propagules and being lost through mortality—both random and selective. The general arena in which much of reproductive activity and genetic movement occurs is called the 'population'—which, for many species, is a 'virtual entity' and difficult to identify in the field.

Genetic diversity also changes over time as a result of random factors. For example, whether a particular seed—with its inherent genetic diversity—germinates and survives depends, to some extent, not only on its compatibility with its environment, but also on the fortuity of being in the right place at the right time. And whether it passes on its genetic heritage to the next generation depends not only on its reproductive output, but also on chance events that influence its mating and the survival of its progeny. With each generation, genes are reshuffled and recombined, to greater or lesser extents depending on breeding systems, population structures, and selection. For plants for example, the longevity and life form of the species (e.g., annual, perennial, long-lived woody species), the ploidy level (e.g., diploid or tetraploid), the mode of reproduction (e.g., asexual, sexual, or some combination; dioecious or monoecious), and the breeding system (e.g., outbreeding, inbreeding, or various combinations) all weigh heavily in determining the movement of genes and the natural amounts of genetic diversity. It is against this dynamic landscape of genetic change, and within the important context of individual species' biology, that we consider the issue of genetic erosion.

The relationship between population size and loss of genetic diversity has been well established and quantified, with Wright's (1931) work being seminal. Generally, small populations tend to lose genetic variation by genetic drift (a random process) much more quickly than larger populations. And the shorter the

generation length (i.e., time to reproductive maturity), the more rapid the diversity loss in absolute time (e.g., Frankham et al. 2004). There is considerable theory and empirical research on the relationship between population size and genetic diversity and a review of that literature is beyond the scope of this paper. See, for example, Falk and Holsinger (1991) and Ellstrand and Elam (1993) for some reviews. This relationship has also been examined at the species level, and various reviews have found restricted or rare species generally less genetically diverse than more common plant species (e.g., Karron 1987, 1991; Hamrick and Godt 1990; Gitzendanner and Soltis 2000; Cole 2003). However, it is important to note that there may be different processes underlying the relationship between genetic diversity and size in populations versus species.

Genetic drift has a second consequence that negatively impacts genetic diversity. Simply put, smaller populations are more likely to have higher rates of inbreeding. Again, considering a sexually reproducing diploid species that is mainly an outbreeder, mating among relatives (inbreeding) is more likely in smaller populations. And the process is cumulative, so that over time matings between unrelated individuals become impossible (e.g., Frankham et al. 2004). Inbreeding also occurs in larger populations, but it occurs less frequently and its impacts take longer to manifest. An increase in the level in inbreeding (in plants that are mainly outbreeders in nature) has profound consequences for the population. This increases the level of homozygosity in the population (i.e., in an individual (diploid) plant, there are two copies of the same allele rather than two different alleles for a given locus), and decreases the level of heterozygosity. In general, increased homozygosity (in particular, of partly recessive, mildly deleterious alleles) leads to reduced reproduction and survival (i.e., lower reproductive fitness) and ultimately to increased risk of extinction (Charlesworth and Charlesworth 1987; Charlesworth and Willis 2009). Hence, this cascade of events that results from increased inbreeding has been described as 'inbreeding depression' (Falconer 1981).

Loss of genetic diversity can occur in restoration or reintroduction projects, where the seed or propagule source included only a small number of parent plants or a small amount of genetic diversity. This change in genetic composition of a population because of an origin consisting of a small number of individuals has been called the 'founder effect'. Such effects often include, in addition to lower genetic diversity, an increase in genetic drift which can lead to an increase in inbreeding, as described earlier. We are aware of founder effects in nature, such as those that occur when a few individuals found new populations as species migrate, over long periods of time, in response to climate change. (e.g., Ledig 1987). But founder effects can occur as a result of human activities, and over a much shorter period of time. For example, in a restoration effort for eelgrass [*Zostera marina* L. (Zosteraceae)], genetic analyses revealed that the transplanted eel beds had significantly lower genetic diversity than natural, undisturbed beds (Williams and Davis 1996). Moreover, subsequent studies showed that the loss of genetic diversity in the restored populations corresponded to lower rates of seed germination and fewer reproductive shoots, suggesting negative consequences for the restored populations (Williams 2001).

Inadequate sampling of genetic diversity in the seed (bulb, ramet, or other propagule) collection can lead to reduced genetic diversity in subsequent populations. For example, a collection consisting of seeds from 10 closely related parent plants would likely have less genetic diversity than one composed of 10 unrelated or more distantly related plants. This applies to plant populations that are strictly or primarily outbreeding. If a species reproduces asexually, reductions in genetic diversity in the genetic collection can occur through inadvertently taking multiple samples (cuttings or other plant part) from the same individual. Depending upon the spatial genetic structure of the plant species, reductions in genetic diversity can also occur by sampling too few populations (relative to what is appropriate for the restoration site). Some references on genetic sampling guidelines include CPC (1991), Guarino et al. (1995), and Guerrant (1992, 1996).

1.2.1 Sources of New Genetic Diversity

New diversity is added to plant populations through mutation—the origin of all genetic diversity—and migration of genes from other populations. New combinations of alleles are formed through recombination. Mutations add genetic diversity to populations very slowly and generally spread slowly through the population and to other populations. The rate of spread is influenced by the reproductive rate, the nature of seed and pollen dispersal, and whether the mutation is affected by selection (for example, whether or not it has adaptive value). In any event, it can take many generations to have an appreciable frequency of the mutation, and this translates into extremely long time periods if the regeneration times are long. Given the potentially long times to introduce meaningful levels of new genetic diversity, any influences that increase the rate of otherwise natural losses of genetic diversity (e.g., through natural selection) can cause a net loss of genetic diversity.

Mutations can have positive, neutral, or deleterious effects for the individuals and populations. Beneficial mutations are those that in some way improve survival or reproductive fitness. Plant species that are largely outbreeding also have some—usually low—level of deleterious alleles. The sum of the fitness-reducing effects from these deleterious mutations is called the genetic or mutation load (e.g., Crow 1993). So even in natural conditions, there is some genetic diversity which is undesirable, or not beneficial to the species.

Although the ultimate source of genetic diversity is mutation, new genetic diversity can be introduced to a population through natural means such as seed dispersal and pollination or through artificial introductions such as transplanting. The former usually occurs slowly and new alleles would normally be in low frequency, at least initially. The latter can occur quickly, and can dramatically change genetic composition. Whether introduced genetic diversity in plant populations is beneficial or detrimental will depend on the context. Some determining factors are the amount of genetic diversity remaining in the resident population, genetic differences between the resident and introduced plants, and breeding system (of both populations, if different).

Models have recently been developed in an attempt to predict when introducing new genetic diversity (and subsequent hybridizations) will be beneficial or detrimental. Key inputs to the models that affect the outcome include (1) divergence between populations, (2) the genetic basis of outbreeding depression (disruption of local adaptation versus intrinsic coadaptation), (3) population parameters such as mutation rate and recombination rate, and (4) alternative management schemes (e.g., 50:50 mixture vs. one migrant per generation) (Edmands and Timmerman 2003).

Hybridization between populations may cause either increased fitness (hybrid vigor) or decreased fitness (outbreeding depression). Translocation between populations may therefore in some cases be a successful means of combating genetic erosion and preserving evolutionary potential (Edmands and Timmerman 2003). For example, supplementing genetic diversity in cases of high environmental variability or uncertainty (e.g., Kitchen and McArthur 2001), or on altered sites, may be advantageous. However, in other cases, it could make the situation worse. If introduced plants are not well adapted in the long term, but do survive to reproductive maturity, then the hybridization between the introduced and resident (or adjacent) plants can lower the fitness of subsequent generations (outbreeding depression) (e.g., Hufford and Mazer 2003). But again, it is context dependent—more likely in cases where the parental populations are outcrossing and genetically distinct. Also, plants are notorious for variability in breeding systems, even within the same species. So uninformed mixing of plant populations—if the species is known to have population variability inbreeding system—could mean that plants with perhaps maladapted breeding systems will get established and disrupt locally developed, specific features of genetic recombination (Linhart 1995). Depending on the breeding system of the populations and the genetic basis of plant characteristics, it is also possible that the specific impacts will vary over time or over generations. So the negative impacts from either inbreeding or outbreeding depression might not occur in the first, but rather subsequent, generations. Alternatively, the negative effects might decrease over time, perhaps the result of natural selection.

1.2.2 Examples of Genetic Erosion in Native Plant Species

For species that have lost large amounts of habitat and census number, it would be expected that considerable genetic diversity would also have been lost. This can be particularly serious for self-incompatible species. For example, loss of variation at loci controlling self-incompatibility in the remaining plants of an Ohio population of lakeside daisy [*Hymenoxys acaulis* (Pursh) Parker var. *glabra* (Gray) Parker (Asteraceae)] reduced mate availability to the extent that the population had produced no seeds for over 15 years (Demauro 1993). In theory, polyploid species may be less susceptible to genetic erosion than diploid species (e.g., Glendinning 1989; Bever and Felber 1992). However, an endangered tetraploid herb endemic to

grasslands of southeastern Australia, *Swainsona recta* A.T. Lee (Fabaceae), suffered considerable genetic erosion despite its polyploidy condition (Buza et al. 2000).

1.3 Genetic Erosion—the Importance of Context

Two frames provide important context for evaluating the significance of genetic erosion and appropriate responses: that of the nature of the species or population and, secondly, the management objective(s). The first frame refers to the status of the species in the wild (abundant to rare) and its degree of manipulation (natural to impacted to domesticated). For example, genetic erosion would have different impacts if detected to be a serious threat to natural populations of an abundant native plant species (i.e., cascading effects throughout the ecosystem) than if detected in a (naturally) rare species. Similarly, genetic erosion is a natural consequence of domestication of wild species, where minimally genetic diversity is 'repackaged' and typically also reduced in the domesticated plant products. Loss of genetic diversity in a wild relative of a domesticated plant, though, could be of concern because of the loss of opportunity it represented in finding valuable new traits or new combinations. In naturally or artificially rare species or populations (e.g., those that have been assessed as 'endangered' or 'threatened' under statutes such as the US. Endangered Species Act), genetic erosion could undermine restoration and recovery efforts, and act as the precursor to extirpation or extinction.

The management objective(s) of the plant species or population is the second frame critical to interpreting the significance of and response to genetic erosion. Even within the context of 'natural areas management' there is a diverse array of objectives including maintaining diversity and ecosystem functioning in natural areas, revegetating after fires or harvests, rehabilitating mine sites or other degraded areas, improving habitat for wildlife, restoring threatened or degraded populations, providing access and infrastructure for recreation and other activities, or serving as a reservoir for species harvested wild from nature and for species useful as gene resources for crop plants. The primary focus for this chapter is natural areas conservation and restoration. If one's objectives differ from maintaining or recreating natural types and levels of genetic diversity in native plant populations, then the discussion and recommendations provided here are not entirely relevant. Furthermore, if the objectives include rehabilitation of degraded sites, then the environment may no longer be completely natural, and the relationship with natural patterns of genetic diversity will have been altered. In those cases, what is 'genetically appropriate' for the sites, at least in the short term, is less clear. Indeed, even the use of nonnative species may be appropriate, at least as a nurse crop to help restore soil stability or quality.

Within this topic of genetic erosion in natural plant communities, one could address maintaining genetic diversity within the populations, reintroducing appropriate levels of genetic diversity in projects involving planting or seeding of native plant species, or monitoring plant populations to detect decreases in genetic

diversity—each a broad topic in its own right. Here, we focus on explaining the importance of genetic diversity and the problems associated with genetic erosion in native plant populations and on suggesting some means to maintain genetic diversity within the context of restoration efforts.

1.4 Genetic Erosion—Management and Mitigation Practices

The most appropriate and effective preventative, management, or restorative practices for the impacts of genetic erosion will depend on context and management objectives. For agricultural crops, solutions or mitigations have focused on facets of ex situ conservation—such as seedbanks, genebanks, in vitro culture banks, and nurseries and gardens. This approach allows genetic diversity to be maintained even if it is not currently represented in agricultural practice. In addition, genetic research on some agriculturally important crops is comparing genetic diversity between modern and historic cultivars and even with the progenitor wild plant species, where possible. This information helps to illuminate current or predict future problems of genetic erosion, allowing an appropriate management response.

For native plant species, the focus is predominantly on conservation of genetic diversity in situ, although ex situ conservation methods are certainly an appropriate parallel conservation strategy, particularly for rare or endangered species or those experiencing high mortality or rapid loss of habitat (see for information on genetically appropriate collection procedures for ex situ genetic collections, Brown and Briggs (1991) and Guerrant et al. (2004)). However, ex situ conservation is not an effective or reasonable substitute for in situ conservation. These are complementary, rather than alternative, conservation strategies (e.g., Falk 1987; Given 1987). Ex situ collections, for example, are only samples of the natural range of genetic diversity in the species and are removed from the influence of natural selection and thus cannot accrue new adaptations over time. They are also vulnerable to financial constraints or downsizing, chronic losses in diversity depending on storage methods, catastrophic losses from equipment failures or fires, among other issues (e.g., Chap. 3 of FAO 2010).

Avoiding losses of habitat or fragmentation of habitat (that can interrupt sharing of genes between populations, for example) are important management practices. In addition to habitat conversion and fragmentation, loss of population size and genetic diversity can also arise through the imposition of additional and incompatible management objectives, or even from unintended consequences. For example, the population size of one of the five extant populations of *Pinus radiata—P. radiata* var. *binata*—on Guadalupe Island, Mexico, had declined to fewer than 500 trees by 1957 (Bannister 1965) and to approximately 200 by 2001 (Rogers et al. 2006) because of introduced goats and extreme grazing pressure that resulted (Fig. 1.1). However, after an extreme effort was successfully launched to remove the goats, natural regeneration resumed although the genetic impacts of

Fig. 1.1 The Guadalupe Island population of *Pinus radiata* was in serious decline at the time of a census in May 2001, with no evidence of any regeneration

this reduction in population size are still being assessed (Fig. 1.2). But this level of stewardship is often beyond the control of natural areas' managers and those other professionals associated with restoration projects. Nevertheless, genetic erosion of native plant populations in protected open spaces or conservation areas can be lessened by practices and policies that promote (genetic) connectivity among habitat fragments. Because of the diversity of jurisdictions involved at the landscape level, government directives (e.g., ordinances) and policies of nongovernmental organizations (NGOs) that have influence on environmental decisions can be important. For example, a large and active NGO in the State of California—the California Native Plant Society—has developed a guidance document that recommends, for horticultural landscaping, the use of known local sources of native plant species, thus providing some genetically appropriate continuity where landscaped areas and private gardens may connect fragments of natural populations (CNPS 2001). At the US national level, protections for endangered or threatened plant species that specifically address genetic considerations such as protection of minimum viable populations, restoration with genetically appropriate materials, and maintenance or establishment of connectivity among populations (when appropriate), can minimize the occurrence of genetic erosion. However, there is rarely sufficient species-specific information to support such policies, where they exist and requirements are often more general. For example, a review of recovery

Fig. 1.2 Following removal of the goats, natural regeneration was evident just a few years later in 2008 (*Photo credit* Richard Hawley)

plans for 24 federally listed plants in California revealed that in only 10 cases was research on genetic issues recommended, in only 7 cases were concerns expressed for contamination of local populations from introductions, and in 1 case was monitoring for genetic variation specifically addressed (D. Elam, unpublished 2005 survey, US Fish and Wildlife Service, Sacramento CA USA).

Revegetation or restoration projects—whether they occur at local or landscape spatial scales—provide a significant opportunity to lessen the risk of genetic erosion. (Alternatively, if conducted without genetic considerations, these projects can represent a major source of genetic erosion.) The genetically appropriate decision is often framed as 'planting local'—which refers to planting or seeding with a genetic source that is locally adapted. Geographic distance is often used as a proxy for genetic distance to give more specific guidance for 'local' collections. However, there is little relationship between the two and more meaningful guidance can be derived from the species' life-history traits. Use of genetically appropriate sources for restoration events can be more important, even critical, for species that are rare, threatened, or endangered. A current effort is underway to develop such specific genetic guidelines for a federal-threatened and state-endangered species in California—San Diego thornmint [*Acanthomintha ilicifolia* (Gray) Gray (Lamiaceae)] (Figs. 1.3 and 1.4). Results from parallel allozyme analysis, ploidy assessment, and common-garden studies suggest strong

Fig. 1.3 San Diego thornmint (*Acanthomintha ilicifolia*) is an annual plant restricted to San Diego County in California USA and Baja California Norte in Mexico. It is endangered because of habitat loss and fragmentation in California (*Photo credit* Sarah Godfrey)

differentiation—with evidence of considerable local adaptation—among populations (Lippitt et al. 2013; Hipkins and DeWoody 2014).

Some loss of seed or seedlings in the window between original collections and planting or seeding on the project site is not necessarily a problem. If the losses are

Fig. 1.4 San Diego thornmint is restricted to heavy clay soils and gentle slopes such as the population in the foreground here. Its habitat requirements and loss of habitat to development have placed the species on the endangered list in California (*Photo credit* Sarah Godfrey)

random (that is, not linked to particular genes or gene combinations), then they will not change the original genetic composition of the sample significantly. High levels of mortality, however, can cause genetic erosion. And any nursery practices that favor some seeds or seedlings over others (i.e., that constitute a 'selection' of some individuals) can cause the resulting genetic composition to differ from that of the original collection. In traditional plant improvement programs, this artificial selection is appropriate and, if successful, results in plants that are better suited to particular goals such as ornamental interest, palatability, or productivity. However, in restoration projects where the goals include restoration of genetic diversity, artificial selection may be less desirable, although it can still occur inadvertently (e.g., Meyer and Monsen 1993). Nursery practices that select for uniformity in individuals (e.g., in seed weight, germination time, or early height growth) can decrease genetic diversity (Kitzmiller 1990; El-Kassaby and Thomson 1996). This phenomenon is known as 'genetic shift'. For example, a shift toward a more uniform germination response in garden-grown versus wild collections of blue flax [*Linum perenne* L. (Linaceae)] provided some evidence of inadvertent selection for nondormant, rapidly germinating seeds under conditions of greenhouse propagation (Meyer and Kitchen 1994). This possible reduction in genetic variability related to germination response could be problematic for restored populations if it represented a critical

mechanism whereby seedbank persistence was ensured under widely different weather patterns. Improper handling or storage can shift the genetic base, if the conditions are such that the more drought-tolerant or cold-tolerant individuals, for example, survive and others die. Some losses during the storage, nursery, or handling activities may simply be early elimination of plants that would die on site in any event. However, if the selection pressures that exerted on the collections are not identical to those experienced in the natural condition, then some valuable genetic diversity may be lost. When seeds are not just grown but produced in the nursery, the physical arrangement of parental plants (if open-pollination is used) will also be important in determining the genetic composition of the seeds (e.g., Reinartz 1995).

A large percentage of angiosperm species have been described as polyploid. Differences in chromosome number may correlate with differences in fitness (Keeler 2004), dispersal (Linder and Barker 2014), or community interactions (Thompson et al. 2004), and may indicate limitations in sexual compatibility (Burton and Husband 2000). Yet the chromosome number of natural populations, whether or not known, is rarely considered in conservation or management strategies. Because chromosome number is not easily predicted (i.e., there can be much variability for chromosome number within a family or genus) and it is not often measured even in the context of genetic studies and thus it is not considered in management or restoration decisions. As a result, planting with the inappropriate cytotype may occur in restoration events, which may result in swamping and eventual erosion of the less common cytotypes or disrupting cytotype-related adaptations. If chromosome numbers cannot be counted directly, relative genome size can be reasonably inferred either from DNA content via flow cytometry (direct evidence) or allozyme banding patterns (indirect evidence).

In managed natural areas or genetic reserves, where inbreeding has been identified as a major risk for the target populations, supplementing genetic diversity may be necessary. Bijlsma and Loeschke (2012) suggest three ways of doing this: increasing gene flow between the target population and nearest other populations of the species (this assumes previous larger undivided population); increasing population size (enlarging the habitat might also achieve this); and facilitating genetic exchange with more distant populations and even populations from different habitats (this is the most extreme suggestion and the risk of swamping local adaptation must be balanced by the current risk of loss of the population due to inbreeding and inability to adapt). Beatty et al. (2014) emphasize the need for monitoring and quick action if augmentations are indicated, since the level of genetic diversity in a population can become too low for such remedies.

1.5 Concluding Recommendations

We offer these recommendations in the context of native ecosystem (or species) conservation—which could include projects that are considered revegetation, restoration, mitigation, or other. If conservation of genetic diversity of native plant species is not a primary management objective, these points will be less relevant.

1. Review the basic biology of the restoration species (in particular, the breeding system, asexual or sexual reproduction, dioecious or monoecious, and general life form—such as annual, perennial, or shrub). A good deal of common sense can be derived from these life-history features that can be applied to genetic collections. For example, if the species is dioecious, genetic collections need to consider a balance of males and females if collecting vegetative material rather than seeds. If collecting from conifers that have serotinous cones held on the trees for years, collecting from cones at different levels in the canopy will sample seeds from different cone crops, and probably reflect more genetic diversity than collecting from one cone crop only. If the plant species is known to reproduce asexually, be particularly careful to not collect seeds or vegetative propagules from just one or a few clones. Species that exhibit a high level of selfing will generally require more genetic samples to obtain the same level of diversity as a comparable species that exhibits a high level of outcrossing (Lawrence et al. 1995).
2. Planning for conservation and restoration activities should include some minimum survey of chromosome number, at least in plants where polyploidy is known within the family, where easily detectable phenotypic differences don't correspond with different chromosome numbers, and especially where rare or endangered species are involved. The most conservative approach would include chromosome number as a criterion for seed collection zones, for example, restricting the transfer of germplasm between populations of different cytotypes.
3. When purchasing plants for conservation-related projects, check with the nursery as to source, collection methods, and conditions for growing out the plants. Even if appropriate (for your project) genetic sources or genetic information are not always available, it is important to fully portray your needs and expectations to those who provide revegetation materials. As noted by Buis (2000), "*They may not know, but if customers keep asking, eventually the nurseries will start answering.*" Create a need; express an expectation.
4. Seed (or other propagule) collection methods should consider not just the locale of the collections, but the number of parent plants in the collection and their distance from one another. For outbreeding plant populations, if there is no evidence to the contrary, assume that plants close to one another might be more genetically similar than those farther apart. Thus, collecting from many adjacent plants would sample less genetic diversity than spacing the collections more widely (e.g., Millar and Libby 1989).
5. If using cultivars of native species, avoid excessive use of one or a few cultivars unless there is reason to believe they contain appropriate levels of genetic diversity for the project site.
6. Nursery activities should aim to maximize the proportion of seeds that become healthy plantable seedlings (Kitzmiller 1990). Good nursery management—that is based on awareness of possible genetic variation in seed characteristics, germination requirements, and growth patterns—can take measures to avoid inadvertent selection and minimize the impact on the genetic diversity of the original collection (Campbell and Sorensen 1984; Meyer and Monsen 1993).

7. Finally, monitoring of genetic diversity is key to recognizing risk of significant genetic erosion and having the (time sensitive) opportunity to mitigate. Although it is neither reasonable nor feasible to monitor genetic diversity in all or most native plant species, it is well recommended in certain situations that could include listed species; species that have recently undergone severe habitat reduction, habitat fragmentation, or loss of census size; populations suspected of possible hybridization with introduced conspecific populations or sexually compatible species; or species that may serve as reflections of change in environmental quality.

General common-sense measures such as these can dramatically decrease the risk or severity of genetic erosion in native-plant populations. Such improvements in the conservation of the genetic diversity of plant populations are not conditional on the development of more quantitative or specific guidelines.

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Chapter 2
Indicators of Genetic Diversity, Genetic Erosion, and Genetic Vulnerability for Plant Genetic Resources

Anthony H.D. Brown and Toby Hodgkin

Abstract This chapter surveys the conceptual basis of indicators of genetic diversity, genetic erosion, and genetic vulnerability. These are summary measures of genetic diversity in cultivated plants and their wild relatives that guide decisions, monitor progress, and warn of emerging issues of genetic resources for resilient agricultural production. Such indicators measure the genetic diversity currently present in agricultural populations on farm and held in germplasm collections, and aim to detect genetic erosion, or serious loss of diversity in time, and to warn of vulnerability due to adverse deployment of genetic diversity in space. While diversity itself encompasses many concepts, *richness diversity*—the number of different kinds of individuals regardless of their frequencies—is the most important theme, followed by *evenness diversity*—how similar the frequencies of the different variants are. Many variables are plausible as indicators of diversity. The more practical are based on number of individuals or area occupied in situ and on the number of accessions and the number of genebanks ex situ. Genetic erosion is measurable as the proportion of richness of genetic diversity no longer existing in current populations, when compared with the crop a decade previously or predicted to be lost in the next decade without remedial action. Genetic vulnerability

This chapter is based on the thematic background study "Indicators of Genetic Diversity, Genetic Erosion and Genetic Vulnerability for Plant Genetic Resources for Food and Agriculture" prepared by the senior author for the second report on the State of the World's Plant Genetic Resources for Food and Agriculture by FAO (Brown 2008) and published only on the internet, http://www.fao.org/docrep/013/i1500e/i1500e20.pdf

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is inversely related to richness diversity that is present locally, particularly if it is known to possess adaptation to exotic or new mutant pathotypes or insect strains or environments. Census information forms the primary data. For cultivated species, these data are based on the farmer's unit of diversity management, most often their named varieties, their number, relative frequencies and divergence over various units of spatial and temporal scale. For wild species, the analogous units of diversity are the lowest recognized (e.g. subspecies, morphological types, ecotypes). Census data should be supplemented and validated using more direct assays at the DNA level with molecular techniques in carefully constructed samples.

Keywords Richness and evenness diversity · Population sizes · Sampling · Varieties · Subspecies · Extinction probability

2.1 Introduction

Plant breeders, farmers and managers of biodiversity continue to make crucial decisions that shape the genetic diversity of crop plants and their wild-related species. Such decisions include the making of genetic resources available to farmers, the defining of broad conservation targets, and the warning of impending genetic impoverishment. These decisions are made at many levels—local, national, and international—and require reliable indicators that measure genetic diversity and how it is responding to human impacts.

Three types of measures are needed in the case of plant genetic resources for food and agriculture (PGRFA). The first type addresses the current state of PGRFA, or the standing **genetic diversity**, including that existing in fields or natural areas in situ, and that stored away from its site of origin ex situ in orchards, seed banks, or gene banks. The second type aims to measure changes in the *status quo* of diversity over time, in particular to monitor the loss of diversity or **genetic erosion**. The third type has to do with deployment of diversity in space, but with particular perspective of **genetic vulnerability**. Such vulnerability arises when genetic homogeneity, or the lack of diversity, renders the crop growing in a region liable to ruin from detrimental environmental changes, or if a new biotype of pest or pathogen were to invade it.

2.1.1 Interpretation

Brown and Brubaker (2002) discussed a number of properties that indicators for managing plant genetic resources should possess. Importantly for reliable interpretation, indicators should be scientifically valid, readily estimable, readily

understandable and aggregative. Once the decision of which indicators to use is taken, the interpretation of the actual estimates will present further challenges. One procedure is to ascribe a meaning or action to a specific '**benchmark**' **value** by having absolute standards (e.g., an absolute limit to land clearing, a minimum number of varieties that should underpin crop production in a given area, or the minimum value of germination for gene bank accessions). Alternatively, the purpose may be to monitor **trends over time**, with desirable or acceptable rates of change specified. The choice of the values that will trigger action requires inputs from both scientist and users so as to assure meaningful outcomes follow from the use of indicators. Even so, there is need for a process to confirm that the indicator actually measures the quantity intended.

2.1.2 Sampling

Because of the constraints of costs, virtually all indicators involve a sampling process to estimate their current values. Sampling is a key step that determines the avoidance of bias and the validity of up-scaling. Stratified random sampling is a basic technique that allows the aggregation of values for heterogeneous strata, and of data from finer scales. In addition, stratified sampling has the advantage that the overall statistics can be disaggregated, to recover the values for contributing strata if targeted action is required.

2.1.3 Aggregation

Aggregation is a common process in obtaining the numerical values for indicators. Aggregation is combining of values for component regions, or time periods, or species. For example, Hamrick and Godt (1989) summed estimates of diversity over different plant species, categorized by breeding system, to obtain overall estimates of genetic diversity in plants. Averaging over unlike entities raises a general problem: should the contributing entities be counted equally, or weighted according to some factor? The weighting factor for each component might be some function of its relative size, frequency, quality, productive capacity, or importance, for example, in weighting the components of a sustaining diet. Alternatively, an appropriate weighting factor might be a relative measure of the economic value of the component. For example, the member crops of a suite of fruit species might be weighted according to their total market value. For studies of trends over time it will be important to retain the component diversity values, or unweighted composite value, particularly, if weighting factors themselves change over time.

2.1.4 Comparability

A second pitfall in making comparisons of averages based on heterogeneous elements is the failure to base comparisons on common elements. An extreme example of this problem would be changes in the average proportions of traditional varieties when estimates for, say, horticultural crops are included in some, but not all, averages. The numbers of traditional varieties of these crops may be an order of magnitude higher than those of major arable crops. Any changes in overall patterns could be due to differences in the composition of the averages, and not to changes in diversity level of any element.

2.2 Genetic Diversity

At the outset, the task of devising a limited set of variables to measure the amount of genetic diversity seems to be straightforward. A manager or decision maker simply wishes to be able to report, for example, that it has stayed at a constant value under the current stewardship. In this way the indicator functions to monitor any change in genetic diversity, or to reflect managerial achievement. Another major use of PGRFA indicators are comparison of variability status of different crops (e.g., is the recently domesticated crop sugar beet, more genetically diverse than pea, an ancient crop?). How do different kinds of crops fruit trees versus field crops, inbreeding versus outcrossing crops differ in diversity patterns? Comparisons within species are also of critical importance. For example, where, if any, are the 'hot spots' of diversity in *Glycine* the wild relatives of soybean (Gonzalez-Orozco et al. 2012).

2.2.1 Location in Plant Genomes of Genetic Diversity

Genetic diversity arises primarily as variants in the linear sequence of nucleotides in DNA. Mutations can happen in the coding region of genes or in the spacer regions within and between genes, in the number of copies of genes, in the patterns of DNA methylation that affect the epigenome, in the linkage relation between several genes or indeed in whole chromosomes. A small portion of these changes translates into protein variation, into marker polymorphisms, into physiological, developmental and morphological variation in agronomic characters and ultimately into varieties given different names by farmers and plant breeders. Some correlation exists between the variations for different characters, but, even so, the choice is unclear of which kind of character is the best for assessing diversity. We cannot rely solely on any one kind, and that it will be important to cross-check major trends in diversity over several kinds.

2.2.2 Diversity Richness and Evenness

The appropriate statistical measures of diversity to use have long been a matter of discussion (Magurran 2003). Indicators of diversity should account for two basic concepts of diversity, namely richness and evenness (see the Appendix for further discussion of these two concepts and how they relate to the so-called evenness index [h] of diversity, which is the complement of the Simpson index [$= D = 1-h$]).

2.3 Genetic Diversity Indicators by Resource Category

In order to devise a set of indicators to measure progress toward the sustainable management of plant genetics resources, Brown and Brubaker (2002) delineated four categories of resources, based on two kinds of gene pools and two conservation strategies (in situ or ex situ). The two kinds of gene pools are broadly distinct: cultivated species with populations that have been deliberately planted; and wild evolutionary relatives or species belonging to the same genus as cultivated species.

This construct focuses primarily on cultivated species, and does not explicitly take account of the many plant species that humans use directly from their wild populations for purposes other than being sources of genes from crop improvement. Such harvested species include forest trees, forage and medicinal plants, and 'keystone' species crucial for ecosystem services or survival (Frankel et al. 1995). However, the indicators of genetic diversity for the natural populations of these extra "plant species that matter" are the same as for wild crop relatives. Indeed monitoring provenance genetic diversity in forest and medicinal species could be even more important than for crop relatives. This is because direct human use of natural populations will inevitably be selective, generating intense selection pressure for desirability or efficacy. The 'best' populations could then become heavily depleted leading to accelerated genetic erosion and heightened genetic vulnerability.

2.3.1 Indicators by Category, Numbers, and Diversity

Table 2.1 is a list of indicators of biodiversity based on the resource categories. The lead indicators for each of the four categories in this table are in essence based on numbers. This reflects the fact the total genetic diversity within a taxon broadly tends to increase with increasing population size, increasing area occupied, or increasing total numbers. Thus, monitoring a change in numbers of populations or numbers of individuals of one species over time usually indicates a trend in the level of genetic diversity they harbor. Comparisons among species are less

Table 2.1 Indicators of genetic diversity in four categories of plant genetic resources for food and agriculture, managed in a particular region or country for conservation and use

Gene pool	In situ	Ex situ
Cultivated	**Number and frequency of landraces, and proportion of the area planted that is growing them**	**• Number of crop species, subspecies or geographic categories adequately sampled in gene banks**
	Environmental amplitude of crop area	**• Number of accessions held in the genebank**
	Number of farmer selection criteria, and evolution of farmer management	**• Number of collections or gene banks**
		• Country distribution of seed gene banks
		• Coverage in collections of crop diversity
Wild	**• Number of species, subspecies or geographic subdivisions of taxa distributed in protected areas, that cover the species environmental range**	**• Number of wild species, subspecies or geographic subdivisions of taxa related to crops adequately sampled in the genebank**
	• Abundance as population numbers and sizes, particularly of rare wild crop relatives, forest trees, forage and medicinal plants	• Coverage of species range
	• Gene diversity, divergence and distribution	• Evolutionary relationships and taxonomic resolution
	• Loss of habitat or land clearing in native range	• Accession viability, documentation and duplication

The **lead** or primary indicator are in **bold**; the secondary or support indicators are important measures that aid the interpretation the values of the primary variables
Source Brown (2008)

clear cut; abundant species may not always be more diverse than rarer species of the same genus. Research is needed to test the reliability and confirm of the relationship between numbers and diversity at and below species level.

2.3.2 Logarithm Transformation

As mentioned above, aggregation is a key feature of indicators and numbers lend themselves readily to summation. However, values for the more abundant species will clearly dominate the total of numbers of entities (accessions, individuals, populations, or subspecies) when the total includes different species. Two individuals from the same population (or species) are more likely on average to share the same gene than are two individuals that come from different populations (or species), because their most recent common ancestor is likely to be closer in time. To reduce this effect, a logarithmic transformation should be applied; the aggregation should be the sum of the log of numerical values for each entity, and the

sum converted back to the numerical scale. The theory of sampling neutral alleles supports such a logarithmic transformation in a hierarchical system (Brown and Hardner 2000). The logarithmic transformation has the virtue of being straightforward, and well known in ecology. Although theoretical distributions or empirical data are generally lacking to establish equivalences among aggregating categories, it is tempting to speculate that the log transform could be extended to each higher level in a hierarchy. Thus, for example, to aggregate values from populations of different sizes, one would use as weights the logarithm of those sizes. Then aggregating species within a genus can be based on the logarithm on the number of populations per species, and in like manner for genera within families.

2.3.3 Wild Relatives

2.3.3.1 Lack of Species Equivalence

While we may treat the wild species related to cultivated plants as entities distinct from crop species, they themselves do not form a single homogenous class. The main problems to note are:

1. The number of taxa involved can be very large. For example, crop wild relatives (CWR species) are said to number 20,000 in Europe alone (Flor et al. 2006).
2. The taxa may differ greatly in their likely importance for the improvement of the related crop. They also differ in their importance to farming communities or to farm management (e.g., some species are key in pastoral ecosystems, others in disease or weed management).
3. The number and conservation status of the subspecific entities, such as ecotypes, morphotypes, outliers, etc. vary widely among genera.
4. The taxa within any one genus differ greatly in their distribution, their population numbers and sizes and the likely viability of their populations.

The oat genus, *Avena*, is a typical example. Some species of this genus are among the world's worst and most abundant weeds, other species are rare and endangered taxa restricted to a few islands. In a simple sum of all wild oat populations, the rare and interesting taxa would be swamped. Autogamous or apomictic species can multiply relatively few genotypes over large areas. The population sizes of such species could mislead as indicators of their standing genetic diversity. For aggregation, we need to build on formally defined genotypic differences within species (subspecies, morphotypes, ecotypes, etc.), despite problems in their recognition. For example, to count the number of morphotypes of the species *Glycine clandestina* (Pfeil et al. 2001) as an indicator of managed diversity is more instructive than knowing the total number of populations of this species complex extant.

2.3.3.2 Management Versus Diversity

Because of the problems just listed, Brown and Brubaker's (2002) previous discussion of indicators for wild relatives focused on two aspects of the management of these resources, and not on diversity per se. Their first indicator was a crisis-based approach applied to populations in situ, and addressed only the rare or endangered elements of wild crop relatives. It borrowed the experience of natural conservation agencies in codifying their 'red lists.' The management indicator for in situ resources was simply the proportion of such elements that were comparatively safe in that they occurred in protected areas such as natural reserves. For examples, Gonzalez-Orozco et al. (2012) measured the degree to which 'hot spots' of perennial *Glycine* species diversity were found in reserves in Australia. The second aspect was applied to samples held ex situ, and emphasized the actual use (use in its broadest sense), or the number of requests to gene banks for wild resources. This too is a resource managerial indicator that aims to display the importance of collections and the need for their continued support. Like the proportion of endangered species or subspecies that is conserved in situ, statistics summarizing use are not measures of genetic diversity.

2.3.3.3 Numbers

A better approach to measuring diversity builds on the basic positive relationship between number (the size of a population or sample) and genetic diversity. Such an approach uses as an indicator, the number of recognizable subspecific taxa or, conceivably, the number of organisms comprising the sample. The subspecific taxa could go beyond the formally described subspecies and include ecotypes, morphotypes, ecogeographic fragments of the full species range, or any reasonably distinct group within the whole species sample. For ex situ collections this would amount to a species or subspecies list together with the total number of accessions for each taxon. Again, the logarithmic transform is available for aggregation of broader categories.

2.3.4 Cultivated Species Germplasm Collections

2.3.4.1 Numbers

The obvious indicator for the management of crop genetic resources ex situ is some function of the number and size of germplasm collections and their spread among countries. The spread of collections among countries is included because it is desirable to have backup, and to have a diversity of agencies and cultures involved. One attractive feature of this measure is that considerable historic data are available both nationally and globally. Working with collection numbers as an

indicator thus affords the chance to exemplify the benefits and pitfalls of indicators. Interpretation can focus on the reliability of the data and the role that subsidiary variables might play to improve interpretation. Considerable thought has been given to the assessment of collections. The International Plant Genetic Resources Institute (IPGRI, now Bioversity International) and FAO have published standards for gene bank management that provide variables and benchmark values for indicators (FAO and IPGRI 1994).

2.3.4.2 Problems in Using Number as a Measure of Diversity

Broadly, two problems are of concern in using the simple number of accessions as an indicator of diversity in ex situ collections. The first is redundancy—the amount of repetition including the level of planned backup duplication within and between collections, and of inadvertent redundancy between very similar or identical samples of an accession. The second is viability and security of accessions. This includes the quality of accessions, especially, the viability of propagating material, the regeneration frequency and strategy, and the housing, staffing, security, and long-term sustainability of the whole collection and the institution that houses it.

2.3.4.3 Supporting Indicators

In principle, each of the collection variables can be handled as a weighting or adjusting factor and combined to yield a 'score' to attach to each accession and to the collection as a whole (Holden et al. 1993). Using fractional weights at the level of the accession, the effective size of a collection can be adjusted for variation in viability, estimated from subsamples of accessions, and taking account of the age of seed from the date of accessioning and known shape of viability curves as a function of seed age. Redundancy can be estimated as a probability of 'identity' for name or origin when two random accessions are compared. This could be refined using such techniques as molecular fingerprinting with an arbitrary level of divergence (e.g., 10 % of fragments different).

2.3.4.4 Aggregating Subspecies or Species Taxa

This leads us to discuss to what extent collection size is a reasonable surrogate measure of genetic diversity present in that collection. Surely the size of a germplasm collection has much to do with the significance of the plant species. The very large global collections of wheat, maize, and rice are not a measure of the inherent diversity of these crops. Hence in Table 2.2, the lead indicator is the number of recognizable taxa (e.g. varieties), which is an echo of the lead one for wild diversity ex situ as discussed above. Yet the number of accessions of a particular taxon is indicative of the intraspecific diversity collected, assuming that extreme

Table 2.2 Estimates of genetic diversity indicators in rice in the three communities in Nepal

Crop species	Rice			17 crop species
	BBara	Kaski	Jumla	25 communities in 8 countries
Site or community				
The sampling base—the total area of a specific crop(s) (ha)	1,034	460	88	63,610[a]
The number of modern varieties available to the community	20	6	0	0.45[b]
Proportion of the farm area growing landraces	27	76	100	92[c]
Number of farms or households sampled	89	161	180	4074[a]
Area of traditional varieties (landraces) per farm (m^2)	3,256	3500	1100	4186[d]
Varietal diversity				
Farm (or household) landrace richness	1.51	3.79	1.09	1.82
Farm evenness (h)	0.15	0.46	0.03	0.26
Community richness	28	63	21	14
Community evenness	0.88	0.93	0.60	0.70
Divergence (between/total %)	83	51	95	63

[a]Grand total, unweighted; [b]Antilog (i.e. exponent) of the average over farms of the log (1 + number of introduced varieties), unweighted; [c]Weighted average over crops where the weights were the log of the total area for each crop; [d]Exponent unweighted average of log of farm areas
Source extracted from Jarvis et al. (2008)

biases of amplification are absent or can be corrected for. The fact that the number of wheat accessions stored globally exceeds 10^7 whereas that for rye is likely to be less than 10^6 is indicative of their comparative levels of stored diversity. This order of magnitude difference supports the suggestion that logarithm transformation should be used for combining sizes over species, regions, countries, etc. from the sizes of heterogeneous units.

2.3.4.5 Breeding System and Numbers

A question of general interest is the effect of the breeding system of a crop species on the assessment of total collection size as an indicator of diversity. In particular, it might be assumed that germplasm collections of inbreeding (self-pollinating) crops contain much less diversity than collections of outbreeding species that are of equivalent total size. However, at the level of comparing different individual accessions, the reduction in effective size of the whole collection due to close inbreeding may not be as marked as implied by the true-breeding tendencies within a line (Frankel et al. 1995). Whereas the individual seeds within an accession are likely to share the same highly homozygous parentage, the seed from different accessions may be unrelated or related through deliberate hybridization in a

breeding pedigree. Overall, self-pollination reduces effective size to some degree (theoretically a halving), and thus reduces genetic diversity, but not by an order of magnitude unless accompanied by severe bottlenecks.

2.3.5 Varietal Diversity In Situ

What are the meaningful indicators of genetic diversity for populations of crop species growing in situ on farm, particularly applicable to traditional varieties or landraces? A complete and detailed inventory of all extant populations of a crop species under study is almost invariably impossible. Instead, we must depend on estimates from a carefully chosen sample of farms, chosen so that it can be reliably up-scaled.

2.3.5.1 Varietal Data Gathering

The steps in the process are:

1. Specify the crop species, the region and the communities, as the basic source from which ideally a random or structured random sample of households is drawn for survey. The number and structure for the farms and the area cultivated are recorded.
2. Define the units of genetic diversity to be assessed, for example, so-called 'farmer managed unit of diversity' or named varieties (Sadiki et al. 2007). This step requires participatory techniques that ask community groups of farmers to agree on their managed units.
3. Sample communities and farms for these defined varieties and estimate the area under each variety.
4. Compute the summary statistics, for example, landrace richness, evenness, and divergence.

A recent synthesis of disparate data on diversity in traditional varieties of 17 field and horticultural crops (27 species) growing in eight countries (Jarvis et al. 2008) illustrates the compilation of simple diversity indicators. Table 2.2 is an extract of these data for rice landraces in Nepal. For comparison and as an example of aggregation, the last column contains the overall estimates for all crops and communities in the study. The data for rice in Nepal were based on three contrasting communities directly representing over 1500 ha of rice fields (line 3). The communities differed in degree of dependence on traditional cultivars (4 and 5), and rice-field size (7). For the rice fields in this study, the richness of diversity at the level of the individual farm (line 9) exceeded one landrace per household, and was very high at Kaski. The evenness index (h) (line 9) was appreciable—two random plants on one farm were almost as likely to belong to different varieties as to the same variety. Substantial differences were evident at the community level (lines 10 and 11).

2.3.5.2 Overall Perspectives on Crop Landrace Diversity In Situ

Most of these variables in the Jarvis et al. (2008) study were readily aggregated to more crops and to higher scales to yield very interesting overall summary measures. The remarkable features to emerge were that the majority of farmers who grew landraces were likely to grow more than one such distinct variety, and that farmers in the same community tended to adopt divergent varietal strategies. Two trends significant for developing indicators were: (1) a close empirical relationship between richness and evenness index (correlations exceeding 0.90); and (2) an appreciable positive relationship between farm field area (log scale) and diversity. These results are important for two reasons. First, farm field area (or population size) within crops, culture, and environments is a valuable, albeit surrogate, comparative indicator for on farm genetic diversity. Second, the evenness index is a good estimator of richness of diversity. The evenness index (h) is assessable in relatively small samples because it converges with the true underlying population value, whereas richness does not reach its population value until the whole of the population is counted. Furthermore, evenness may be less sensitive than richness to problems of accuracy, homonymy, or synonymy in the naming varieties and to telling rare varieties apart.

2.3.5.3 Farmer-Named Varieties and Diversity

Statistics based on farmer-named variety are questionable as valid measures of genetic diversity. For example, Nuitjen and van Treuren (2007) urge caution and question their validity when lists include homonyms or synonyms or when minor discrepancies or inconsistencies at the DNA level between variety names and the genes they contain. Clearly, variety names are assessable rapidly over a wide sampling base, enabling the testing of broad hypotheses for the distribution of diversity. Many farmer managerial decisions are made at the varietal level, and many modes of selection (such as climate, soil, elevation, maturity time) operate on the whole field. By planting a reputedly tolerant variety in a stressful situation, farmers reinforce the attributes of the varieties they recognize as units of diversity. They directly benefit from correct decisions based on names, and suffer the consequences of poor ones.

Reliable, consistent recognition of identities of types (subspecies, variety names) and differences is a key assumption for indicators based on these units. Ideally, this is true not only within communities, but at broader spatial and temporal scales. Molecular techniques have a role in testing the limits of this assumption. Likewise, one issue for indicators of genetic erosion is the matter of identifying locally common alleles that are important for adaptation. Molecular techniques have a possible role in assessing the uniqueness of such alleles in test samples. In addition, molecular fingerprinting of a current and a past sample of varieties could in principle measure proportionate declines in genomic diversity. Such an approach requires the benchmarking of the significance of observed

decay rates of molecular diversity. For genetic vulnerability, it is important to add data on performance in assays of biotic and abiotic stress to measures of varietal homogeneity.

2.4 Genetic Erosion

Genetic erosion is the process of the loss of portion of the gene pool of a species in a specific region. Here, we use the term in the sense of loss of particular alleles, or the loss of genotypes, subspecies, or varieties, while acknowledging some conservationists use it to describe species extinction. The principal concept is the depletion of genetic richness. Maxted and Guarino's (2006) definition (see also Guarino 1999) specifies "the permanent reduction in richness (or evenness) of common local alleles, or the loss of (local) combinations of alleles over time in a defined area." In focusing on alleles that are highly restricted to a few populations and only there are relatively frequent, this definition underlines the aspect of local adaptation. However, it is not clear why a definition should add reductions in evenness. Changes in evenness alone without any loss of richness are unlikely because evenness and richness are correlated empirically (Jarvis et al. 2008) and theoretically (see the Appendix).

An indicator should reveal trends in time and be most sensitive to the changes of concern. If the conceptual basis of an indicator is overly inclusive, it is likely to meet the following problems:

1. It may be hard to estimate and to aggregate over different species, areas, or aspects of diversity.
2. Neutral or trivial changes could mask critical changes when summed over loci, genotypes, populations, or species. For example, the loss of a few alleles at a highly polymorphic microsatellite locus is likely to be of trivial or no importance compared with the loss of disease resistance alleles.
3. Emphasis on combinations of alleles can be confusing in sexual species where the genome multilocus genotype of an individual is unique and ephemeral. Thus, when a claim is made that some percentage of distinct clones or genotypes has been lost from a region or a species, this is not necessarily genetic erosion. The life of each genotype is finite in sexually reproduced species, although vegetative reproduction might prolong that life (such as in named cultivars of fruit trees). A reduction in population size, and not increased recombination, is the primary agent of erosion.
4. Above all, the need is for practical ways of monitoring genetic erosion so that it is clear when and where it is occurring.

As a process, genetic erosion is difficult to quantify in an index. To monitor changes in the rate of genetic erosion strictly requires directly comparable measures of the state of a system at several points in time. Alternatively, it is possible to measure the major agents of erosion (e.g., deterioration or destruction of habitat

due to urbanization, land clearing, overgrazing, salinization, drought, climate change, etc.). However, such indirect measures are very broad and have other and possibly more profound impacts than causing loss of diversity.

2.4.1 Erosion in Retrospect or in Prospect

Relevant measures of genetic erosion will often include some subjective assessment based on expertise and local knowledge on the significance of any loss. The inclusion of such evaluative information in measuring erosion is desirable. The challenge is to format it in such a way that at least a tentative quantitative treatment is possible. The FAO survey and database of reported instances of genetic erosion has the potential to provide the basic information for constructing such measures (Diulgheff 2006). Many of the records so far assembled are in descriptive, narrative style of local expert opinion. Summing these stories over crops or regions or time periods requires their conversion to quantitative estimates, which is a significant challenge.

We should adopt a procedure that can look back (retrospective) or look forward (prospective). In the former case, the researcher has before him or her a gene pool containing some variation and asks the question as to what proportion remains of the diversity that was known or assumed to have been present a decade ago. The estimate of the richness diversity that was previously extant should rely on as much evidence as possible.

Alternatively a predictive or prospective view could be appropriate. In this case, two quantities are essential for any reported instance. These are:

1. A measure of the significance of the loss of the gene pool in question. This is approached by estimating the extent of the total similar diversity that is at risk. This could in turn be based on the area cultivated or the number of varieties or populations with a factor of 0.20 as an estimate of the proportion of all diversity (in this case allelic richness) that is locally common (Brown and Hardner 2000). Suppose 20 % of the area or of the varieties are deemed to be at risk. Then this amounts to $0.2 \times 20\ \% = 4\ \%$ of the species genetic diversity imperilled.
2. A category of the likelihood of loss under the current situation, with no intervention (in some time period such as one decade) Classes: C = Almost certain ($P > 90\ \%$); L = likely ($P > 50\ \%$), U = unlikely but threat is still real ($P < 50\ \%$), V = very unlikely ($P < 10\ \%$). The actual area growing these varieties may affect such opinions.

Both of these quantities are subjective estimates, but ideally could be based on local knowledge of the specific crop and threats to it. Any existing survey data can be used within the above framework to support the estimates. While individual estimates and predictions may be prone to error, the framework is a way to codify the best opinion and the averages will converge to give a trend. Finally, the

predicted erosion is estimable as the proportion of the resource under threat of erosion multiplied by the estimated probability of loss.

Prospective studies encounter the problem of how to foretell future climates and future responses of plant populations. On the other hand retrospective studies many lack accurate samples and information of what diversity existed in the past. They may also be subject to the bias of not knowing what has gone completely extinct and left no clear evidence of prior existence.

Examples of studies using farmers' assessment to provide data on losses include the following. Willemen et al. (2007) interviewed 285 cassava farmers in Ucayali, Peru and matched their perceived trends in diversity trends in the preceding decade, with current levels diversity for groups of varieties defined by cluster analysis of 23 morphological characters analysing socioeconomic and environmental predictors of erosion. Actual estimates of varieties and proportions lost were not reported. In a comparable study, Kombo et al. (2012) used participatory appraisal in 21 villages in the Republic of Congo. Groups of farmers assessed cassava variety diversity as for number of varieties per farm growing and those they recall as recently abandoned. The estimated rate of loss of landraces was around 30 %. While the time period is not specified, we might assume one generation of three decades.

Two studies in which landrace samples were available at both the beginning and the current time point gave results that appear to counter prevailing expectations. Teshome et al. (2007) resurveyed 260 sorghum fields in five communities in Ethiopia. They found that the overall average field size had fallen 50 %, but with little consistent change in landrace variety richness per field. Bezancon et al. (2009) revisited 79 villages in Niger that had been surveyed for sorghum and millet landrace diversity 26 years earlier. They also found no erosion of variety richness; indeed the number of varieties had apparently doubled in this time in both crops. They noted little consistency in naming varieties and "new varieties" could arise from renaming earlier ones "for ethnic reasons." Both studies paid tribute to the resilience of farmers' selection criteria in maintaining diversity.

2.4.2 From Narrative to Estimate

The basic task in estimating values for erosion measures is to convert a series of descriptive narratives of the state of a variety of gene pools into numbers that can be compared in time and among cases. Table 2.3 (from Brown (2008)) gave some examples from the FAO database of this process. In addition to the two erosion variables, several parameters specified the geographic sampling space and the three categories of aggregation: the kind of management (cultivated versus wild used versus wild unused), the taxonomic level of loss and the major kinds of threat. As date-marked reports accumulate in the database over time it should be feasible to summarize trends in estimated rates of realized erosion or predicted rates of erosion in prospect for various categories of crops within decade

Table 2.3 Measuring genetic erosion: illustrative examples of quantitative estimates of potential erosion or the rate of erosion, based on survey reports

Variable	Definition or description	Examples			
Year	Year of observation	1998	2001	2001	Unknown
Region	Sensible groupings of countries		Pacific Islands	Caucasus	Pacific Islands
Country		Ecuador	Fiji	Azerbaijan	Fiji
Area	Geographic region of observation (name/km^2)	3 provinces	Most	5	Most
Crop group and management type[a]	Cereal, pulse, fruit tree, root, vegetable, harvested wild, unused wild	H	R	F	F
Taxon	Name of taxon	*Vasconcella*	*Colocasia*	*Prunus avium*	*Cocos nucifera*
Threatened entity or taxon[b]	Genus, species, ssp., cultivars or populations (number)	2 spp.	28 cvs	2 cvs	4 cvs
Fraction threatened[c]	Proportion of the total, e.g. in first case 2 spp. threatened of a total of 7 spp.	2 of 7 = 0.29	28 of 112 = 0.26	2 of 8 = 0.25	4 of 14 = 0.29
Likelihood of loss (cf. IUCN species categories)[d]	Probability of loss under the current situation, with no intervention (in one decade)	0.95	0.50	0.95	0.05
Predicted erosion[e]	Proportion of resource × probability of loss	0.28	0.13	0.24	0.015
Kinds of threat[f]	New varieties; other species; major abiotic change; major biotic change; loss of farming area or wild habitat	A	NV	NV	NV
Data source	FAO database	*	†	‡	†

[a]Management class—*C* cereal, *R* root, *F* fruit tree, *H* wild harvested or used, *W* wild and unused by humans. Example categories for aggregation
[b]Level of potential loss or extinction and category for aggregation
[c]Proportion of the total number of kinds of the higher category—order of magnitude is sufficient
[d]Category of estimated likelihood of loss: Classes: Almost certain ($P > 90$ %), Likely ($P > 50$ %), Unlikely but the threat still real (<50 % but >10 %), Very unlikely (<10 %). We adopted the most conservative value for each class
[e]Predicted erosion = proportion of resource × probability of loss × 0.20 (locally common genes)
[f]Kind of threat: *NV* new varieties; *OS* other species; *C* major abiotic change; *D* major biotic change; *A* loss of farming area
[*]http://www.pgrfa.org/gpa/ecu/quesreport.jspx?quesno=1&rowno=4&instid=S-58-2&tablename=xmlanswers&iterationno=1 and R. Morales (personal communication)
[†]http://www.pgrfa.org/gpa/fji/quesreport.jspx?quesno=1&rowno=3&instid=S-66-8&tablename=xmlanswers&iterationno=1 and T. Kete (personal communication)
[‡]http://www.pgrfa.org/gpa/aze/quesreport.jspx?quesno=1&rowno=4&instid=S-52-6&tablename=xmlanswers&iterationno=1 and Z. Akparov (personal communication)

intervals or due to various agents. The erosion indicator is the proportion of variants (alleles, genotypes, or populations) lost or likely to be lost in a given time period (for example, a decade). Such estimates can be combined as weighted or unweighted averages.

The four essential elements of the procedure are

1. Specifying the sample basis that is the subject of the inferences. The specifications will guide the aggregation of estimates.
2. Estimating the diversity previously present.
3. Estimating the extent or fraction of the diversity that is at risk.
4. Estimating the likelihood of the loss occurring.

The key assumptions and problems of this model are that:

- diversity is uniformly spread (but overall, at risk 'hot spots' probably balance very safe ones);
- the likelihood of loss cannot be estimated retrospectively as the taxon is known to be present today. Past erosion rates will require guesses about what has disappeared; and
- the fraction of diversity that is 'localized' will increase as the proportion of threatened resource increases.

2.4.3 Role of Molecular Markers

The few "quasi quantitative" estimates of genetic erosion as outlined support generally the concern for crop genetic resources at risk. They help to address the lack of evidence that van de Wouw et al. (2009) discussed recently. However, they leave open many questions of the dynamics of diversity underlying these changes

in farmers' use of landraces, and to landrace displacement by the varieties of modern plant breeding.

Clearly, the newer technologies offer increased precision of estimates of genetic diversity and understanding of its structure in populations. Molecular techniques have the power to monitor genetic variation at the elemental level of DNA sequences. They offer a fundamental gain in genetic knowledge; not only is it possible to prove that two individuals or two gene copies differ, but they can be placed in a phylogenetic hierarchy of relationships based on their recency of a shared ancestor. Once this is done, the phylogenetic diversity of the collection can be estimated (Crozier 1997). They have an obvious role in the field of genetic indicators (Brown and Brubaker 2002; Brown 2008). Molecular techniques therefore have a secondary, but nonetheless important, role in indicator development. They enable a deeper appreciation of the recognition of taxa and hence provide a ground-truthing of the diversity units monitored at the phenotype level. Sequence changes introduce a temporal perspective (coalescent theory) of evolving relationships and the measurement of evolutionary processes such as migration and breeding systems.

There are a growing number of research reports that have used genetic markers (allozymes, AFLP, microsatellites, SNPs) and morphological characters along with variety statistics to assess genetic erosion. Table 2.4 is a list of such recent studies that have used data on marker genes to assess the extent of genetic erosion in the crop systems. From these studies, a disparity has emerged between hypotheses of erosion expected from varietal or morphological statistics on the one hand and the levels or patterns of molecular marker diversity on the other. The studies have employed measures of total diversity and comparison within more or less advanced varieties. These may not capture the loss of particular alleles or characters, especially those likely to feature in the displacement of landraces. Obtaining valid historical samples is itself a major challenge.

Suppose that gene marker data are available for diversity within and between populations as valid samples in historical time. Can we combine varietal or morphological class statistics with estimates of molecular identity? Bonneuil et al. (2012) have constructed an indicator of crop genetic diversity that aims to integrate varietal richness, evenness, between variety genetic divergence and within-variety genetic diversity. Their approach employs Nei's gene diversity statistics both within and between populations. It includes richness because as we have already noted, richness is related to the gene identity measures. Thus, it may offer a good summary measure for following erosion, because it also gives greater weight to loci that are showing population divergence (i.e., loci with locally common alleles). The authors applied their measure to microsatellite data in wheat varieties planted in Eure-et Loir, France since 1878, and compared trends with those for five varietal diversity statistics. The results give conflicting evidence of erosion. The number of varieties (richness) appeared to be constant around 10 for a century then suddenly achieved new levels of around 60 in the last two decades. Varietal evenness measures showed no strong temporal trend. Bonneuil's composite indicator which incorporates marker gene identity measures, declined from a

Table 2.4 Recent examples of monitoring of genetic erosion

Species/region breeding system	Circumstance, issue or concern	Genes	Diversity indicators	Conclusions regarding change with time	Authors
Maize/France outbreeder	Variety catalog 1990 inbred lines grouped in up to 5 decades	- 34 morph traits - 17 allozyme loci	Nei diversity index for gene loci and morph class	No temporal change in gene or morphological diversity	Le Clerc et al. (2006)
Peas/France inbreeder	Variety catalog 578 lines grouped in up to 5 decades	- 61 traits - 8 allozyme loci	Nei diversity index for gene loci and morph class	No temporal change in gene or morphological diversity	Le Clerc et al. (2006)
Rice/The Gambia inbreeder	- Name diversity versus morph - Whether PPB maintains diversity	- 15 morphological traits - 92 variable AFLP fragments	Shannon Index of diversity in Famer varieties versus modern varieties	Synonymy of farmer names (i.e. name divergence without gene divergence) masks little genetic change in space	Nuitjen and van Treuren (2007)
Millet/The Gambia outbreeder	- Whether breeders versus farmer better retain diversity	- 13 morphological traits - 70 variable amplicons	Shannon Index of diversity in Famer varieties versus modern varieties	Breeding system crucial. Introduction of modern varieties likely has increased diversity within varieties	Nuitjen and van Treuren (2007)
Durum wheat/Italy inbreeder	158 genebank samples from 5 breeding period, from before 1915 until after 1970	- Gliadins - 10 SSR loci	Allelic richness and Nei gene diversity	Slight decline in richness and evenness diversity detected	Figliuolo et al. (2007)
Rice/South East Asia inbreeder	5641 landrace accessions collected over 33 years, grouped to 22 populations	- 12 allozyme loci	Allelic richness and Nei diversity index	No apparent loss over time in the year total marker diversity	Ford-Lloyd et al. (2008)
Rice/Guinea inbreeder	Landrace collected in 6 villages around 1980 compared 2003	- Named varieties - 10 SSR loci	Variety and allelic richness, Nei gene diversity	Changed sampling affected results. No erosion: 46 % more varieties, changes in allelic composition and increase in 50 % richness	Barry et al. (2008)

(continued)

Table 2.4 (continued)

Species/region breeding system	Circumstance, issue or concern	Genes	Diversity indicators	Conclusions regarding change with time	Authors
Sorghum/Niger	Changes in diversity in 28 years, 71 villages	- Racial characters - 28 SSR loci	Allelic richness, Nei gene diversity	Low divergence between 1976 and 2003, allelic richness and evenness diversity were stable or increased	Deu et al. (2010)
Pearl millet/Niger	Variety and genetic changes in 79 villages in the arid Sahel	- 136 paired samples of 192 - 25 SSR loci	Allelic richness, Nei diversity, landrace richness	No change in the major varieties. No erosion in allelic richness or gene diversity. Early flowering allele at PHYC locus increased	Vigouroux et al. (2011)
Wheat/France inbreeder	305 cultivars grown since 1878 in Eure-et-Loir region. Temporal trends	- Named varieties - 35 variable AFLP amplicons	5 genetic statistics and new composite H* measure	Apparent fivefold increase in cv richness since 1980; yet H* halved since 1878 and stable	Bonneuil et al. (2012)
Lettuce/France and Holland inbreeder	878 cultivars listed in 225 French and Dutch seed catalogs since mid 19th C	- Named cultivars - 100 variable AFLP amplicons	Nei diversity index	1960 turning point—seed companies and varieties increase with UPOV rules. Cv richness increased 4×. AFLP declined 10 %	van de Wouw et al. (2013)

historical maximum of over 1.0 to levels of half this value that seem relatively stable since the 1960s.

2.5 Genetic Vulnerability

Whereas genetic erosion is a key aspect of the dynamics of diversity in time, the phenomenon of genetic vulnerability arises from patterns of deployment or impoverishment of genetic diversity in space. Populations of a crop species are said to be genetically vulnerable if they lack the diversity necessary to adapt to a biotic challenge or to an abiotic stress that is likely to intensify. The concept of vulnerability implies a lack or low level of genetic diversity, most graphically realized when vast areas of a region are a monoculture of a single variety. If one plant succumbs to a newly arriving disease, to a new biotype or to a new extreme of climatic stress, all the fields of the region respond similarly because of their shared genetic heritage particularly for the genes involved in the host–plant's susceptible (or 'compatible') response. The concept of 'vulnerability' could apply to a whole range of adverse situations arising from the precariousness of living systems. It is arguable that for vulnerability to be 'genetic' requires that other varieties or populations exist elsewhere that contain resistance or tolerance genes that would have moderated the loss in yield if they had been present. Thus, the concept of genetic vulnerability should go beyond mere genetic uniformity per se. Ideally, genetic vulnerability should add the notion of genotype × environment interaction, i.e., not all genotypes (and in particular not all populations or varieties from other regions) succumb as readily as the home population to the new threat to yield. Indicators of genetic vulnerability should therefore include:

1. A measure of the lack of genetic diversity, particularly for resistance genes affecting host–plant response to major likely diseases; and
2. A measure of lowered diversity of host-pathogen interactions and differential responses to different biotypes, with some spatial structure.

Here, we first consider indicators for genetic vulnerability to biotic challenges, and then assess the extension of this framework to indicators for vulnerability to abiotic stresses such as climate change.

2.5.1 Kinds of Genetic Vulnerability

Table 2.5 lists four kinds of genetic vulnerability upon which indicators can be framed. The first of these is genetic homogeneity. Losing diversity from the current cropping region increases vulnerability. Strictly, the diversity should refer to the genes determining plant response to disease. It is insufficient to have a large number of named varieties as a hedge against crop failure if they share the same

genes for resistance. This was the case in the USA, where male-sterile yet disease-susceptible cytoplasmic DNA was shared among many maize hybrid varieties in the field at that time, resulting in them all being vulnerable to the southern corn leaf blight. However, knowledge of the comparative resistance structure of the varieties available to farmers is generally lacking, so that a census of variety names may be the only readily obtainable information.

2.5.1.1 Richness and Evenness of Varieties as Indicators of Genetic Vulnerability

The indicator for the first concept of genetic vulnerability in Table 2.5 is varietal diversity measured as both richness (the number varieties per crop, reduced if any are known to be closely related) and evenness (as measured by the evenness index). Computing the latter requires estimates of the area planted to each variety. High scores of richness imply there are many future varietal options near at hand and that seed is available for increase if needed. High richness implies insurance against pathogen evolution. In some cases, richness is high but a large portion of the region is planted to a single dominant variety. When the dominant variety succumbs to a new disease biotype, losses will be incurred for a few seasons until more resistant varieties are multiplied and deployed. A further danger inherent in this pattern of varietal diversity deployment is that the dominant susceptible variety would allow the build up of large amounts of pathogen inoculum with increased likelihood of evolving virulence on the rare previously resistant varieties. On the other hand, high evenness (lack of dominance) implies resistance diversity is already deployed to meet a new stress, and could save the farmer from severe immediate loss. It is therefore arguable that a high value for evenness diversity (i.e., low dominance) is a better indicator of low genetic vulnerability than is a high richness score.

2.5.1.2 Mutational Vulnerability

The second type of vulnerability listed in Table 2.5, mutational vulnerability, specifically aims to conceptualize vulnerability to a new virulence mutation in a pest organism. Strictly speaking, the pathogenic properties of a future new virulent mutant are unknowable. One approach to a quantitative measure is to test the responses of the present cultivar(s) to a random sample of distinct isolates or defined pathotypes. From these data it is possible to compute the probability of infection or the average level of damage caused by nonlocal isolates. The scores for each pathotype are not weighted by the pathotype frequency of occurrence. The indicator is thus the probability of disease (or the measured adverse effect caused by the disease) in nonlocal environments. Clearly, this indicator requires experimental measurement, essentially the assessment of the performance of a representative sample of local material in alien stress-prone environments. Many

Table 2.5 Indicators of genetic vulnerability

Concept of genetic vulnerability	Theoretical measure	Indicator
(1) *Genetic homogeneity*—The standing crop consists of a single genotype or few varieties or genotypes	The diversity of resistances in host population. Richness diversity represents diversity near at hand that could be deployed. Evenness diversity or low dominance indicates diversity deployed to meet the current pathogen population	- The number of varieties per crop present on farm or in a region - Evenness index—more important for disease vulnerability
(2) *Mutational vulnerability*—The standing crop consists of genotypes that require a single mutation in the pathogen for virulence	The fraction of nonlocal pathotypes that can attack a random plant	Probability of disease (or quantitative adverse effect) when tested with a set of distinct experimental isolates
(3) *Migrational vulnerability*—The standing crop consists of locally resistant genotypes that are susceptible to a new migrant strain of a pathogen or pest	The probability that a random migrant pathogen propagule will succeed in causing disease on a random healthy plant in the population in question. This assumes the environment is favorable to the pathogen, and is calculated by integrating the frequency of particular compatible (diseased) interactions between alien disease strains on local crop genotypes, and could be distance-weighted. Ideally the statistic is also weighted by the relative frequency of pathotypes	Proportion of plants that become diseased when grown in other disease-prone environments
(4) *Environmental vulnerability*—The standing crop consists of genotypes that are adapted to the current abiotic environment (climate, soil) but lack adaptation to environmental stresses that are intensifying with time	The stress-induced yield depletion of current varieties relative to the performance of resistant nonlocal varieties that exhibit stress tolerance adjusted for the likelihood of degrees of stress, and for the frequency of local variety occurrence	- Relative sensitivity of local varieties when grown in clines of increasing stress - Proportional loss of cropping area for specific varieties following the increase of regions inhospitable due to climate change

Concept 1 is a crop-plant diversity concept; concepts 2 and 3 are defined on host–parasite interactions; and concept 4 deals with the physical abiotic environment

breeders routinely conduct trials for many crop-disease or pest situations, but the data are dispersed and rarely synthesized. The summing of averages of individual variety scores, weighted by the current frequency of the varieties on farm in a given region, would provide a synthetic overview of mutational vulnerability. Technical consistency of approach is obviously necessary for the comparison of estimates over time and over different locations.

2.5.1.3 Migrational Vulnerability

The idea behind recognizing migrational vulnerability as distinct from mutational vulnerability is to divide future risks into two categories. Defining the specific actual agent of risk in the mutational case is virtually impossible. The nature of a new mutant pathotype of a disease (its virulence spectrum or aggressiveness) in the future cannot be known for certain. Therefore, we cannot test specifically for genetic diversity to meet such a possible future challenge. The only strategy for unknowable risks is to retain as much diversity as possible. On the other hand, migrational vulnerability refers to pressures that are currently absent from a certain home environment, but are foreseeable as inevitably arising from an alien source at some future date if unchecked, e.g., the Ug99 pathotype of wheat stem rust (Singh et al. 2006).

2.5.1.4 Environmental Vulnerability

Abiotic environmental stresses that arise from prolonged unidirectional changes in the physical environment, such as global warming, increasing regional aridity or increasing climatic variability are another threat to crop production. Changes in the farm environment over time resemble the threats from the invasion of pest organisms of known virulent strains (e.g., Ug99). In pearl millet on farms in the Niger, a shorter cropping cycle has evolved to meet increasing aridity. This case exemplifies the importance of specific adaptive allelic diversity in crop populations to allow evolutionary change (Vigouroux et al. 2011). In this example, the comparable samples of pearl millet in 1976 versus 2003 showed adaptation by way of an increased frequency of the early flowering allele at the PHYC locus without any general change in the main varieties or their levels of genetic diversity. As with biotic stresses, the degree of vulnerability to future threats can be measured experimentally by the performance or response of a local sample of varieties to specific pressure. The values of the likely impact of several separate risks on productivity could then be integrated, weighting by an estimate of the likely probability of each threat.

Although this fourth type of vulnerability resembles migrational vulnerability in Table 2.5 it is worthwhile to recognize that it merits developing separate indicators because of the topicality of climate change, the marked difference in spatial scales, in how the stresses increase and in how agencies will respond to

such data. Plant ecologists (e.g., Gómez-Mendoza and Arriaga 2007) are developing approaches to model changes in the natural geographic distribution of species under various scenarios of future climate. These authors used current distributions to predict decreases of between approximately 1 and 50 % for different species of *Pinus* and *Quercus* in Mexico as a result of climate change. They use these estimates as measure of differential species vulnerability and recommend conservation priorities.

2.5.2 Off-Site Testing—Pursuing Measurement of G × E

It may seem to be overly problematic, unduly complex, and impractical to attempt a systematic, detailed risk, and genetic remediation analysis to derive measures of vulnerability. The need to attempt such computation arises from the limitation of relying on estimates of varietal richness diversity alone. Such counts lack a test of relevance of that diversity, i.e., whether, it will help cope with future threats to productivity. As mentioned at the outset of this section, the unifying concept underlying reduced genetic vulnerability is the provision of a diversity of interactions. Whether this can be measured satisfactorily by the tools of genotype × environment (G × E) analysis in plant breeding remains to be investigated. In this case 'genotype' represents the suite of available varieties and 'environment' the different pathogen populations or abiotic stress levels. Situations of low genetic vulnerability are obtained when the G × E component of variance accounts for a large fraction of the overall performance variance, particularly, when different cultivars are resistant or perform better in different stress states. Another indicator is the character of the variance–covariance matrix of performance across environments. Situations of low risk are associated with negative covariance values. This result is analogous with modern investment portfolio theory of market economics, in which risk (i.e., vulnerability) is minimized when the total investment is made over a diversity of the stocks whose performance patterns in the past feature negative covariances. A portfolio of stocks that have responded differentially provide the best hedge against risk.

2.6 Conclusions

Indicators have a clear and increasing role in the management of the genetic diversity of crop plants and its deployment within and among farmers' fields. They are needed to guide decisions on using and conserving genetic diversity and tell us where problems exist; problems of the loss of diversity in time and problems of production increasingly vulnerable to ecological change. In this chapter, we have argued for the utility of primary measures based on population size, or the extent

of field plantings. Counts may be of individual or of the lowest of individual groups—varieties, morphological ecological types, subspecies.

Having decided on the key primary indicators of diversity, it is important to develop a set of subsidiary supporting indicators to test the reliability of the primary measures. These could range from information on population or ecological history in the target area on the one hand, to in depth genetic DNA marker data. For genetic erosion in particular, which is a process in time, explicit methods are needed to incorporate more subjective data and expert opinion into measures of erosion.

The literature attempting to amass evidence of genetic erosion in gene pool groups falls into two major kinds. The first (e.g., Bisht et al. 2007) are reports based on surveys that concentrate mainly on the extent of plantings of each of three categories: landrace varieties, improved farmers varieties derived on farm from them, and varieties that are exotic, or that are the products of modern plant breeding. The second are more detailed studies of genetic markers (Table 2.4) and changes ***within*** these three categories. In many studies, there seems to be a gulf between landscape versus genome evidence. Genetic erosion that was evident from studies in the statistics on varieties planted and was therefore expected, but was not seen in genomic studies.

Yet neither of these two approaches on their own is sufficient and the challenge is to choose sensible sampling and experimental procedures to give better insights into the process of genetic erosion. Crop systems, countries and trends are so varied as to defy simple overly general statements (such as a global loss of diversity from major crops since the 1960s). We need meta-analyses of data based on many cases so as to pinpoint where and when genetic erosion is a significant serious problem and the factors linked to them. For such analyses the value of indicators will be crucial.

Caution is needed when using biodiversity indices, as they are merely attempts at simplifying complex systems and may often misrepresent what they are meant to simplify. Yet major management decisions have to be made, and indeed are being made. Such decisions can either invoke diversity criteria, e.g., saving endangered gene pools, or will be made on grounds other than the biological well-being of the system. Our task is to decide on the best, simplified measures, which may be less than desirable but still ensure the most important outcomes. A clear need and golden opportunity exists for research to develop the indicators proposed, and to test them with suitable databases.

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Appendix Richness Diversity and Evenness Diversity

The measurement of diversity requires an understanding of the different concepts or meanings that belong to the statement: "Population A is more diverse that population B". One concept is that population A harbors more recognizable, distinct types than does population B. This we call **richness diversity** and refers to the number of different kinds of individuals regardless of their frequencies. Another related concept, **evenness diversity**, refers to the similarity in frequencies of the types in population A compared with population B. Low evenness indicates the dominance by one or two types. If the frequencies of the different types in A are very similar, the variance in their frequency is lower compared with that in B.

The measure of richness is, straightforwardly, the number (k; $k = 1, 2, 3 \ldots$) of types in a sample. Its dependence on sample size can be corrected using resampling techniques. Evenness, on the other hand, is less obvious. A standard, conceptual parameter for measuring variation in biology is the coefficient of variation of the frequencies of types, where the coefficient of variation ($\mathrm{CV}[p_i]$) is the square root of the variance divided by the mean frequency ($p = 1/k$). If all the types in the population are equally frequent, then the variance of their frequencies is very low or zero, and the evenness diversity would be high. The evenness index commonly used in genetics is Nei's ($h = 1-\Sigma p_i^2$; $0 \leq h \leq 1.0$) is also called the genetic diversity index. It is the complement of the Simpson index of dominance ($D = 1-h$) in ecology. The symbol h signifies the close parallel with expected heterozygosity in population genetics. Despite these potentially confusing names, h is perhaps the most understandable measure of evenness diversity. This is because h is the average chance that two gametes drawn at random from the population will differ at a locus.

Because of close parallel with expected heterozygosity for a single gene polymorphism in a random-mating population, we use the symbol h. It is known that the evenness index (h) is a simple function of the variance evenness and richness measures:

$$h = 1 - \left\{1 + \mathrm{CV}^2[p]\right\}/k$$

This formula shows that this evenness index (h) increases as the richness (k) increases, and as the coefficient of variation of the frequency of types decreases. Yet, in general, h is more a measure of evenness than it is of richness. Numerically, h is largely determined by the frequency of the most frequent, or dominant type. (Hence the Simpson Index is sometimes called the dominance index.)

There are in theory other additional concepts and measures of genetic diversity (Brown and Weir 1983; Brown and Hodgkin 2007) that could serve as indicators. However, the two measures (k and h) discussed here are the most useful and readily understandable, and these two concepts are fundamental to the present discussion.

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Chapter 3
Genetic Diversity and Modern Plant Breeding

Stephen Smith, David Bubeck, Barry Nelson, Jason Stanek and Justin Gerke

Abstract Genome sequence data provide new capabilities to characterize genetic diversity across a comprehensive range of plant germplasm including breeding materials, modern cultivars, landraces, and wild and weedy ancestors. This sequence "language" allows breeders to monitor, help identify, and select for useful diversity thereby developing new improved varieties. Although much genetic diversity in wild ancestral species was not transferred into domesticated species, surprisingly high levels of diversity have been retained during the past century, a period of intensive selection for increased productivity. Diversity in modern varieties exhibits temporal flux associated with bottlenecks due to grain quality or specific introductions of germplasm. There is no evidence over many decades in the twentieth century of a narrowing of the genetic base. Diversity has increased in some crops due to conscious sourcing of landrace diversity. Finding useful diversity to provide successful genotype by environment (G × E) interaction remains both the essential challenge for plant breeders and an assurance that new genetic diversity must continue to be sourced in order to allow continued genetic gain in a dynamic agricultural environment. Plant breeders can never afford to be complacent about stewardship and use of genetic diversity. Trends of genetic diversity

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usage should be regularly monitored in breeding programs and in commercial agriculture.

Keywords Agriculture · Conservation · Cultivar · DNA sequence · In situ · Ex situ · Gene × environment interaction · G × E · Genetic diversity · Genome · Intellectual property protection · Molecular marker · Mutation · Plant breeder · Plant variety protection · Productivity · Utility patent · Yield

3.1 Introduction

The conduct of agriculture creates a mutual codependence for humankind with domesticated animal and plant species (Harlan 1992; FAO 1997, 2010; Zeder 2006; Vaughan et al. 2007; Purugganan and Fuller 2009; Rottenberg 2013). It is vital to practice intelligent management and use of genetic diversity to sustain agricultural productivity (Stuthman 2002). Persistent narrowing of germplasm diversity would inevitably lead to a litany of undesirable consequences including reduced potential to improve crop production, increased susceptibilities to pests and diseases, reduced potential to adapt to changing weather patterns, greater instability in agricultural production, and loss of genetic resources (National Research Council 1972, 1993; FAO 1997, 2010; Brown-Guerdia et al. 2000).

Modern plant breeding has been attributed as contributing to the reduction of genetic diversity in agriculture (Vellve 1992; Clunies-Ross 1995). Most concerns arise from a focus on the initial changeover in cultivation from landraces to "modern" varieties and from comparisons using surrogate data; e.g., numbers of varieties rather than genetic diversity data per se (Meul et al. 2005). Any discussion of genetic diversity in agriculture is meaningless unless productivity gains are also considered (Fig. 3.1). Productivity gains in major US field crops are reviewed in Smith et al. (2014b).

Plant breeding contributes from 50 to 88 % of increased yield production due to genetic gain (Duvick 2005; Mackay et al. 2011; Smith et al. 2014a, b). In Iowa, the contribution of genetic gain to increased productivity was 79 % during 1930–2011 (Smith et al. 2014a). Achievement of genetic gain is dependent upon access to and effective management of genetic diversity contributing to quantity and quality of agricultural production. Future needs for increased productivity contributed by genetic gain and crop management will be very challenging to achieve. For example, the BBSRC (2011) states that: "Total wheat grain production over the next 50 years must exceed that previously produced over the last 10,000 years." It is likely that plant breeders will be called upon to contribute an even greater proportion to improved farm productivity as gains from other inputs plateau or decline. A lack of useful and well-adapted genetic diversity will undermine abilities of plant breeders and farmers to achieve these important societal goals.

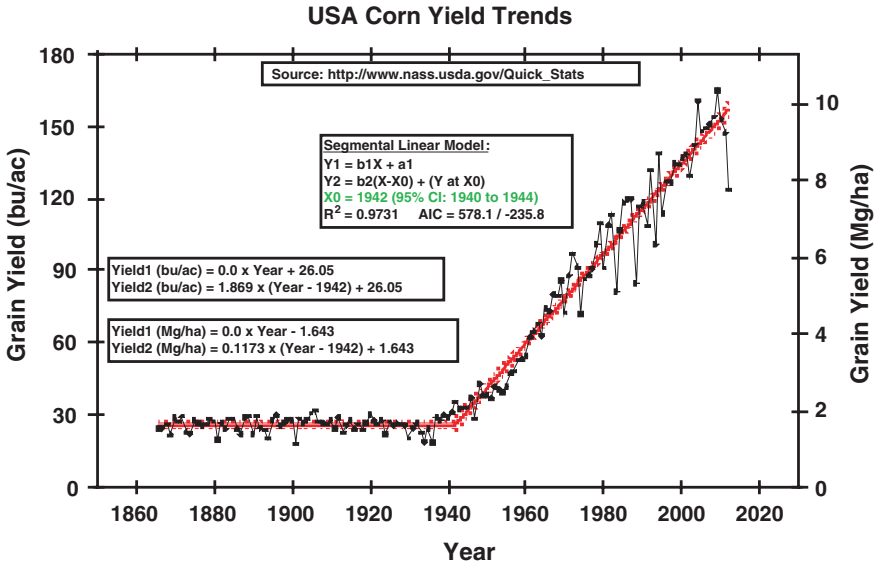

Fig. 3.1 U.S. maize yields 1865–2012 (with permission from Smith et al. 2014a)

3.2 Modern Plant Breeding

Because of the focus on genetic diversity, we adopt the definition of "modern plant breeding" or "scientific breeding" (FAO 1997) as: The act of using genetic diversity to improve the agronomic performance of plants conducted as a formal endeavor and according to scientific principles. We agree with Cooper et al. (2014) who describe modern plant breeding as an "integration of quantitative genetics, statistics, gene-to-phenotype knowledge of traits embedded within crop growth and development models"… to "advance our understanding of functional germplasm diversity." A schematic of hybrid maize breeding is presented in Fig. 3.2.

Genetic diversity changes as represented by arrays of molecular marker founder haplotypes through four generations of pedigree breeding for two representative maize chromosomes are presented in Fig. 3.3.

Diversity changes occur as a result of crosses with other parents, segregation, recombination, genetic drift, and selection by breeders. Our definition of "modern plant breeding" is broad, and we do not regard use of a specific technology as definitional of "modern." We are well aware of the debate regarding the use of genetically modified organisms (GMOs) (Gurian-Sherman 2009; Brookes and Barfoot 2014; Heinemann et al. 2013). In this respect, we recommend that definitions be based upon scientific principles. Consequently, the use of GMOs and organic methods could coexist and would not preclude the use of best principles from either fields. Bt toxin is included in many "organic approved" pesticides and was recommended for environmentally friendly pesticide use (Carson 1962).

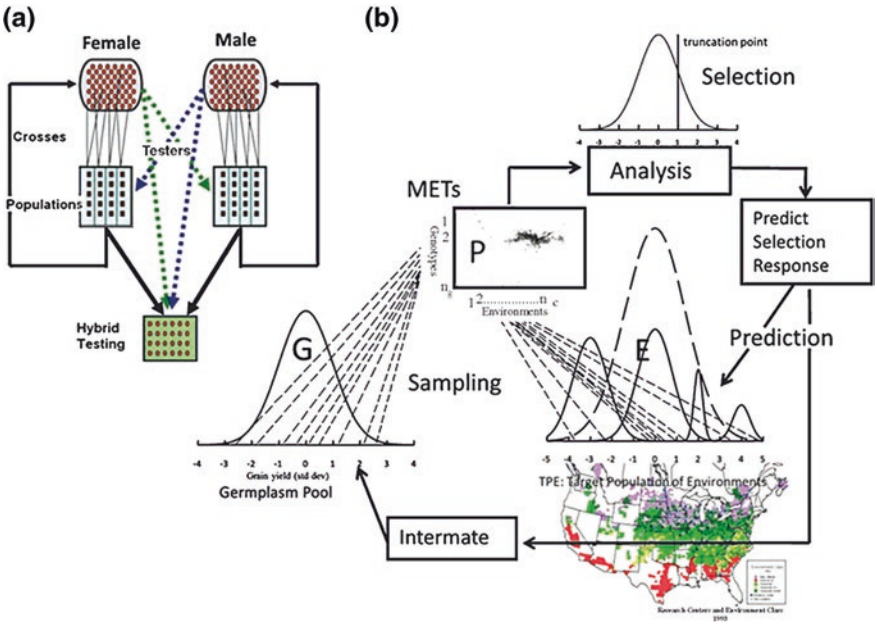

Fig. 3.2 **A** Schematic of a hybrid maize breeding program: *a* Schematic of a hybrid breeding program; *b* Schematic of the major steps undertaken within a cycle of a breeding program (by kind permission, Fig. 2 from Cooper et al. 2014). **B** Schematic of a large-scale commercial hybrid breeding program operated as a coordinated network of breeding programs. Germplasm and genetic information from experiments conducted in any cycle '*t*' are shared among breeders and breeding programs to create new inbreds and hybrids in future cycles '$t + 1$' (by kind permission, Fig. 3 from Cooper et al. 2014). **C** Use of pedigree, marker haplotypes, and phenotypic data in a breeding program: *a* germplasm universe depicting pedigree relationships between founders to modern elite inbreds: the breeding germplasm pool from which the inbreds were sampled, with the pedigree trajectory that contributed to a specific elite individual highlighted; *b* specific inbred pedigree: an extract of the highlighted pedigree that leads from founder ancestors to the highlighted elite inbred, with the founder contribution depicted for a particular 10 cM chromosome region during the pedigree history from founders to elite inbred; *c* identity-by-descent (IBD) profiles: the IBD founder haplotype diversity among a set of elite inbreds for a particular chromosome position; *d* founder haplotype frequencies: the change in frequency of alleles for two QTLs where the alleles are defined in terms of IBD to defined founder ancestors in the pedigree history (by kind permission, Fig. 13 from Cooper et al. 2014)

We regard the partitioning of yield as either "intrinsic" or "operational" (Gurian-Sherman 2009) to be a false dichotomy. The genetic basis for crop yield increase comes from either increased stress resistances or a greater relative partitioning of photosynthates to the harvested organs. Neither of these sources of yield gain could be regarded as "intrinsic" as photosynthesis per se has not increased. Further, protecting yield is as fundamentally important as creating the genetic basis for yield potential. Protecting yield from insect attack using native maize genes was considered an example of genetic gain (Duvick 2005). Protection of harvested produce from spoilage is equally important.

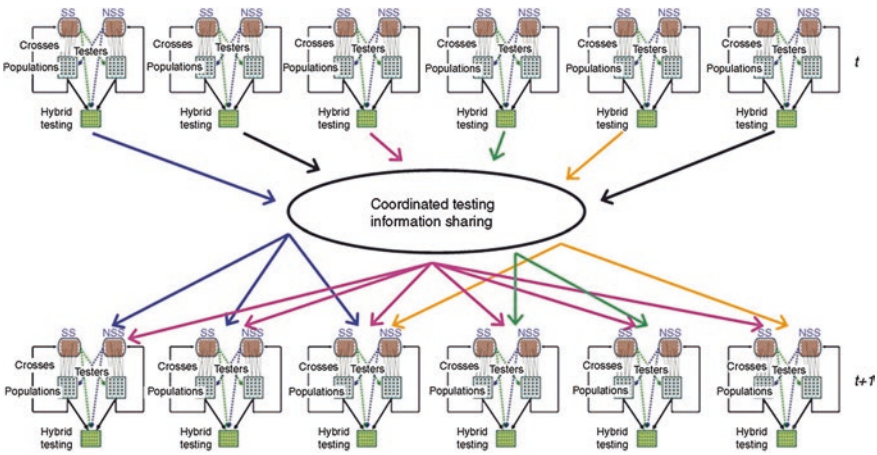

Fig. 3.2 (continued)

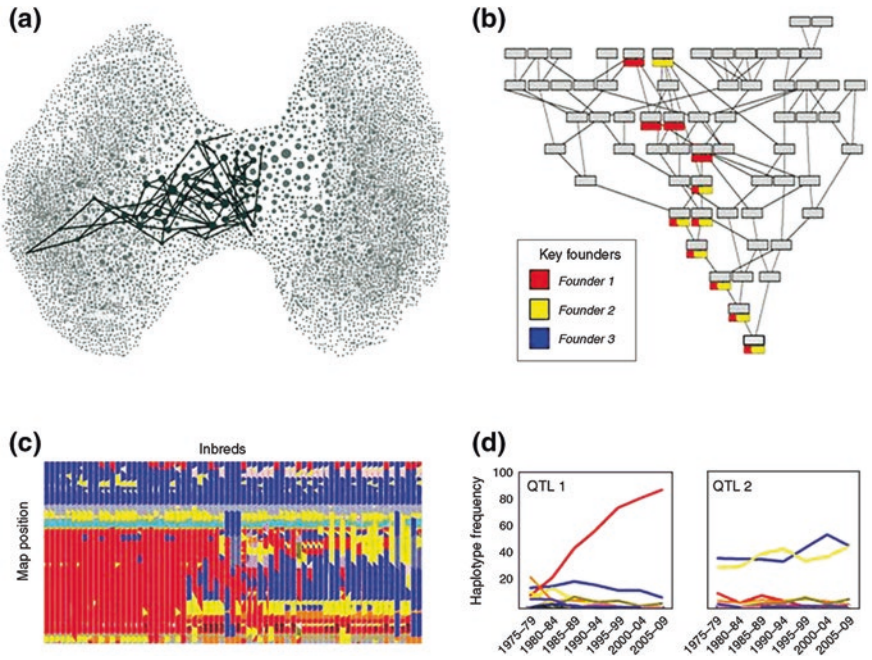

Fig. 3.2 (continued)

As Charles Darwin well understood, the development of domesticated varieties provides powerful examples of evolution in progress. Each variety interacts with the environment creating selection pressures upon pest and disease organisms, especially when a variety is cultivated over a large area. Varieties that were once

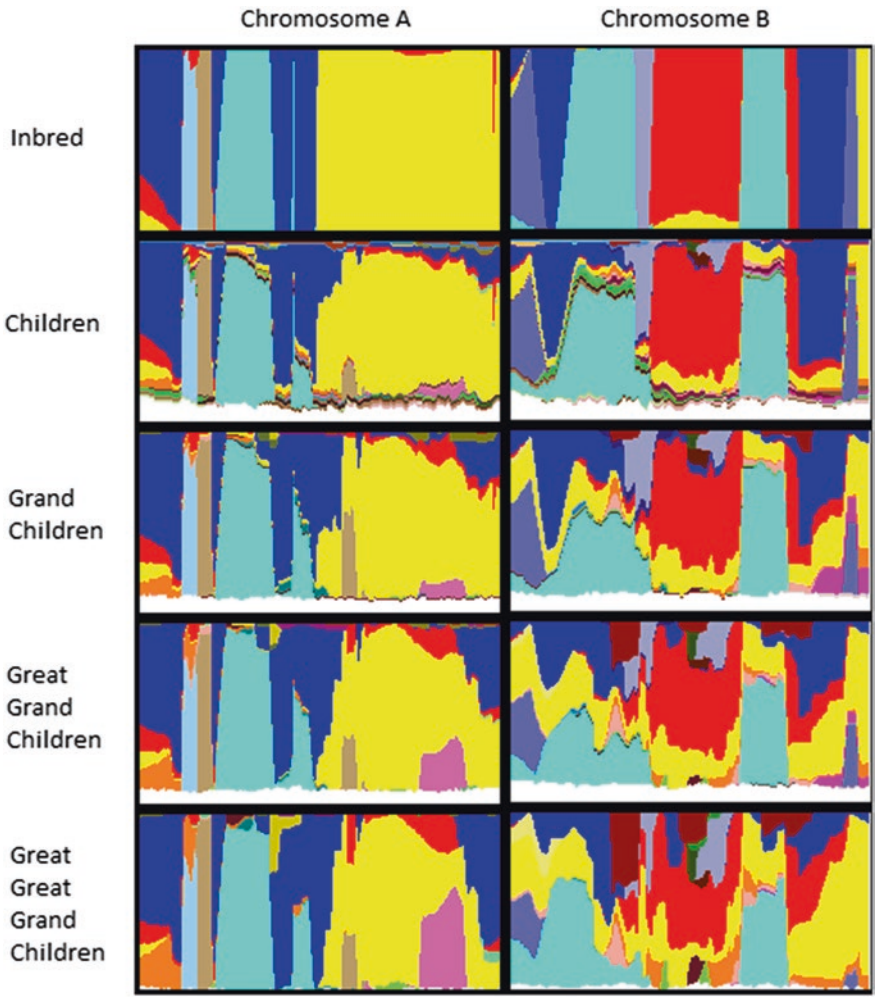

Fig. 3.3 Genetic diversity in time. The evolution of genomic constitution during the breeding process as shown by SNP-founder haplotypes coded by color from an initial parental inbred line through 4 generations of progeny; examples from 2 representative chromosomes of maize are shown. For example, on chromosome A the genomic region represented by the *yellow* haplotype becomes more diversified through the progeny as does the red region on chromosome B. The *dark gray* haplotype on chromosome B diminishes through the progeny lineage

best adapted rarely remain so for long. Varietal inadequacies are revealed by pest and disease pressures or changes in crop management. Examples include: During the 1920s–1940s maize was selected for ease of hand harvesting; however, selection then had to be reversed to adapt maize for machine harvesting. Increasing planting density using narrower rows was made possible by improved precision planting equipment and is associated with genetics conferring more upright leaf canopies. Higher plant populations put increased pressure on stalk and root

lodging which forced breeders to place more emphasis on these traits. Extreme weather events put increased pressure on stalks and the need for drought resistance. Herbicide-resistant crops facilitate no-till which makes the spring planting soil environment colder, wetter, and with increased disease carry-over forcing additional selection by breeders for "defensive" traits. Improved moisture conservation allowed maize cultivation to be feasible on land that was previously drought prone; development of drought-resistant maize hybrids has accelerated this trend. Sorghum breeders have had to source new germplasm to develop varieties that are better adapted to either dryland or irrigated conditions as farmers in the U.S. and Australia transition management practices.

3.2.1 Modern Plant Breeding and Consequences of Role Specialization

Prior to the advent of modern plant breeding farmers exercised three roles: (1) food production, (2) stewardship of genetic resources, and (3) the improvement of crop performance by agronomic practice and varietal selection. Critical changes occurred once farmers chose to use new varieties developed by plant breeders. Change may be gradual, over many decades, where farmers allow landraces to be hybridized or mixed with breeder sourced varieties (Brush 1991, 1995; Hammer et al. 1996) or can occur more swiftly during a few years or decades (Duvick 2005; Negri 2003). Landraces persist in cultivation in less favorable environments due primarily to stability of performance, consumer preference, or lack of breeding support rather than because of intrinsic abilities for high yield potential (Zeven 1998; Almekinders et al. 1994; Newton et al. 2010). Increased availability of resources and specialization of skill sets emphasize the complementary roles of farmers as producers and plant breeders as developers of varieties. Consequently, landrace genetic diversity that is not transferred into formal breeding programs will be lost unless the role of conservator is consciously taken up.

3.2.2 New Arrays of Genetic Diversity as a Result of Modern Plant Breeding

How genetic diversity is arrayed in space, and time changes when farmers transition to using newly bred varieties. Formal plant breeding systems and less formalized systems including via networks of growers and breeders facilitate international access and use of genetic diversity provided phytosanitary requirements and terms relating to access and benefit sharing are met. For example, prior to the establishment of formal maize (*Zea mays* L.) breeding programs in the U.S. the most widely used open-pollinated variety (OPV) in the central Corn Belt was

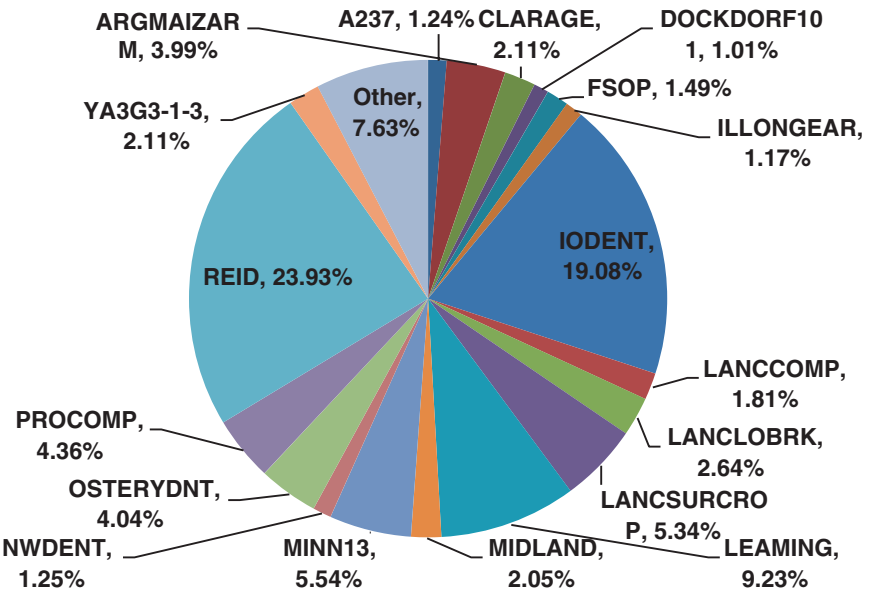

Fig. 3.4 Mean contribution by pedigree of founder genotypes to 14 Pioneer hybrids that were widely grown in the central Corn Belt of the U.S. By contrast, the landrace Reid Yellow Dent was the primary germplasm cultivated on farms prior to the advent of hybrid maize

Reid Yellow Dent (RYD). In contrast, maize farmers in this region today cultivate a broader sampling of landrace diversity. Figure 3.4 shows the mean contribution by pedigree of major founder genotypes for a set of 14 Pioneer brand hybrids that were widely cultivated in the central Corn Belt during the 2000s. The most widely grown OPV, which is grown in the central Corn Belt prior to the 1930s (RYD), now represents 24 % of diversity due to the introduction of inbred lines with pedigrees tracking to other founders. Farmers cultivate a greater diversity of founder germplasm as a result of networking among plant breeders. A typical modern variety of spring wheat released in developing countries may be derived from 45 to 50 landraces and a modern rice variety from 25 or more landraces (Morris and Heisey 1998).

Most diversity within landraces resides between individual plants. In contrast, pedigree or reciprocal recurrent selection breeding schemes partition diversity among different varieties. The inescapable biological reality is that genotype × environment (G × E) interactions condition phenotype (P). Since agroecological environments vary according to weather, maturity, and soil type, successful varieties must demonstrate genetic diversity in space (Fig. 3.5).

Genetic diversity is also arrayed in time (Figs. 3.3 and 3.6) as fresh diversity, which underpins genetic gain, is created by breeding through recombination and including diversity sourced from other geographic regions.

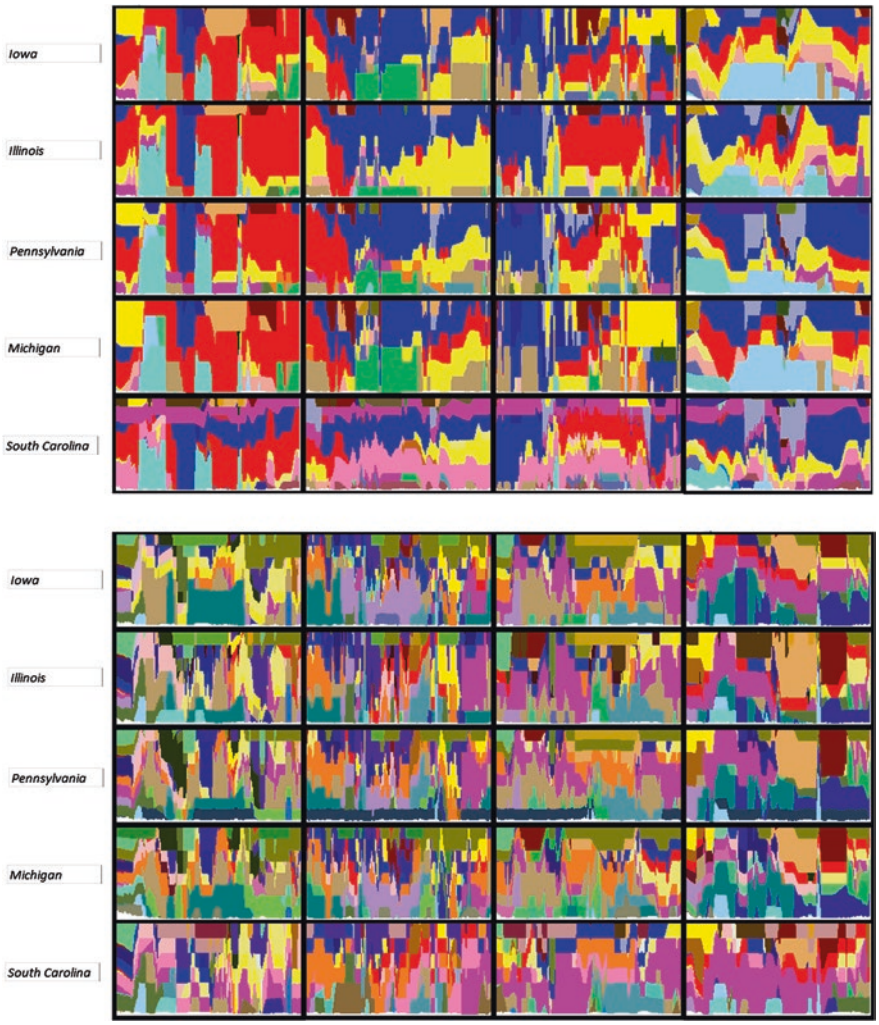

Fig. 3.5 Genetic diversity in space. Mean SNP-founder haplotype profiles for female (*upper panel*) and male (*lower panel*) parental lines of Pioneer maize hybrids that were widely cultivated during the 1990s for each of the named U.S. states. Examples from four chromosomes representing the range of diversity change are presented

3.3 Measuring Genetic Diversity

It is important to characterize and monitor genetic diversity, not only quantitatively and qualitatively, but also temporally and spatially (Morris and Heisey 1998). Comparisons of varietal names provide no diversity metric. When improved varieties are used on farms alongside traditional landraces, then diversity

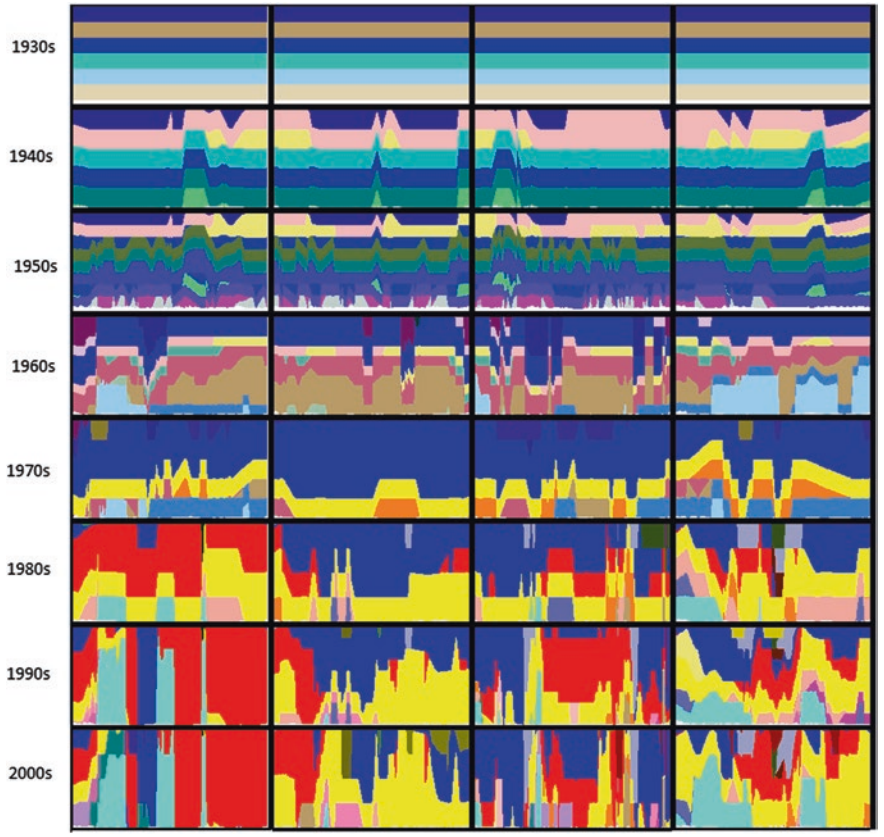

Fig. 3.6 Genetic diversity in time. Mean SNP-founder haplotype profiles for female (*upper panel*) and male (*lower panel*) parental lines of Pioneer maize hybrids that were widely cultivated during decades from the 1930s–2000s and which comprised the germplasm used to measure genetic gain (Smith et al. 2014a). Examples from 4 chromosomes representing the range of diversity change are presented

changes can be complex (Brush 1991, 1995; Bellon and Brush 1994; Louette 1995; Louette et al. 1997: Brush and Perales 2007).

An ideal parameter might be to measure diversity for agronomically important traits. However, useful genetic diversity usually remains hidden from casual observation. For example, Jack Harlan (1975) noted: "A wheat (*Triticum* spp.) I collected in a remote part of Eastern Turkey in 1948… is miserable looking…, tall, thin-stemmed, lodges badly, is susceptible to leaf rust, lacks winter hardiness yet is difficult to vernalize, and has poor baking qualities. Understandably, no-one paid any attention to it for some 15 years. Suddenly, stripe rust became serious in the northwestern states and (it) turned out to be resistant to four races of stripe rust, 35 races of common bunt, ten races of dwarf bunt and to have good tolerance

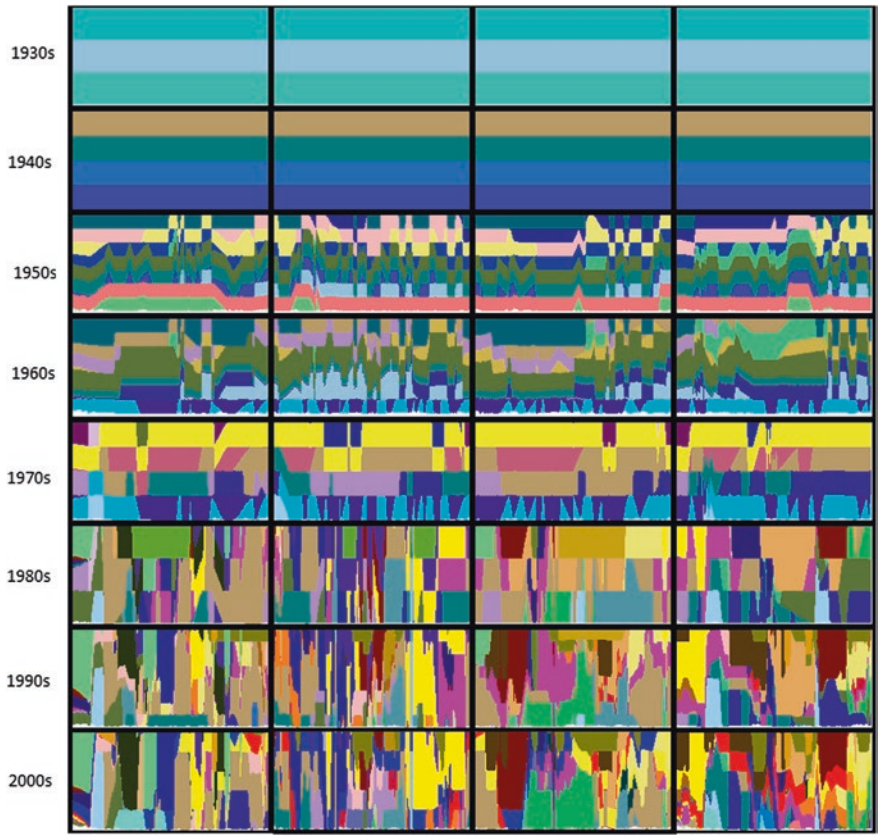

Fig. 3.6 (continued)

to flag smut and snow mould." The range and variance of genetic diversity underlying agronomic traits are important to ascertain for they determine whether sufficient progress can be made through selection. For example, ear length is a component of yield in maize yet selection of increased ear length did not result in higher yield due to negative correlations with other yield traits (Ross et al. 2006).

Other sources of data have been used to characterize genetic diversity including morphology (Dillmann et al. 1997; UPOV 2009), pedigree (Delannay et al. 1983; Cox et al. 1985), heterosis (Smith and Smith 1992; Gizlice et al. 1993), and molecular markers, (Donini et al. 2000; Kim and Ward 2000); see reviews by Rauf et al. (2010) and van de Wouw et al. (2010). Pedigree data are subject to error due to incorrect or missing data and cannot reflect selection but can reveal trends. Molecular marker technologies have evolved rapidly, from allowing 20–25 genes to be interrogated during the 1980s to today, when assays of thousands or millions of single nucleotide polymorphisms (SNPs) are routine. Molecular marker or sequence data provide the most useful means to measure genetic diversity. These

data provide a common diversity "language" encompassing wild and weedy species, domesticated landraces, and new varieties. Nonetheless, the complexities of genetic, regulatory, creation of de novo diversity (Hopkins et al. 2013), and other mechanisms (e.g., epigenetic) systems still render a comprehensive understanding of agronomic performance in terms of genetic sequence or methylation data as far from complete (De Koeyer et al. 1999; Lucas et al. 2013).

3.4 Patterns of Change in Genetic Diversity

3.4.1 Bottlenecks Where Genetic Diversity Can Be Lost in the Continuum from Crop Domestication, Through Use of Landraces, to Modern Plant Breeding

Table 3.1 presents loss of diversity during domestication 8–10,000 years ago for several plant species. For most cultivated species, approx. 65–70 % of diversity in the wild species transferred through the domesticated genepool. For soybean, the domestication process was responsible for a relatively low reduction in diversity. However, the wild species (*Glycine soja*) has unusually low levels of sequence diversity. In contrast, wild barley and wild maize (teosinte) have

Table 3.1 Estimated % genetic diversity not transferred as a result of domestication bottleneck(s) during selection from respective progenitor wild species some 8–10,000 years ago

Species	Common name	% Genetic diversity not transferred	Data source	References
Zea mays L.	Maize or corn	35	All nucleotide sites	Wright et al. (2005)
		38	Silent nucleotide sites	Tenaillon et al. (2004)
		17	Nondomestication genes	Hufford et al. (2012)
		43	SNPs in 774 gene fragments	Wright et al. (2005)
		24	Number of SSR alleles	Vigouroux et al. (2005)
		12	Genetic Diversity of SSR alleles	Vigouroux et al. (2005)
		12	SSR Gene Diversity	Matsuoka et al. (2002)
Medicago sativa	Alfalfa/Lucerne	31	All nucleotide sites	Muller et al. (2006)
		31	Silent nucleotide sites	Muller et al. (2006)

Table 3.1 (continued)

Species	Common name	% Genetic diversity not transferred	Data source	References
Helianthus annuus	Sunflower	55	All nucleotide sites	Liu and Burke (2006)
		59	Silent nucleotide sites	Liu and Burke (2006)
		27	Nucleotides	Kolkman et al. (2007)
Pennisetum	Millet	33	Silent nucleotide sites	Gaut and Clegg (1993)
Glycine	Soybean	34	All nucleotide sites	Hyten et al. (2006)
		36	Silent nucleotide sites	Hyten et al. (2006)
Hordeum	Barley	57	All nucleotide sites	Caldwell et al. (2006)
		62	Silent nucleotide sites	Kilian et al. (2006)
		20	Nucleotides	Morrell et al. (2013)
		45	Nucleotides	Glemin and Bataillon (2009)
Triticum spp.	Emmer Durum and Hexaploid bread wheat	65	All nucleotide sites	Haudry et al. (2007)
		70	Silent nucleotide sites	Haudry et al. (2007)
		84	All nucleotide sites	Haudry et al. (2007)
		69	All nucleotide sites	Haudry et al. (2007)
Avena	Oats	34		Murphy and Phillips (1993)
Oryza	Rice ssp. indica	29	SSRs	Gao and Innan (2008)
	Rice ssp. japonica	38	SSRs	Gao and Innan (2008)
Sorghum	Sorghum	34	Isozymes	Morden et al. (1990)

4× and 5× the amount of diversity, respectively (Hyten et al. 2006). Cultivated sunflower (*Helianthus annuus*) and barley (*Hordeum vulgare*) suffered higher losses of germplasm diversity (55–60 %) although more recent data for barley (Morrell et al. 2013) indicate a relatively low loss (20 %). Cultivated wheat species (*Triticum* spp.) suffered the greatest losses of diversity (65–84 %) compared to wild ancestral species.

3.4.2 Changes in Genetic Diversity from Landraces to Well-Adapted Inbred Lines and Varieties

3.4.2.1 Case Study: Soybean

The cultivated genetic base of U.S. soybean accounts for 47 % of global soy production (Wilcox 2004). Relatively few founders (17) contributed to the U.S. breeding base. However, this bottleneck was moderate for 80–87 % of nucleotide diversity in the landrace class was maintained among these founders. Nonetheless, 78 % of rare landrace alleles were lost. Another potential bottleneck occurred as elite cultivars were bred using the variety founder base. However, Hyten et al. (2006) found elite cultivars retained 83–97 % of nucleotide diversity in the founders. Nucleotide diversity among elite soybean cultivars was "similar to values reported for humans, lower than that of *Sorghum bicolor*, and an order of magnitude lower than modern maize inbreds." Hyten et al. (2006) concluded that "modern soybean breeding has minimally affected allele structure of the genome compared with the other historical bottlenecks (i.e., during initial domestication process." Hyten et al. (2006) considered that it would be unlikely to add new diversity by "randomly adding 100 new Asian landraces to the elite pool," rather it would be more effective "to introduce on a per need basis." Genetic diversity in wild *G. soja* should also be ensured by conservation.

3.4.2.2 Case Study: Maize

Vigouroux et al. (2008) examined almost the entire set of approximately 350 races of maize native to the Americas (Matsuoka et al. 2002). Landraces associated into four major groups: Highland Mexico, Northern U.S., Andean, and Tropical Lowland. Highland and tropical lowland races encompassed most diversity. Nonetheless, Northern U.S. landraces expressed 88 % of the gene diversity and 71 % of the number of alleles/locus compared to the most diverse set (Highland Mexico).

Matsuoka et al. (2002) and Liu et al. (2003) estimated that 101–206 inbred lines collectively retained 98 % of gene diversity, from 76–93 % of the number of alleles, and 73 % of the number of alleles per locus compared to landraces. The USA set (54 inbreds) retained 93–98 % of alleles present in landraces. Compared to the wild ancestor, the US inbred set retained 84 % of the genetic diversity and 67 % of the number of alleles (Matsuoka et al. 2002). In contrast, Liu et al. (2003) showed a greater reduction of diversity; inbreds had 76 % the number of alleles, 73 % the number of alleles/locus, and 98 % of overall gene diversity compared to landraces.

Liu et al. (2003) categorized 260 inbreds into three major groups; non-stiff stalk (NSS), stiff-stalk (SS), and tropicals. Tropical inbreds originated mostly from tropical lowland (66 %) and tropical highland (18 %) races. NSS and SS

originated mostly from Southern Dent (37–38 %) and Northern Flint (23–27 %) which together form the Corn Belt Dent race of maize, the most productive and globally widespread maize race. The NF and SD races are radically different in their morphology, isozymic constitution, and cytology. Doebley et al. (1988) described them as "representing the opposite ends of the spectrum of variation in maize" and Anderson and Brown (1952) considered them to be so different that "relative to the variation found within the wild grasses, they would be considered different species and possibly members of different genera."

Liu et al. (2003) concluded that tropical highland diversity was not well represented in the inbreds. Consequently, tropical highland maize and tropical inbreds could be useful candidates for broadening the diversity of the elite germplasm base. Highland races of maize showed evidence of introgression from their wild ancestor teosinte and so represent sources of both wild and cultivated exotic diversity (Hufford et al. 2013).

During the early phase of the Pioneer corn breeding program in the 1920s and 1930s, the Director of Corn Breeding, Raymond Baker, encouraged Pioneer corn breeders to source diversity from a broad base of OPVs. Considerable genetic diversity can exist within a single maize OPV. For example, results from Illinois long-term selection studies in maize that began in 1896 continue over a century later to show responsiveness to selection, including reversible responses. These results indicate high levels of complex genetic diversity contributing to oil and protein levels (Lucas et al. 2013). Lu and Bernardo (2001) compared diversity among 40 U.S. inbred lines and concluded that genetic diversity had declined at the gene level but had been maintained at the population level. We also found that genetic diversity is reducing within individual heterotic groups, but diversity overall is maintained by increased separation between heterotic groups (Figs. 3.2 and 3.7). Duvick (1984) and Morris and Heisey (1998) refer to cultivated genetic diversity being arrayed in space and in time. There is abundant evidence of temporal diversity (Figs. 3.3, 3.4, 3.6 and 3.7) reflecting the contribution of genetic gain to increase on-farm productivity (Smith et al. 2014a). There is also evidence of spatial genetic diversity (Fig. 3.5) showing that different arrays of genetic diversity are required to allow optimum phenotypic expression in different environments (Smith et al. 2006).

Romay et al. (2013) compared 2815 maize inbreds using over 680,000 SNPs. They found that: "Although all of the major private seed companies are represented within each group (consistent with the small value of divergence between companies), Pioneer germplasm is represented more in the Iodent group and more of its germplasm falls outside the three main clusters." The signature of diverse germplasm sourcing is still reflected in more recently developed germplasm (Fig. 3.4) and indicates that the U.S. maize germplasm base is broader than just Reid and Lancaster OPVs (Troyer 1999, 2004). On the other hand, there is no justification for complacency. A trend of genetic diversity being depleted by only breeding with and commercializing the best performing varieties would be a natural outcome of selection if conducted in a closed system. Plant breeders must therefore actively manage germplasm diversity to provide potential for continued

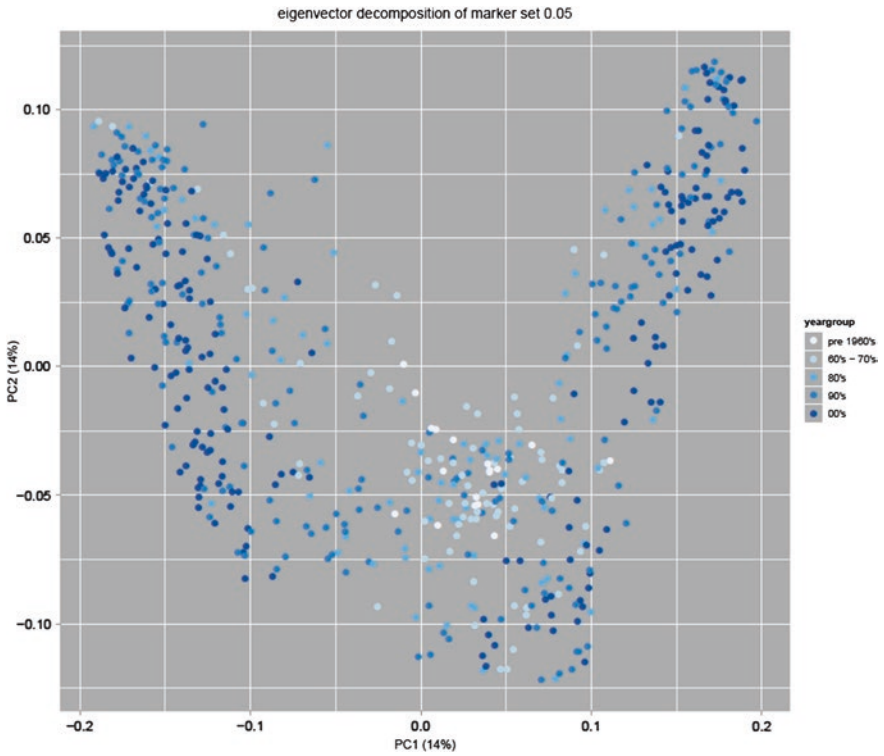

Fig. 3.7 Associations of inbred lines represented by colored dots representing different eras of breeding based upon genetic distances calculated from comparisons of SNP profiles. The pattern of association shows increasing divergence in genetic diversity between inbreds that are allocated into either female or male heterotic pools as breeding has progressed from the 1960s to the 2000s

realization of genetic gain. Furthermore, there is no reason to suppose that all useful genetic diversity present in *Zea* should reside in one race, even one with very divergent origins. Programs dedicated to evaluating exotic maize germplasm in the U.S. have proven this assertion (Lewis and Goodman 2003; GEM 2014).

3.4.3 Changes in Genetic Diversity Across Decades in Varieties Deployed in Agriculture

Diversity generally decreased during the initial transition from landraces to the first cycle of varietal breeding. During subsequent decades, there have been temporal fluctuations in diversity (Rauf et al. 2010; FAO 2010; van de Wouw et al. 2010). Fluctuations are associated with bottleneck effects due to demands for high malting, brewing, or baking qualities. Additional temporary bottlenecks

were associated with the introduction of new germplasm, e.g., semidwarf wheat and wheat-rye (*Triticum-Secale*) translocation stocks (Orabi et al. 2014). In contrast, introduction of GMO herbicide resistance did not cause a bottleneck in soybean (Sneller 2003). Some breeding programs have reversed the post-landrace trend, reaching higher levels of diversity. For example, Ren et al. (2013) observed a significant increase in wheat diversity for cultivars released during 1980–2009. Parker et al. (2002) concluded that genetic diversity in 124 Australian wheat varieties increased over time. Rauf et al. (2010) showed introduction of CIMMYT wheat lines with diverse landrace pedigrees increased diversity surpassing that of the pre-Green Revolution era by 1991–2000. Smale et al. (2002) concluded that diversity trends for spring bread wheat cultivars released after 1965 were "not consistent with the view that the genetic diversity of modern semidwarf wheat grown in the developing world has decreased over time." Orabi et al. (2014) found that during 1886–2009 diversity in European wheat varieties had declined by the 1940s as farmers moved away from cultivating landraces. However, during the 2000s, European wheat varieties reached higher levels of diversity than was exhibited by landraces. Van de Wouw et al. (2010) showed a decrease of diversity for 8 field crops from the 1950s to the 1960s with a subsequent increase in diversity. Orabi et al. (2014) credited the use by breeders of adapted germplasm from different regions, exotic germplasm, landraces, and wild relatives.

3.5 Measures to Conserve and Source Additional Genetic Diversity

The successful deployment of genetic diversity in plant breeding and agriculture to achieve sustained improvements in productivity depends upon the continued sourcing, creation, and deployment of new useful diversity. While there is evidence for de novo creation of diversity (Hopkins et al. 2013), it would be foolish to restrict access by repeatedly sourcing only widely used well-adapted varieties. Temporal fluctuations in genetic diversity reflect the dynamic nature of the agricultural environment and abilities to successfully adapt to both challenges and opportunities provided by ever-evolving weeds, pests, and diseases, unstable and unpredictable climates, changing management practices on farms, and changing consumer preferences. Primary sources of potentially useful new diversity include well-adapted varieties from adjacent regions, less immediately well-adapted or "exotic" varieties including landraces (Warburton et al. 2006), de novo generation of allelic variation in well-adapted varieties including via gene mutation or through the alteration of gene expression, crop-related wild and weedy species, and "trans-genic" diversity sourced from other genera or phyla and incorporated using molecular engineering.

3.5.1 Concerns About Genetic Uniformity; Examples Where Single-gene (or Cytoplasmic) Resistances Have Broken Down

Concerns about loss of diversity were heightened in the 1960s when a high yielding rice cultivar IR8, susceptible to the bacterial leaf blight pathogen (*Xanthomonas oryzae* pv. Oryzae) was widely planted throughout Southeast and South Asia. Outbreaks of bacterial blight cut yields by 20–50 % and as high as 80 % (American Phytopathology Society 2014). Additional concerns arose when Southern Leaf Corn Blight caused by Race T of the fungus *Bipolaris* (*Helminthosporium*) *maydis*, struck the US maize crop in 1970. In this case, uniformity was associated with a specific cytoplasm rather than the nuclear genome. Yield losses reached 50–100 % in some areas with economic losses of about 1 billion dollars (American Phytopathology Society 2014). "*Never again should a major cultivated species be molded into such uniformity that it is so universally vulnerable to attack by a pathogen, an insect, or environmental stress. Diversity must be maintained in both the genetic and cytoplasmic constitution of all important crop species.*" (Ullstrup 1972). Since maize breeders had elite inbred lines in other cytoplasms, they were able to rapidly "unmold" nuclear genomic diversity using N, C, and S cytoplasms. Similarly, soybean (*Glycine max*) variety BR16 dominated use in Brazilian agriculture until stem canker (*Diaporthe* spp.) essentially wiped it out in 1996; a single-resistance gene was incorporated and a modified version of BR16 was introduced. Soybean rust (*Puccinia pachyrhizi*) also had devastating effects and multigenic sources of particle resistance coupled with fungicides currently control the disease.

Brown (1983) reminded that diversity per se provides no guarantee of resistance to pests or diseases. For example, the American chestnut (*Castanea dentate*) was decimated by blight (*Endothia parasitica*) in two decades and the highly variable American elm (*Ulmus americana*) is very susceptible to Dutch elm disease (*Ophiostoma* spp.). Prior to the advent of plant breeding, there were attacks by the potato blight fungus (*Phytopthora infestans*) in Ireland during the 1840s and in Germany during World War I. Attacks of ergot (cased by the fungus *Claviceps purpurea*) in the Rhine valley occurred during 857 AD–1300 AD, in England during 1355, and in Russia during 1926–1927. In 1916, wheat rust (*Puccinia graminis*) caused significant yield losses to U.S. landraces. During the early 1950s, the tropical rust fungus (*Puccinia polysora*) spread across Africa on maize OPV cultivars that otherwise sequestered much genetic variability. Mercer and Perales (2010) expressed concerns that highland maize landraces could be vulnerable to loss of diversity and even potential extinction due to relatively poor performance as climates warm.

It is to be expected that farmer demand for a variety with outstanding agronomic performance will lead to its wide cultivation. FAO (1997) noted that "uniformity per se need not be dangerous, for some crop cultivars are remarkably stable." For example, for the last 40 years, Azul has been the only variety

of *Agave tequilana* permitted in the production of tequila (Valanzuela 2011). Brazilian orange (*Citrus sinensis*) production is based on few varieties (Machado et al. 2011), and use of a narrow genetic base is not restricted to humans. The Monarch butterfly (*Danaus plexippus*) feeds only on *Asclepia* spp. (milkweed), while the fastest mammal, the cheetah (*Acinonyx jubatus*), is well adapted to a predatory lifestyle, its lack of genetic diversity (Menotti-Raymond and O'Brien 1993) makes the species quite unadaptable. Overreliance upon a successful variety or reliance upon specialized behavior is a potential Achilles heel for any cultivar or species. For example, mounting selection pressures that result in pathogens overcoming varietal resistance creates a "social trap" (Morris and Heisey 1998). Biological interactions are inevitable and must be managed as integral components of plant breeding. For example, Panama disease, which caused the export banana (*Musa acuminata*) trade to be decimated at the beginning of the twentieth century, is now reappearing in Cavendish banana, the sole banana variety that is grown for export (Van der Wal and de Groot, n.d.). Outbreaks of Corn Northern Leaf Blight (*Exserohilum turcicum)* in the Alsace-Rhine valley were countered by breeding in additional resistance genes. The susceptibility of the widely sold U.S. corn hybrid B73 × Mo17 to stalk, disease, and drought was countered by development of improved varieties. B73, in particular, has been one of the most widely used parental lines and productive breeding materials in U.S. maize history with numerous improvements made by breeders to its agronomic deficiencies. Leading sunflower varieties in Argentina and a leading U.S. maize hybrid were replaced by further breeding when susceptibilities to stalk and leaf diseases were exposed as the planting environment changed. Each new variety or "genetic solution" can only be temporary as the on-farm environment is dynamic. Dynamic change requires the creative use and deployment of useful genetic diversity in breeding programs.

3.5.2 Conservation of Genetic Resources

Conservation of genetic diversity is akin to a long-term insurance policy for potential future resource use and thus a long-term public good. Conservation of plant genetic resources for food and agriculture is usually considered as occurring "on-farm" (in situ) or "off-farm" (ex situ). Ex situ gene banks are the last repository for conserving genetic diversity that otherwise would be lost as farmers transition from using landraces to cultivating varieties developed by plant breeders, including via participatory plant breeding where farmers play a more intimate role in selecting progeny. Needs to access a broad base of genetic diversity lead to the establishment of the US Plant Exploration program in 1898 (Williams 2005). Pioneering research and germplasm collection expeditions were carried out in the 1920s and 1930s by N.I. Vavilov with specific goals to better understand and thereby utilize global genetic diversity of cultivated species which included the establishment of genebanks (Vavilov Institute 2014). Additional genebanks were

established during the late 1940s–1950s in the US, Mexico (Taba et al. 2004; Williams 2005). Concerns about loss of landrace genetic diversity during the expansion of area planted to newly bred varieties during the Green Revolution lead to the establishment of additional genebanks, notably those under the auspices of the Consultative Group on International Agricultural Research (CGIAR). Globally, there are over 1300 genebanks. Of these, only 56 % accessions are stored in medium to long-term facilities, 8 % are in short-term, 10 % are in field conditions, and 25 % have no information (Scarascia-Mugnozza and Perrino 2002). Fears of loss of germplasm lead to the establishment in 2004 of the Global Crop Diversity Trust (GCDT) (www.startwithaseed.org) through a partnership with the Food and Agriculture Organization (FAO) of the United Nations (UN) and the CGIAR. Goals of the GCDT are to raise an endowment sufficient support a rational, efficient, and sustainable global system of genebanks (Raymond 2004). CGIAR collections are held as a "common responsibility of humankind" under the auspices of the FAO. These genetic resources are conserved in a multilateral system thereby recognizing the collective primary benefit of enabling access for potential further use in food and agriculture for all countries. Benefits reside in the assured ability to access a broad range of genetic diversity beyond that present in any single country or region. No single country or region, even those where crops were initially domesticated or later developed increased diversity, is or can be self-sufficient for its supply of crop genetic resources (Fowler et al. 2001; Voysest et al. 2003; Fowler and Hodgkin 2004). An alternate scenario of finding unique germplasm source in only one location of very high monetary value that could be readily realized elsewhere was acknowledged not to be concordant with the monetary worth and geographic distribution of genetic resources useful for food and agriculture (Gollin 1998; Voysest et al. 2003; Fowler and Hodgkin 2004) as witnessed by the establishment of the FAO International Treaty (FAO 2009).

Strategies to conserve germplasm ex situ have been criticized because they effectively place germplasm in cold storage and so halt further evolution of the variety in response to pests, diseases, or climate, which are under constant change. This criticism is misguided; however, for opportunities to generate new diversity occur when plant breeders access and use this germplasm, creating more new diversity by crossing and selection and at a faster rate than would otherwise have occurred even if the initial landrace diversity could have been maintained on farm. Ex situ germplasm conservation allows genetic diversity that would otherwise have been lost to be still used in plant breeding programs. Most successful plant breeding programs have to be in situ due to the effects of gene × environment interaction. The notion of in situ conservation also requires careful scrutiny. Studies of genetic diversity in farmer managed agriculture and seed supply systems indicate use of fairly sophisticated practices to maintain varietal quality and to re-invigorate existing diversity (Brush 1991, 1995; Bellon and Brush 1994; Louette 1995; Louette et al. 1997: Brush and Perales 2007). When more productive germplasm is introduced then farmers will, according to their needs, utilize that germplasm per se, or for outcrossing crops, introduce some of that "new" genetic diversity by hybridization. Thus, even if the quantitative level of genetic

diversity is maintained some of the diversity present in the native landraces is vulnerable to loss. Consequently, use of the term "conservation" in conjunction with "in situ" can be a misnomer and might better be described as "in situ crop management" or "plant breeding." Genetic diversity used on farms should be regularly monitored regardless of the source or type of germplasm (i.e., landraces, landraces with additional introduced germplasm, or varieties developed in breeding nurseries).

3.5.3 Programs to Broaden the Genetic Base in Breeding and Agriculture

There are significant challenges to evaluate exotic, including wild germplasm that is unadapted to a target production environment (TPE). Challenges include (1) deciding which of many accessions to evaluate and (2) adapting exotic germplasm by breeding with adapted germplasm to allow trait performance to be fairly tested.

Information on genetic bottlenecking helps (1) prioritize germplasm as a source of additional diversity and (2) indicate the challenges that need to be overcome to evaluate that germplasm for its potential utility. For example, maize landraces distributed in the Mexican highlands and tropical inbred lines are high priority candidates as they carry much diversity and neither has recent phylogenetic relationships with the Corn Belt Dents. For soybean, landraces would be most effectively sourced by evaluation schemes that target specific traits, whereas most additional diversity resides in wild soybean.

The conduct of germplasm introduction and evaluation programs has usually been undertaken through public or private–public partnerships. The U.S. sorghum crop is highly dependent upon recently introduced exotic germplasm. Sorghum (*S. bicolor*) was introduced into the U.S. from Africa during 1874–1908 through few, yet diverse founder cultivars (Milo, Guinea Kafir) (Klein et al. 2008). By the early 1960s, it was realized that the U.S. sorghum germplasm pool was very narrow with limited opportunities to increase adaptation to U.S. farms. A sorghum conversion program involving the USDA and Texas A&M University was initiated in 1963 to broaden the germplasm base by removing the photoperiod bottleneck. More than 840 sorghum lines with tropical germplasm were converted. A dramatic increase in marker haplotype diversity validated the introduction of new genetic diversity (Klein et al. 2008).

Dr. Major Goodman directs a program at North Carolina State University that incorporates exotic tropical maize germplasm into U.S. breeding materials. Estimated exotic contributions into new inbred lines are from 32–70 % with the best testcrosses out-yielding well-adapted check hybrids by up to 11 % (Lewis and Goodman 2003). The Genetic Enhancement of Maize (GEM) consortium comprises 17 universities, 7 USDA-ARS units, 3 international collaborators, 27 US companies, and 9 international companies. GEM utilizes germplasm from over 12

countries and has surveyed accessions from 24 maize races resulting in 190 germplasm releases (GEM 2014). Useful genetics in GEM releases include improved yield as well as resistances to insects and diseases, high protein, oil, and starch.

The UK Biotechnology and Biological Sciences Research Council (BBSRC 2011) is broadening the genetic base of wheat. Three strategies are being used: source from landrace and locally adapted varieties, make new hexaploid wheats from crossing tetraploid x diploid progenitors, and transfer small genetic segments from wild relatives into hexaploid wheat.

Van Esbroeck and Bowman (1998) cited the infrequent use of exotic germplasm in US cotton (*Gossypium hirsutum*) breeding for cultivars released during 1972–1996. They conclude that "unless methods are improved to transfer useful allelic variation from diverse to adapted germplasm without negative agronomic effects, cotton germplasm resources will remain largely under-used and the trend toward increased genetic uniformity will probably continue." A comprehensive USDA-ARS research program to broaden the genetic base of U.S. cotton was initiated (Wallace et al. 2008). Important useful germplasm releases include nematode resistance and improved fiber quality (Texas A&M 2010).

3.5.4 Generating Additional Diversity

Methods to generate additional diversity include inducing new mutants and the incorporation of specific gene sequences from other unrelated genera or phyla. Plant breeders usually draw upon diversity using the native germplasm base for their specific crop of interest to develop a variety that is well adapted to the TPE and outperforms earlier bred varieties. Targeted use of additional diversity can then be used to modify or better adapt the existing variety. Modified varieties can be developed by (1) crossing the initial variety to another variety that contains the additional diversity of interest (the donor source) and then (2) perform repeated generations of "back-crossing" using the initial (recurrent) parent while simultaneously selecting for the specific donor genes. The goal is to recover a modified variety that is as closely genetically similar to the initial variety as possible with the sole exception of the added desirable genes from the donor source germplasm. Once desired additional genes are incorporated into well-adapted varieties breeders also have the option to forgo use of donor germplasm and instead use the modified varieties as breeding parents per se (forward breeding).

3.5.4.1 Use of Induced Mutations

The fundamental cause of all genetic diversity is mutation which can be defined as "any change in nucleotide composition of the genome." Using the products of induced mutation in breeding was initiated by Stadler (1928a, b) in barley and maize using X-rays and radium. Advances in mutation breeding include the ability

to make sequence site or gene directed mutants e.g., via RNA interference, site-specific mutagenesis, use of zinc-finger nucleases, and more efficient means to identify new, including recessive alleles via the process of Targeted Induced Local Lesions IN Genomes (TILLING) and transcriptome analyses (Phillips and Rines 2009). Mutation breeding plays an increasingly important role in providing plant breeders with potentially useful sources of new genetic diversity and is particularly useful for the improvement of asexual crop species (Ishige 2009) and species with limited diversity (Shu 2009). Over 2700 new crop varieties encompassing 170 species have been developed and released to farmers including rice, wheat, barley, apples, citrus, sugar cane, and banana (Lagoda 2009). Many varieties bred using induced mutation have had significant positive economic benefits to farmers. Examples include brewing barley varieties 'Diamant' and 'Golden Promise' annually contributing $20 million annually and the Japanese pear variety 'Gold Nijesseiki' contributing $30 million annually. The Chinese rice variety 'Zhefu 802' contributed a yield increase of 10.5 % between 1980 and 1995 which is equivalent to feeding an extra 2 million people each year. A thorough review of the use of mutation breeding is provided by Shu (2009).

3.5.4.2 Use of Germplasm from Other Genera or Phyla (Transgenic)

As of July 2014 ISAAA (2014a, b) listed as present in one or more counties 31 genetically modified (GM) traits of which 8 (27 %) conferred herbicide resistance and 3 (10 %) conferred insect resistance. Twenty-seven crops were listed as having varieties that included GM traits. Seven (23 %) traits were listed as being commercially available in one or more countries. These traits comprised drought resistance, altered growth/yield, disease resistance, herbicide tolerance, insect resistance, modified product quality, and pollination control system for the production of hybrid seed. The first commercially available herbicide (glyphosate)-resistant soybean varieties were released in 1996. Maize hybrids resistant to the European Corn Borer (ECB) (*Ostrinia nubilalis*) through integration of a toxin producing gene from *Bacillus thuringiensis* completed regulatory approvals in 1996. Bt toxin had already been used as a pesticide in Europe in 1920 and is espoused as an environmentally friendly pesticide (Carson 1962).

Prior to the development of GMO approaches, maize breeders had found genotypes that were highly toxic to ECB. However, even after 7 decades of breeding with some success in developing improved tolerance (Duvick 1984, 1997), maize hybrids remained highly vulnerable to significant economic loss annually exceeding $1000 million in the 1990s (Mason et al. 1996). Hutchinson et al. (2010) estimated that $6 billion in economic benefits had accumulated over 14 years in suppressing ECB in the major US Corn Belt; an additional $1.9 billion had also accrued to non-Bt maize growers. Herbicide-resistant varieties have facilitated the more widespread use of conservation tillage, which helps protect against soil erosion and compaction and reduces fuel use thereby reducing costs and carbon emissions (Fawcett and Towery n.d; So et al. 2001; Holland 2004; Green 2012).

It is important to recognize that current transgenic approaches to achieving herbicide and insect resistance remain as chemical solutions directed by single genes using the plant as a source of energy to drive chemical manufacture. They offer the same advantages and disadvantages of any single-gene approach. They provide highly heritable and significant effects but they are liable to loose efficacy due to high selection pressure on pathogens or weeds to overcome the resistance mechanism. Transgenic approaches have also been used commercially to change oil profiles and to control pollen development. There were concerns that adding additional classes of single genes by backcrossing and transgenic modification would increase potential bottleneck effects and thereby reduce genetic diversity in commercially available varieties (Sneller 2003). However, the diversity of US cotton increased during the introduction of Bt varieties and the diversity of US soybeans was maintained as glyphosate-resistant varieties were introduced. There was an initial reduction of diversity in Indian cotton varieties with the introduction of Bt varieties. However, diversity increased as more Bt varieties became available (Carpenter 2011).

It is only very recently that single-gene approaches to agronomic traits such as drought resistance have been developed and commercially released. Even as knowledge of the complex physiological and genetic mechanisms underlying quantitative traits increases, it will still remain challenging to find single-gene or "silver-bullet" solutions either by insertion of exotic genes or though mediated expression of single native genes. To date, 100 % of the genetic potential for yield increase comes from developing new arrangements of native diversity (and possibly via some contribution of the de novo generation of diversity). To date, genetic modifications whether they be contributed by mutation breeding, changing gene expression, or through the integration of exotic germplasm via transgenic methods have served to protect or enhance quality of the existing genetic potential for yield. Such contributions can be highly significant economically, environmentally beneficial, and are in high demand by the farmer. More effective insect control makes more accessible germplasm that hitherto had been precluded from use due to its inherent limitations. Improved drought resistance and nitrogen use efficiency can contribute to a more sustainable agriculture. However, use of one approach should not preclude another including complimentary use of best practices from "organic" and other "non-organic" approaches. Plant breeders and farmers will need to use all available tools to sustainably improve productivity. Nonetheless, we anticipate that primary drivers in achieving increased yield productivity will be as a result of more efficient breeding and selection using native germplasm to further optimize genotype x environmental interactions (Smith et al. 2014a).

3.6 Intellectual Property Protection and Genetic Diversity

Seeds of self-pollinating species and vegetatively propagated plants can be readily copied. Consequently, in order to secure further investments into privately or commercially funded plant breeding, it is obligatory that new parental lines of hybrids, varieties, and other research-based products such as novel traits or breeding methods, can be eligible to be protected as intellectual property. Misappropriation also contributes to a narrowing of the genetic base and misleads farmers who wish to diversify their cultivated genetic base so as to spread maturities and to help guard against potential weather, insect, or pest-related risks associated with growing any single or narrow germplasm pool.

The most widely used form of protection is Plant Breeders' Rights (PBRs) or Plant Variety Protection (PVP). PVP was developed specifically for plant breeding under the auspices of the Union for the Protection of Cultivated Plants (UPOV) and is implemented via national or, in the case of the European Union, regional legislation. The UPOV Convention was adopted in 1961, came into force 1968 and revised in 1972, 1978, and 1991 (UPOV 2014). Briefly, PVP provides time-limited (usually 20 years) protection during which the owner has a monopoly on the sale and commercial exploitation of the variety per se. However, during that period there is an exception allowing others to breed with the protected variety and (under laws compliant with UPOV 1991) to freely commercialize the resultant progeny providing they meet the UPOV requirements for distinctness, uniformity, and stability (DUS), and they are not so similar to the initial variety as to be declared essentially derived varieties (EDVs). There may also be exceptions that allow farmers to save seed for use on their own farms; royalties may be levied for commercial scale use of farm saved seed, but this is not the case in the U.S. The U.S. initiated IPP for vegetatively propagated nontuberous species through the US Plant Patent Act of 1930 which provides PVP-style protection. The U.S. has allowed Utility Patents on biological inventions since 1980 including plant varieties per se since 1985; reinforced by the US Supreme Court (Baird 2002). Eligibility requirements for patentability of a variety include showing of DUS plus demonstration of utility, novelty, nonobviousness or inventive step, and a written description. A seed deposit completes the requirement of written description for patents on varieties per se. Additional forms of IP include trade secrets and contract law e.g., via use of bag-tag notices.

The policy goal of intellectual property protection is to increase social welfare by encouraging the development of new and useful products that otherwise would not have been created (Lence et al. 2005). The means by which PVP and patents seek to achieve net positive social welfare is to provide time-limited protection for the initial developer. PVP adds an exception allowing further breeding and commercialization in countries where the variety PVP applies. Europe does not allow utility patents on varieties per se but can allow patents on genes and associated markers, although there is intense discussion on the latter subject and the long-term situation in Europe remains unclear (EPO 2013). Whether trait

patents extend to the scope of the plant depends upon specific claims and on country or regional patent laws. The challenge, in respect of IPP and social welfare, is to provide an appropriate balance between time and level of protection in regard to quality of innovation and access to the variety or invention. Utility Patents have been criticized because, in contrast to PVP, they only allow licensed access during the period of protection. In contrast, following expiration of patent protection the previously protected object (including patented parental lines of hybrids) is placed into the public domain, whereas under UPOV such public access is not mandatory but is required by the USDA in the U.S. Consequently, European plant breeders are more reliant upon trade secrets than are US plant breeders to protect parental lines of hybrids; a practice that can have negative impact on social welfare. Furthermore, in contrast to Europe, the U.S. regularly makes available through the NPGS off-patent and off-PVP germplasm to the FAO ITPGRFA multilateral germplasm system.

IPP in respect of plant breeding exists within an environment that is continuously changing in terms of technical capabilities. For example, the effective time period of protection once afforded under PVP has been reduced as new breeding methods shorten cycle times by 2–5 years. Reduced cycle times are most dramatic for hybrids because appropriate heterotic groups for progeny can now be more readily identified. In contrast, breeding from many hybrids was precluded previously because optimum line combinations to allow expression of heterosis could not be so readily determined. Consequently, PVP now provides an even lower level of incentives to invest in more time consuming and risky breeding activities including to find, adapt, and incorporate new exotic genetic diversity. Countries that do not allow utility patents on varieties already limit the potential of commercially funded enterprises to access and utilize exotic germplasm. Meanwhile, the full potential breeders could apply to developing new adapted germplasm from exotic sources that could be supported by utility patents has possibly not yet been fully taken advantage of in the U.S. Social welfare goals of patents are increased when companies can agree to licensing terms. U.S. farmers have access to germplasm and traits developed by competing companies as a result of licensing. Transparency of patent information helps breeders license traits, or to breed traits out in order to access underlying germplasm.

IPP is often wrongly criticized as precluding commercial use of heirloom varieties when it is seed registration or certification laws that limit or preclude their use. Furthermore, it is often not IPP that acts as a barrier for access to varieties or traits but rather the needs to satisfy regulatory requirements associated with genetically modified traits. The types of IPP that countries utilize are very dependent upon their state of economic development and cultural heritage. UPOV periodically revises the PVP system to take account of new technological developments. Scope of patent claims is under constant scrutiny as the boundaries of scientific knowledge expand and so change definitions of innovation and nonobviousness. Policy measures relating to IPP are under discussion and debate within academia and by policy makers, legislatures, and courts. Ultimately each country will decide the types of IPP instruments to deploy according to its economic and cultural

needs and in the framework of global economic and trade agreements. What will be important is to monitor the degree of success contributed by different forms of IPP in achieving genetic gain and increased sustainable productivity in agriculture and to implement change as needed.

3.7 Conclusions

There is an ongoing change in plant breeding facilitated by technological advances in diverse fields such as information management, statistics, and mechanization fueled by increasing knowledge of the physiology and genetic basis of important agronomic traits. One goal remains fixed. That is to ever more effectively and efficiently find the best possible fit of G × E, including by crop management. Breeders understandably preferentially use advanced cultivars and recycle advanced breeding lines. Duvick (1984) referred to three sources of genetic reserves that are immediately available to breeders; (1) varieties on farms, (2) cultivars in advanced yield trials, and (3) cultivars in preliminary trials. Breeders are well aware that continued cycles of pedigree breeding in a closed system inevitably narrows diversity thereby reducing future potential for continued genetic gain. Looking beyond the genetic diversity present in a commercial pipeline of new varieties and their immediate ancestors, there is a staggeringly large potential of genetic diversity that remains potentially available for future use. For example, if one takes a conservative and admittedly overly simplistic view of genetic diversity and assumes there is an average diversity level among the entirety of maize races of 10 different allelic types at just 60 % (20,000) of the 32,000 protein coding genes found to date in the maize genome (Schnable et al. 2009), then it could be possible to create 10^{20000} unique genotypes. To place this number into context, approximately 2,000 two-row plots can be accommodated in 1 hectare so planting out this number of unique genotypes would require $10^{19996.7}$ ha, which is an area many orders of magnitude greater than the land area of the earth (14.8 billion hectares). Successful plant breeders must therefore balance short-term needs to deliver new varieties that meet or exceed the performance expectations while also generating and evaluating new diversity. This balance can be achieved by integrating "new" diversity from national or international breeding programs and from exotic germplasm adaptation and evaluation programs. Determining which germplasm to use from the vast array array of available diversity and developing best strategies to effectively explore and integrate that diversity into improved cultivars represent both critical challenges and opportunities for plant breeders.

The diversity of environments within and among farms and across broader eco-geographic areas provides a challenge to plant breeders; the requirement to have useful genetic diversity ready at hand or imminently accessible from more exotic sources. As a result of G × E and the need to achieve genetic gain, there is diversity in space and in time as new generations of varieties are developed to fit their individual areas of adaptation. The description of "massive

monocultures" of maize and soybean as described by Heinemann et al. (2013) is a misnomer because these varieties exhibit genetic diversity temporally and spatially. Nevertheless, we and others (Meul et al. 2005; van de Wouw et al. 2010) concur with the National Research Council (1993) that "the potential for crop vulnerability must be nationally and globally monitored."

The increasingly important and routine use of molecular marker data provides opportunities to facilitate the more effective use of genetic diversity. First, to identify genomic regions where there is a trend toward reduced diversity. Second, to identify important genomic regions for targeted selection. Third, to help identify new and potentially useful sources of genetic diversity. Stronger public funding is required to support international germplasm conservation, evaluation, and prebreeding programs. Strong public support is also needed to support breeding programs especially for regions and crops that do not fit the business model of commercially funded breeding programs.

There is arguably no higher priority or conceivably better form of social welfare or public good than to provide excellent stewardship and optimum use of genetic resources in the service of agriculture. The ample supply and availability of high quality food provides the basis for health, economic welfare, prospects for increasing global environmental sustainability, and a civilized society (Bronowski 1973). An initial application of molecular marker data was to better comprehend the domestication of cultivated varieties; a process that occurred some 8–10,000 years ago. Radically improved versions of these technologies now offer prospects to help more effectively conserve and sustainably utilize genetic diversity that resides, not only in domesticated species, but also in their wild progenitor species; genetic diversity that our ancestors did not source during the dawn of agriculture. Huge challenges are being placed upon agriculture to improve global productivity in a sustainable manner. There will be increased expectations that the more effective use of plant genetic resources can help meet these goals. There is a long-term public good in better conserving and making more accessible a broader base of wild and domesticated genetic resource diversity (McCouch et al. 2012). In terms of finance, the need is minor compared to other public expenditures. For the continued well-being of society, the need is ineluctable (Serageldin 2002; Ehrlich and Ehrlich 2013). Modern plant breeding will have an even more important role to play in the future.

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Chapter 4
Genetic Erosion Under Modern Plant Breeding: Case Studies in Canadian Crop Gene Pools

Yong-Bi Fu and Yi-Bo Dong

Abstract There is long-standing concern that modern plant breeding reduces crop genetic diversity. Such reduction may have consequences both for the vulnerability of crops to biotic and abiotic stress. To understand the impact of plant breeding on diversity, we conducted a series of genetic diversity analyses from 1999 to 2009 on existing Canadian gene pools of flax, oat, wheat, soybean, potato and canola. Here we summarize these analyses, highlight major findings, and discuss related issues. These gene pools displayed variable patterns and degrees of genetic diversity decline over the past 100 years of Canadian breeding efforts. Significant allelic loss and genetic shift were found in the wheat and oat gene pools. Such diversity declines underline the need for continuous efforts in conservation of improved crop germplasm and in the diversification of plant breeding materials for sustainable breeding programs.

Keywords Plant breeding · Canadian crop gene pool · Genetic diversity · Genetic erosion · Allelic change · Genetic shift · Genetic marker

4.1 Introduction

Concern has been frequently expressed that modern plant breeding reduces crop genetic diversity (National Research Council 1972; Duvick 1984; Vellve 1993; Clunier-Ross 1995; Tripp 1996; Tanksley and McCouch 1997), as modern crops have become phenotypically more uniform and genetically less heterogeneous (Duvick 1984; Brush 1999). The genetic vulnerability of crop uniformity has been well documented with the abundant history of epidemics such as the Irish potato

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blight in the 1840s and the U.S.A. corn blight in the 1970s (National Science Council 1972). The threat of the extremely virulent new race of stem rust Ug99 from East Africa to genetically uniform wheat is a current concern (Borlaug 2007). Theoretically, selective breeding within a narrow range of plant germplasm could eliminate rare alleles, change allele frequencies, reduce genetic diversity, and increase linkage disequilibrium (Allard 1999; Hedrick 2000).

Efforts have been made to assess genetic diversity changes in major agricultural crop species (Duvick 1984; Swanson 1996; Tripp 1996; Donini et al. 2000; Fu and Somers 2009). Early assessments were largely based on phenotypic (Rodgers et al. 1983) and pedigree data (e.g., Cox et al. 1985) and have their limitations either due to environmental influence or biased inference of parental contribution. Over the last two decades, more informative assessments have been made using molecular markers such as random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), and simple sequence repeats (SSRs) (Fu 2006). However, no consensus has been reached as to the overall impact of modern plant breeding on crop genetic diversity (Fu 2006; Rauf et al. 2010; van de Wouw et al. 2010).

From 1999 to 2009, we conducted a series of genetic diversity analyses of existing Canadian gene pools of flax, oat, wheat, soybean, potato, and canola, using different molecular markers, as part of the effort to monitor genetic erosion in crop gene pools and to understand the impacts of plant breeding on crop genetic diversity (Fu 2005; Fu and Gugel 2010). In this chapter, we summarize these diversity analyses, highlight the major findings, and discuss some key issues associated with these analyses. It is our hope that this summary helps to paint a clear picture of the genetic diversity changes in the Canadian crop gene pools established over the last 100 years of plant breeding.

4.2 Plant Breeding in Canada

Canada is a comparatively young country, but it is one of the few countries with a long-term, continuous, and complete modern plant breeding history (Campbell and Shebeski 1986). In 1874, the first agricultural college was established at Guelph, Ontario, to train professionals in agricultural science. In 1886, the act creating the system of federal experimental farms was passed and the first experimental farm was established at Ottawa. In 1888, William Saunders, the first director of the Central Experimental Farm, Ottawa, initiated a wheat breeding program and his son, Charles Saunders, released the famous cultivar Marquis in 1909. While plant breeding efforts were expanded in the federal department of agriculture, the provinces, universities, and private companies were slow to initiate cultivar development programs. After the Second World War, many veterans received training at agricultural colleges as professional breeders (Archibald 1949), and the breeding efforts accelerated with many cultivars released and more breeding programs were established across Canada. Rapeseed oil seed breeding was a good example of the

modern plant breeding expansion in Canada with success to improve oil quality (Stefansson and Downey 1995). The legislation of Plant Breeders' Rights legislation in 1990 further enhanced the breeding efforts in Canada (Slinkard and Knott 1995).

The breeding efforts for the six crops are briefly summarized in Table 4.1. Some variation exists among different crops in breeding history, method, goal, and output; however, the breeding methods were similar, particularly before 1940s. The four basic breeding methods of introduction, selection, hybridization, and backcrossing, were widely applied. Introduction and selection within landraces played an important role in the early breeding efforts. Hybridization is a procedure of deliberate intraspecific or interspecific breeding to generate variability and was widely applied to develop new cultivars. Recurrent selection with backcrossing, since the 1940s, was successful in genetic improvement of disease resistance. Since the 1990s, biotechnology and marker-assisted selection has become available and used to a limited extent in cultivar development.

The major breeding targets of all crops were adaptability, yield, early maturity, disease resistance, and quality, but they varied among the different crops and changed over time (Table 4.1). For example, spring wheat breeding before 1928 was aimed at early maturity, yield, and baking quality. With the rust epidemic in 1920–1930, the breeding targets were shifted toward developing cultivars with disease resistance. Since then, breeding efforts for disease resistance has intensified, particularly in oat (McKenzie and Harder 1995). While yield has always been the primary goal of any breeding program since the 1950s, end-use quality traits have also been considered. For example, one of the main goals of rapeseed breeding was to decrease erucic acid content and improve rapeseed oil/meal quality for human consumption (Downey 1964). In wheat, there are many classes of eastern and western Canada wheat, respectively, to address requirements of the end-users (http://www.grainscanada.gc.ca/wheat-ble/classes/classes-eng.htm).

4.3 Case Studies in Canadian Crop Gene Pools

We have performed 10 genetic diversity analyses using different sets of molecular markers to monitor six crop gene pools (Table 4.2). The basic procedure for these analyses was to select representative cultivars from a germplasm collection held at Plant Gene Resources of Canada, sample seeds randomly from each cultivar, plant them in a greenhouse, collect leaf tissue, extract DNA, perform PCR using selected primers, collect marker data, and analyze marker data for various diversity components. These analyses may have differed in cultivar sampling, marker application, and diversity estimation. All of the analyses were centered on addressing a key question of the crop gene pool: Does modern plant breeding in Canada reduce allelic diversity at individual loci, shift the genetic background, or narrow the genetic diversity?

Table 4.1 Brief summary of breeding efforts before 1996 in Canada for the six crops analyzed in this chapter

Crop	Brief history	Breeding method	Primary breeding focus		Released cultivars (years)
			Agronomy/quality	Resistance	
Wheat	Wheat production as early as 1605; public breeding in 1886	Introduction; selection; hybridization; double-haploid; marker-assisted selection	Yield; early maturing; milling quality; protein	Rust; bunt; loose smut; *Fusarium*; stem sawfly; drought	89 (1845–1994)
Oat	Oat production as early as 1605; public breeding in the late 1800s	Introduction; selection; hybridization; backcrossing	Yield; early maturing; protein; milling quality	Smut; rust; BYDV	43 (1886–1992)
Flax	Flax cultivation as early as 1617; public breeding in early 1900s	Introduction; selection; hybridization; double-haploid; biotechnology	Yield; oil quality	*Fusarium* wilt; rust	34 (1910–1995)
Potato	Potato production in 1888; public breeding in 1934	Introduction; selection; hybridization; clonal propagation	Yield; disease-free stock; processing quality	Late blight; mosaic; common scab; leaf roll	59 (1888–1982)
Soybean	Public breeding in 1893	Introduction; selection; pedigree; backcrossing; modified single seed descent	Yield; early maturing; natto-type; tofu type; yellow hilum; protein	Root rot	191 (1923–1992)
Canola	Rapeseed production in 1939; public breeding in 1944	Recurrent selection; selfing, synthetic and half-seed techniques; male sterility	Yield; early maturing; oil quality	White rust; blackleg; triazine herbicide	15 (1954–1988)

Note The information in this table was largely obtained from Anstey (1986), Marshall (1989), and Slinkard and Knott (1995)

Table 4.2 List of 10 assessments from 1999 to 2009 on genetic diversity changes in six Canadian crop gene pools

Analysis	Crop[a]	Marker	Diversity change[b]	Allelic reduction	Shift[c]
Fu et al. 2005	Wheat, 75, 1845–2004	31 SSRs	R by AMOVA*, but M by similarity*	After 1970s*	GS
Fu et al. 2006	Wheat, 75, 1845–2004	37 EST–SSRs	R by AMOVA*	After 1990s*	GS
Fu et al. 2009c	Wheat, 75, 1845–2004	370 SSRs	R by AMOVA*	After 1930s*	GS
Fu et al. 2003a	Oat, 96, 1886–2001	30 SSRs	M by similarity*	After 1970s*	GS
Fu et al. 2004	Oat, 96, 1886–2001	442 AFLPs	M by PFL*	–	–
Fu et al. 2003b	Flax, 28, 1910–1998	84 RAPDs	M by PFL*	–	–
Fu et al. 2009a	Canola, 10 (*B. rapa*), 1940–2001	18 SSRs	R by similarity*	After 1960s	–
Fu et al. 2010	Canola, 10 (*B. napus*), 1943–1992	22 SSRs	R by similarity*	After 1960s	–
Fu et al. 2007	Soybean, 45, 1934–2001	37 SSRs	I by AMOVA*	–	NGS
Fu et al. 2009b	Potato, 68, 1919–2002	36 SSRs	M by AMOVA*	–	NGS

[a]The number of assayed cultivars is shown after the crop, followed by the period of cultivar release
[b]*R* reduction, *M* maintained, *I* increased, *AMOVA* analysis of molecular variance, and *PFL* proportion of fixed loci
[c]*GS* genetic shift and *NGS* no genetic shift
*Result with a statistical test of significance. – Result not available

4.3.1 Wheat

Wheat is the most important cultivated crop in Canada. Wheat breeding began in 1886, has so far released hundreds of cultivars, and has generated a significant impact on Canadian agriculture (DePauw et al. 1995). Briefly, breeding goals in wheat have changed from adaptation and quality before 1940, resistance to biotic and abiotic stresses such as rust from 1940 to 1990, to end-use quality such as increased grain protein after 1990. The accompanying breeding methods ranged from introduction, mass selection, hybridization, backcrossing, and double-haploidy to marker-assisted selection.

We selected 75 Canadian hard red spring wheat cultivars released from 1845 to 2004 and performed three diversity analyses using (1) 31 genomic SSR markers

(Fu et al. 2005), (2) 37 EST-derived SSR markers (Fu et al. 2006), and (3) 370 genomic SSR markers (Fu and Somers 2009). The assayed cultivars represented early introductions and those cultivars released over the more than 100 years from several main wheat breeding programs in Canada. To facilitate the diversity analyses, these cultivars were grouped based on their periods of release (prior 1910, 1911–1929, 1930–1949, 1950–1969, 1970–1989, and 1990–2004).

The diversity analyses revealed several patterns of genetic changes over time. First, significant allelic reduction started as early as the 1930s (Fig. 4.1). Considering 2010 SSR alleles detected in 20 of the earliest released cultivars, 38 % of them were retained, 18 % are new, and 44 % were lost in the 20 most recent cultivars. The net reduction of the total SSR variation in the 20 recent cultivars was 17 % (Fu and Somers 2009). Interestingly, allelic reduction occurred in every part of the wheat genome and a majority of the reduced alleles resided in only a few early cultivars. Second, a significant genetic shift was also observed in the gene pool in response to the long-term breeding pressure (Fig. 4.2a). Third, these allelic changes in the gene pool were associated with long-term wheat trait improvements (Fu and Somers 2011). Comparing results among three analyses revealed essentially the same patterns of genetic changes in the gene pool; however, the EST-derived SSR markers displayed smaller allelic changes than the genomic SSR markers (Fu et al. 2005, 2006) and the genome-wide sampling increased the resolution for assessing allelic changes over time (Fu and Somers 2009).

These patterns of genetic changes in the wheat gene pool are not surprising, as the breeding was largely limited to a narrow range of wheat germplasm with only three major introgressions as reflected in three ancestral families (Marquis in 1908; introduced Thatcher introduced in 1935; and Neepawa in 1969) and the selection was intensified over time to meet market class requirements of bread wheat (McCallum and DePauw 2008).

4.3.2 Oat

Oat breeding in Canada began in the late 1800s to meet the demand of the growing Canadian livestock industry (McKenzie and Harder 1995). Selection and hybridization from the 1900s to the 1930s generated several highly productive cultivars such as ‘Liberty’ and ‘Legacy.’ Backcrossing of rust resistant genes into ‘Rodney’ and ‘Pendek’ in the 1960s greatly improved rust resistance in cultivars such as ‘Harmon,’ ‘Dumont,’ and ‘Robert.’ Introduction of germplasm from other oat species than *Avenasativa* in the 1970s further enhanced the development of many cultivars with genes for resistance to both stem rust and crown rust. Overall, the breeding efforts have developed over 43 registered cultivars, many of which have made significant impacts on the economy of western Canada (McKinnon 1998).

We selected 96 Canadian oat cultivars released from 1886 to 2001 and performed two diversity analyses using 30 SSR (Fu et al. 2003a) and 442 AFLP markers (Fu et al. 2004), respectively. These cultivars were selected based on

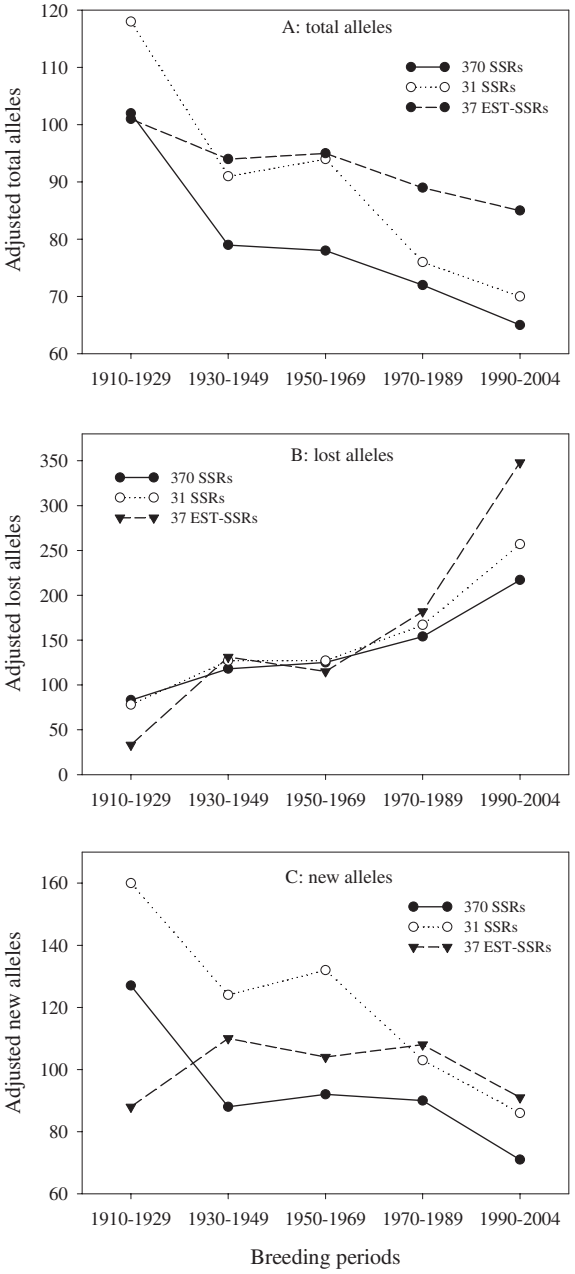

Fig. 4.1 Allelic changes in 75 Canadian hard red spring wheat cultivars released over the six breeding periods, as revealed by three genetic diversity analyses (see Fu et al. 2005, 2006; Fu and Somers 2009). For ease of diversity comparison among cultivar groups of variable size, the adjusted alleles are presented. They are calculated as the observed allele count adjusted by 100 over the expected allele count under a random scenario with a given group size, in which the expected allele count was obtained from 10,000 random permutations

pedigree, agronomic and economic importance, and representation of different eras of oat breeding in Canada. The cultivars were grouped based on their release periods (pre-1930, 1930s, 1940s, 1950s, 1960s, 1970s, 1980s, and 1990s) for diversity analyses.

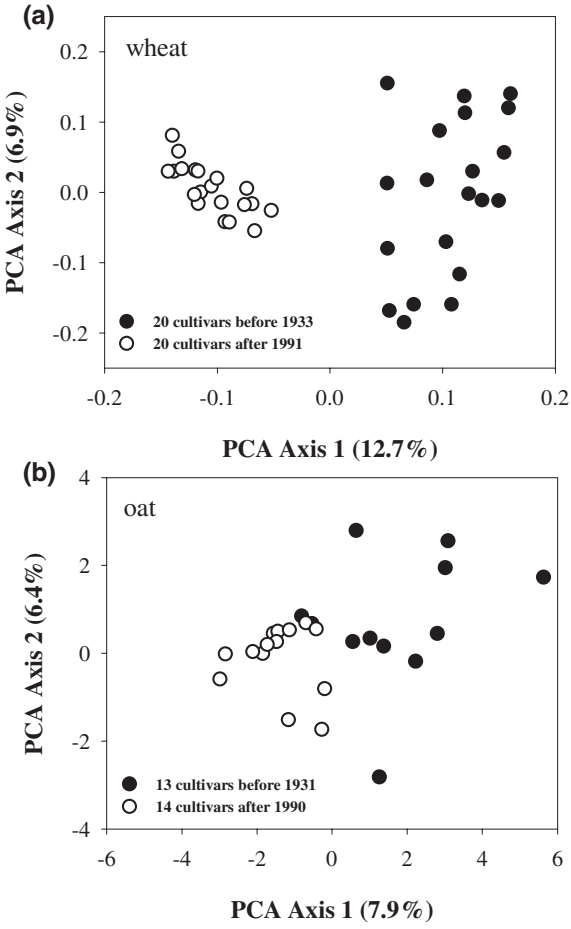

Fig. 4.2 Genetic shift detected from early to recent cultivars in the Canadian improved gene pools of wheat and oat, as illustrated in the principal component plots of respective SSR data (adapted from Fu et al. 2003a; Fu and Somers 2009)

The diversity analyses revealed three major findings. First, there were four marked patterns of allelic change detected at single loci over the past century of the Canadian oat breeding: Decreasing, Shifting, Increasing, and Random (Fig. 4.3). More loci with decreasing allelic pattern supported the reduction tendency of genetic diversity in the Canadian oat gene pool over years (Fu et al 2003a). Agenetic shift occurred in the oat gene pool (Fig. 4.2b), as evidenced with some loci (Fig. 4.3d). Second, a significant allelic reduction was found for the cultivars released after 1970 (Table 4.2; Fu et al 2003a). Third, the SSR analysis (Fu et al. 2003a) was more informative than the AFLP analysis in the assessment of diversity changes, and the allele-based diversity measure is more informative than those similarity-based diversity estimates (Fu et al. 2004).

The oat SSR analysis in 2003 revealed the first, clear-cut molecular evidence for the negative impacts of modern oat breeding on the oat genetic diversity. Genetic changes occurred at individual loci and allelic loss started after 1970. The evidence, along with those in the Canadian wheat gene pool (Fu and Somers 2009), supports the argument that modern plant breeding has reduced crop genetic diversity.

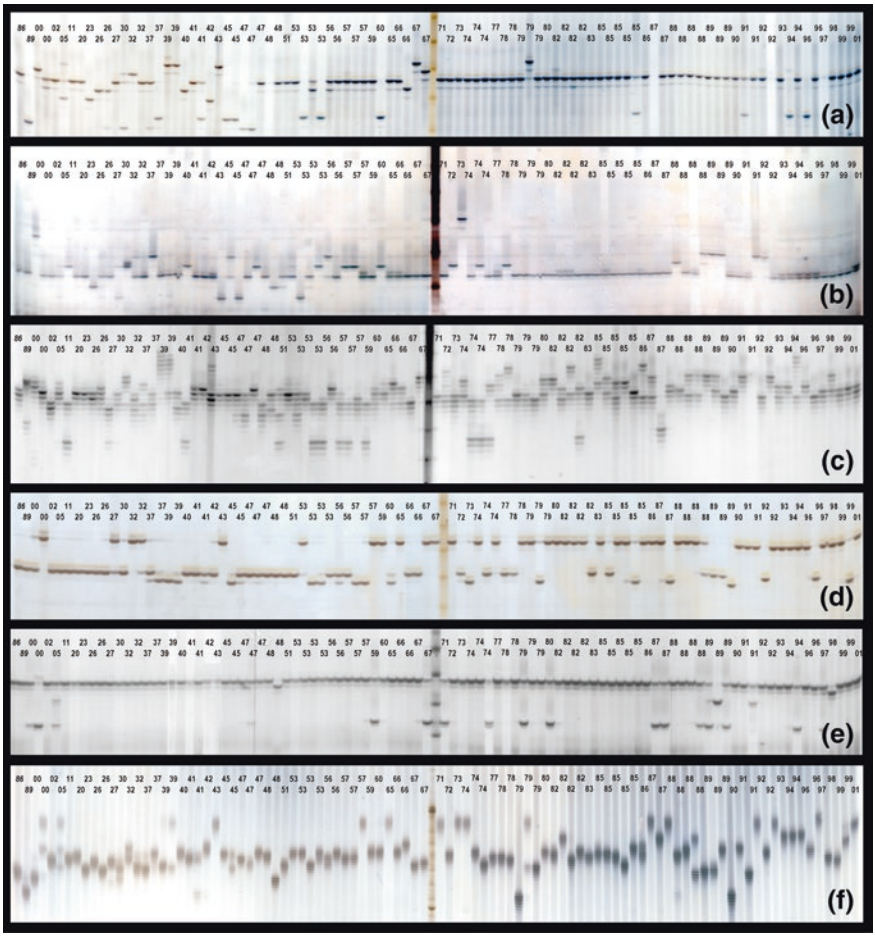

Fig. 4.3 Seven silver staining gels that illustrate four patterns of allelic change over the past 115 years of Canadian oat breeding. **a**–**c** Decreasing (detected by SSR primers AM31, AM38, AM1, respectively); **d** Shifting (by AM42); **e** Increasing (by AM102); and **f** Random (by AM3). In each gel, samples for 96 Canadian oat cultivars are arrayed from left to right in a chronological order, from 1886 to 2001; only the last two digits of the release year are given for each cultivar. The DNA ladder is shown between two cultivars released in 1967 and 1971 (adapted from Fu et al. 2003a)

4.3.3 Flax

Canadian flax breeding started in the early 1900s at the Central Experimental Farm, Ottawa, with release of three cultivars in 1910. The breeding effort was expanded into western Canada in the 1920s to develop cultivars with increased seed yield and improved oil quality. Introduction, selection and hybridization were among the major breeding methods applied to develop cultivars, although biotechnology was applied in the late 1990s to develop genetically modified flax.

The long-term breeding efforts toward increasing seed yield, improving oil quality, selecting for resistance to rust and wilt, and decreasing days to maturity, have produced more than 30 registered cultivars (Kenaschuk and Rowland 1995).

A RAPD analysis of 28 Canadian flax cultivars released from 1910 to 1998 was performed in 2002 (Fu et al. 2003b). The assayed cultivars represented the effort in three breeding periods: Period 1 for the cultivars released before 1932; Period 2 from 1932 to 1980; and Period 3 after 1980. However, these cultivars displayed only a trend of genetic erosion over the 90 years, measured by the proportion of fixed recessive RAPD loci. Specifically, the intensive selection for rust resistance after 1947 increased the average proportion of fixed recessive RAPD loci in the cultivars released later. In addition, the average RAPD similarity in the flax gene pool was continuously decreasing over the past century (Fu et al. 2003b). These findings suggest that genetic narrowing occurred in the Canadian flax gene pool.

4.3.4 Potato

Potato breeding in Canada began in 1888 with the goal to improve potato yield of early introductions (Turner and Molyneaux 2004). Since then, the breeding efforts have gone through several major stages from early selection and adaptation, selection for disease resistance, obtaining disease-free stock, to selection for processing quality (Anstey 1986). Substantial improvements have been made in traits associated with production, utilization, disease control, in vitro culture, and processing quality (Tarn et al. 1992). Hundreds of potato cultivars have been developed, many of which have had significant impacts on Canadian agriculture (Anstey 1986; Turner and Molyneaux 2004).

We assayed 114 Canadian and 55 exotic potato accessions released from 1910 to 2002 using 36 SSR primer pairs (Fu et al. 2009). It was found that the proportion of total SSR variation occurring between Canadian and exotic germplasm was 0.6 %; among the Canadian cultivars of four major breeding periods 2.7 %; among heirloom varieties, modern cultivars and elite breeding lines 4 %; and between tetraploid and diploid lines 3.7 %. Slightly more diversity was found for exotic, than the Canadian, germplasm. The modern cultivars displayed slightly more diversity than the heirloom varieties and the early cultivars revealed slightly more variation than the recent ones. The analysis with only the Canadian cultivars and breeding lines revealed only a trend of genetic erosion over the whole modern breeding history (Fu et al. 2009). These findings demonstrate the narrow genetic base of the Canadian potato germplasm. Interestingly, such genetic narrowing persists in spite of the use in breeding of Andean cultivated potatoes of groups Phureja, Andigena, and Tuberosum and as many as 14 wild species (Spooner and Salas 2006; Hirsch et al. 2013).

4.3.5 Soybean

Soybean breeding in Canada began in 1893 with the goal to improve forage yield of early introductions (Beversdorf et al. 1995). Since then, the breeding efforts have gone through several major stages from early selection and adaptation, to expansion and to commercialization. Substantial improvements have been made in disease control, maturity, seed yield, and quality traits (Morrison et al. 2000). So far, hundreds of soybean cultivars have been developed, many of which have had significant impacts on the economy of eastern Canada (Beversdorf et al. 1995).

We selected 45 Canadian soybean cultivars released from 1934 to 2001 and 37 exotic germplasm accessions as the comparison for a diversity analysis using 37 SSR markers (Fu et al. 2007). It was found that the proportion of total SSR variation residing between exotic and Canadian germplasm was 9 %; among the Canadian cultivars of three breeding periods 10 %; and between the cultivars of maturity groups 0 and 00 4 %. Greater allelic diversity was found for exotic, than the Canadian, germplasm. More diversity was observed in the cultivars of the recent, than the early, breeding period. More specifically, the cultivars released after 1990 had slightly more diversity than those prior to 1970. These results indicate that the Canadian soybean gene pool, although genetically narrow, maintained a broad degree of genetic diversity.

4.3.6 Canola

Canola, a low erucic acid, low glucosinolate form of rapeseed (Stefansson and Downey 1995), is a great achievement of modern plant breeding in Canada (Busch et al. 1994). Small-scale *Brassica napus* breeding efforts started in 1944, but it was expanded significantly in the 1960s to include other *Brassica* species (Stefansson and Downey 1995). The first Canadian licensed *B. napus* and *B. rapa* cultivars were Golden in 1954 and Echo in 1964, respectively. The public breeding programs released more than 15 cultivars before 1996. Since the 1990s, the canola breeding in private sector has accelerated and produced more than 100 herbicide-tolerant cultivars.

The rapeseed oilseed crop in western Canada has undergone extensive genetic modifications on oil quality traits through conventional breeding methods (Stefansson and Downey 1995); however, the developed rapeseed germplasm may have reduced genetic diversity. We selected 10 elite *B. napus* summer rape and 10 elite summer *B. rapa* cultivars from 1940 to 2001 for diversity analyses with 18–22 SSR markers, and found that the overall genetic diversity was largely unchanged over the years of cultivar release (Fu and Gugel 2009, 2010). However, significant decreases in the number of SSR alleles and average dissimilarities were found over the 60 years of breeding outcrossing *B. rapa*. Similarly, a trend of decline in SSR variation was also observed over the years of breeding

self-compatible *B. napus*. These findings are aligned with the argument that the extensive selection in conventional canola breeding for oil quality traits could narrow the crop genetic diversity.

4.4 Limitation and Implication

Our genetic diversity analyses have revealed variable patterns and degrees of diversity decline in the six Canadian crop gene pools. How general are these findings with respect to other plant breeding programs? What can be learned from these diversity analyses? To answer these questions, we will discuss the related issues with our diversity analyses and their implications for future efforts.

4.4.1 Issues

Our diversity analyses have several major issues that may affect the interpretations of our research findings. First, these analyses applied different marker systems that would affect the comparison of genetic changes within and among different gene pools. For example, the SSR analysis of the oat gene pool showed a better resolution of genetic changes than the AFLP analysis (Fu et al. 2003a, 2004). The genomic SSR markers (Fu et al. 2005) were more sensitive for wheat genetic change assessment than the EST-derived SSR markers (Fu et al. 2006). Second, insufficient genome coverage with application of a limited number of markers may have diluted the diversity analyses. For example, the analysis of 370 wheat SSR markers that were widely distributed over all 21 wheat chromosomes (Fu and Somers 2009) was more informative in detecting the allelic changes than those with 31 genomic SSR (Fu et al. 2005) or 37 EST-SSR markers (Fu et al. 2006). Third, not all of the diversity measures used were equally informative, and the measure of allelic change was more informative than the similarity-based diversity measure. For example, a significant decrease in SSR alleles was found over the 60 years of breeding outcrossing *B. rapa*, but the overall genetic diversity was largely maintained in these assayed cultivars (Fu and Gugel 2009). Fourth, bias existed in cultivar representation and grouping for breeding periods, and unbalanced group sizes may have also introduced bias into the assessment of diversity changes.

Considering these issues, we would argue for the need to establish a standard procedure for a crop genetic diversity analysis of this nature to make the diversity comparison among crop gene pools more feasible. Ideally, allelic diversity should be measured and compared (Caballero and Rodríguez-Ramilo 2010), and a genome-wide sampling should be done with informative genetic markers such as SSR or single nucleotide polymorphism (Jiao et al. 2012; Hirsch et al. 2013; Fu et al. 2014). To make a crop genetic diversity analysis more informative,

we may need to consider all the related issues discussed in detail by Fu (2006), minimize possible biases at every aspect, and focus on specific long-term breeding programs. With the advances in next generation sequencing, it is more feasible nowadays to perform a genome-wide diversity analysis of a crop gene pool through genotyping by sequencing (Fu and Peterson 2011; Poland and Rife 2012; Fu et al. 2014) for a better understanding of the diversity impacts of modern plant breeding.

4.4.2 Implications

Given that these limitations may have accounted for much variation in revealed patterns of diversity change, it is difficult to generalize these diversity patterns to other breeding programs in Canada, and even more challenging to do this for programs in other countries. However, several lines of reasoning for it are worth mentioning here. First, the six assayed crops represent cereals, oilseeds, and specialty crops, and the established gene pools should generally reflect the genetic consequences of the long-term breeding efforts in Canada. Second, the assayed gene pools largely represent self-fertilizing crops. The Canadian breeding efforts for these crops applied essentially the same breeding methods to reach similar goals, particularly before the 1990s. Thus, they should have similar breeding history and compatible genetic consequences, as revealed with the wheat and oat crops. Third, the breeding efforts and selection intensities varied among different crops over various breeding periods. Such variation may help to explain in part the variable patterns of diversity changes revealed from these analyses. Fourth, some of these diversity patterns may be found in the crop gene pools established in other developed countries, as the cereal breeding in Canada may not differ much in breeding method and goal from those in other countries such as the USA oat breeding program (Holland 1997).

The revealed patterns of genetic erosion are consistent with our common knowledge about the diversity reduction from modern plant breeding. These findings have significant practical implications for both plant germplasm conservation and genetic improvement. The need is obvious for continuous efforts to conserve released cultivars and germplasm from public breeding, especially older cultivars. Public gene banks like Plant Gene Resources of Canada have and will continue to play an important role in acquisition and conservation of elite germplasm released over the years. Substantial genetic variation still exists within some gene pools such as wheat and oat, even soybean, and selections within these gene pools are still possible, although it may be limited in the gene pools of canola and potato. Thus, it is important to widen the genetic bases of these breeding gene pools for sustainable agriculture. Fortunately, Canadian breeders have made some efforts to build more robust genetic bases, as an example, in the potato and soybean breeding programs (Fu et al. 2007, 2007).

4.5 Conclusions

Our genetic diversity analyses, although with some limitations, have revealed a new, clear picture that the Canadian crop gene pools displayed variable patterns and degrees of genetic diversity decline over the past 100 years of breeding effort. Substantial allelic reduction at individual loci was observed and genetic shift usually accompanying the reduction of genetic diversity was detected, particularly in the gene pools of wheat and oat. These patterns of genetic erosion support the argument that modern plant breeding reduces crop genetic diversity. The revealed erosions have significant practical implications for both plant germplasm conservation and genetic improvement. There is a need for ongoing efforts to conserve released cultivars and germplasm from public breeding programs, especially older cultivars, so that the purged alleles are protected. With such protected resources, plant breeders have the means required to widen the genetic base of the crop breeding gene pool for sustainable agriculture.

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Chapter 5
Molecular Marker Based Assessment of Genetic Diversity in Rye

Hanna Bolibok-Brągoszewska and Monika Rakoczy-Trojanowska

Abstract Genetic variation is a fundamental resource in crop improvement programs and thus a detailed knowledge of genetic relationships among accessions is a prerequisite for successful exploitation of genetic resources in breeding and for efficient genebank management. DNA markers are a convenient and powerful tool for assessment of genetic diversity. Over the years, several molecular studies were undertaken to characterize genetic relationships in various *Secale* accessions: wild species, landraces, varieties and inbred lines. Valuable, and sometimes surprising information on the extent and the structure of genetic variation was obtained, which can be crucial for the preservation of the genetic diversity of rye germplasm and its efficient use in rye improvement. DNA marker based studies of rye germplasm revealed, among others, the influence of life cycle differences on genetic relationships among *Secale* species, a great genetic potential of landraces for detection of unexplored alleles for broadening the genetic diversity in current breeding programs, and, very importantly, a narrow genetic diversity of advanced varieties, indicating their common genetic background. Possible influence of reproduction methods on the observed diversity patterns, as well as locations of genome regions targeted by selection during domestication and current breeding programs was also found. Nevertheless, the rye accessions characterized to date with molecular markers constitute a very small fraction of the worldwide genetic resources and further research is needed, involving, among others, the creation of a rye core collection. Efforts should be also made to facilitate the use of unimproved accessions in targeted broadening of the genetic diversity in breeding germplasm.

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5.1 Introduction

5.1.1 Molecular Markers in Analyses of Genetic Diversity

Genetic variation within a species is a fundamental resource in crop improvement programs and thus a detailed characterization of genetic diversity and understanding of the genetic relationships among accessions are prerequisites for successful exploitation of genetic variation contained in germplasm collections in breeding and for efficient genebank management.

Conventionally, morphological and agronomic traits, or biochemical tests have been used to assess intra species genetic variation. DNA markers provide convenient and powerful alternative for these methods, since they are not subject to environmental effects and are independent of the developmental stage of the plant.

Numerous methods of detecting DNA polymorphism were established over the years, such as: Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Inter Simple Sequence Repeats (ISSR), Random Amplified Polymorphic DNA (RAPD), Selective Amplification of Microsatellite Polymorphic Loci (SAMPL), Sequence Specific Amplification Polymorphism (SSAP), Simple Sequence Repeats (SSR), Single Nucleotide Polymorphism (SNP), Diversity Arrays Technology (DArT) (Karp et al. 1996; Rakoczy-Trojanowska and Bolibok 2004; Gupta et al. 2008).

Whereas at the advent of molecular fingerprinting the labor-intensive and time-consuming procedures allowed for sampling of only relatively limited numbers of accessions and loci, the recent advances in high throughput genotyping technologies, such as fluorescence-based SSR detection on automated sequencers and highly parallel SNP genotyping assays, along with the establishment of high throughput DNA isolation protocols (Bashalkhanov and Rajora 2008) enabled extensive characterizations of whole germplasm collections (Upadhyaya et al. 2008; Lv et al. 2012; Emanuelli et al. 2013). In consequence molecular markers became an indispensable tool of assessing genetic diversity that supplements morphological evaluations.

Over the years several molecular studies using various markers and involving also analyses of chloroplast and mitochondrial genomes were undertaken to asses genetic diversity and relationships in various *Secale* accessions: wild species, landraces, varieties, and inbred lines (Myśków et al. 2001; Persson et al. 2001; Persson and von Bothmer 2002; Ma et al. 2004; Chikmawati et al. 2005; Bolibok et al. 2005; Shang et al. 2006; Isik et al. 2007; Chikmawati et al. 2012; Bolibok-Brągoszewska et al. 2012, 2014).

5.1.2 Rye—General Characteristics and Genetic Resources

Presently four species are recognized in the genus *Secale*: *S. sylvestre* Host, *S. vavilovii* Grossh—both annual and self-pollinating, *S. strictum* (C. Presl.) C. Presl.—perennial and open-pollinating and *S. cereale*—annual and open-pollinating. There are eight subspecies in *S. cereale* and five in *S. strictum* with *Secale cereale* ssp. *cereale* L. being the only cultivated rye (Shang et al. 2006).

Cultivated rye ($2n = 14$, RR) is a temperate zone cereal with low water and soil fertility requirements and excellent tolerance against many abiotic and biotic stresses. Consequently, rye is relatively high yielding under environmental conditions in which other crops perform poorly, even with low chemical inputs, such as fertilizers and pesticides, enabling ecologically and economically sound cultivation. It is an important crop in several Eastern, Central, and Northern European countries. Worldwide approximately 16.6 Million tones of rye were produced in 2013, and the biggest share of the production was realized in Germany (ca. 27 %) [http://faostat3.fao.org]. The primary uses of rye include bread making, alcohol production, and animal feed.

Rye is also a source of variability for triticale (x *Triticosecale* Wittmack) and wheat (*Triticum* ssp.) breeding, since it is a donor of the R genome of the triticale and the 1BL.1RS or 1AL.1RS translocation is present in hundreds of wheat cultivars (Lukaszewski 1990).

It appears that the center of origin of cultivated rye is located in the Mount Ararat and Lake Van area of eastern Turkey. Two, possibly parallel, migration routes of the species into Europe are proposed: the first route via Russia to Poland and Germany, from where rye was subsequently distributed throughout most of Europe, and the second route via Turkey and across the Balkan Peninsula. From Europe rye was introduced into America, with the first European settlers. Chinese and subsequently Japanese ryes originated from Turkey (Ma et al. 2004; Isik et al. 2007).

Traditional rye varieties are open-pollinated varieties (OPVs), characterized by high levels of heterozygosity and heterogeneity (Chebotar et al. 2003). One of the more successful varieties in rye breeding history was Petkus and many OPVs worldwide include Petkus in their ancestry or are selections from Petkus (Persson and von Bothmer 2002; Fischer et al. 2010).

Over 20 years ago hybrid varieties were introduced. Based on diallel variety crosses (Hepting 1978), Petkus and Carsten gene pools were chosen for the development of seed- and pollinator lines, respectively (Geiger and Miedaner 2009). Rye F_1 varieties quickly gained popularity due to considerable increase in grain yield.

Presently, the "Common Catalogue of Varieties of Agricultural Plant Species" (European Commission 2013) lists 157 varieties of rye, among them: 122 OPVs, 20 hybrid varieties, and 12 conservation varieties. Worldwide there are approximately 90 rye germplasm collections, maintaining in total ca. 21,000 accessions. The largest rye genebanks include: N.I. Vavilov Institute of Plant Industry

(Russian Federation), Polish Academy of Sciences Botanical Garden (PAS BG), (Poland), Plant Breeding and Acclimatization Institute, Radzików (Poland), Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben (Germany), National Small Grains Collection (USA), Plant Gene Resources of Canada, Saskatoon Research Centre (Canada) and Institute for Plant Genetic Resources «K. Malkov» (Bulgaria). Wild species, landraces, and advanced cultivars constitute, respectively, 6, 29, and 15 % of accessions maintained at rye genebanks (FAO 2010).

5.2 Genetic Relationships Among *Secale* Species Reflect Life Cycle Differences and *S. cereale* Is the Youngest Species of the Genus

Several studies deploying molecular markers were carried out to elucidate phylogenetic relationships between the species of the genus *Secale*.

In an AFLP markers based study 29 accessions representing the most commonly recognized rye species and subspecies were analyzed (Chikmawati et al. 2005). The AFLP markers, generated using 18 primer combinations, turned out to be a suitable tool for resolving phylogenetic relationships among *Secale* taxa, because they were able to differentiate all accessions studied and to separate them into tree distinct groups, with group I containing *S. sylvestre*, group II containing all perennial taxa, and group III containing all annual taxa. These results were thus consistent with the revision of the genus *Secale* by Frederiksen and Petersen (1998), who recognized only 3 species within it: *S. sylvestre*, *S. strictum* (syn. *S. montanum*), and *S. cereale*. The distinct separation of annual and perennial forms indicated that life cycle probably played an important role in determining the relationships among *Secale* species. The AFLP data demonstrated also clearly that *S. sylvestre* is the most ancient *Secale* species, very distinct from remaining accessions that split off first from the common ancestor, whereas *S. cereale* evolved most recently. Interesting results were obtained for the perennial *S. ciliatoglume*—it did not cluster together with the other perennial *Secale* accessions, but stood alone between annual and perennial taxa in cluster analyses. Nevertheless, the principal coordinate (PCO) analysis placed *S. ciliatoglume* in the same quadrant with the other perennial taxa (Chikmawati et al. 2005).

The next study of phylogenetic relationships in the genus *Secale* was done with the help of 24 SSR markers and involved 30 accessions representing different *Secale* taxa. The obtained results were in good agreement with the outcome of the AFLP-based study, as, again, the annual accessions were well separated from the perennial ones and the six S. *sylvestre* accessions analyzed were very divergent from the other taxa. The perennial *S. strictum* ssp. accessions were relatively diverse, whereas the *S. cereale* ssp. and *S. vavilovii* accessions displayed higher similarity and were grouped in one cluster. The study provided also information on

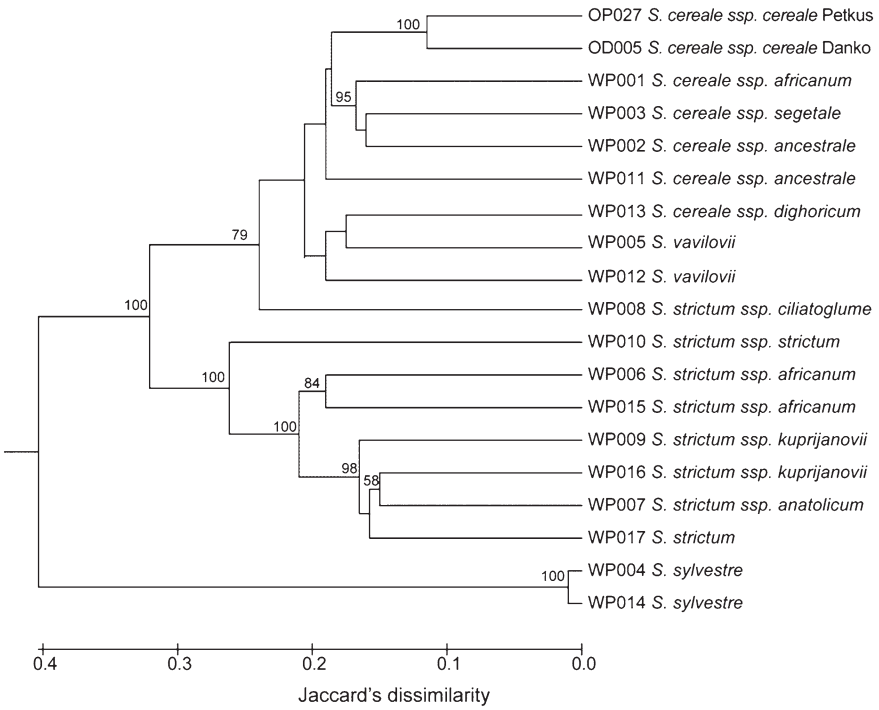

Fig. 5.1 Dendrogram demonstrating genetic relationships among wild rye accessions based on Jaccard's dissimilarity matrix. Bootstrap support values are shown if greater than 50 % (Bolibok-Brągoszewska et al. 2014)

the degree of genetic diversity of wild *Secale* accessions with respect to cultivated rye, because, in parallel 47 accessions of *S. cereale* spp. *cereale* were analyzed with the same set of SSR markers. Not surprisingly, the average Genetic Similarity (GS) index was lower for the 30 different *Secale* taxa (0.633) than for the 47 cultivated rye accessions (0.773), indicating that the genetic diversity in the *Secale* as a whole is more extensive than that in cultivated ryes (Shang et al. 2006).

A recently published genome–wide study on genetic diversity of rye, based on DArT markers, essentially confirmed previous findings concerning genetic relationships in the genus *Secale* (Bolibok-Brągoszewska et al. 2014), with the cluster analysis separating 19 accessions representing different *Secale* species and subspecies into three groups: one of them, very divergent from the rest, comprised *S. silvestre* accessions, the second comprised only *S. strictum* accessions (perennial), the third cluster consisted of *S. cereale* subspecies, *S. vavilovii* accessions and *S. strictum* ssp. *ciliatoglume* (Fig. 5.1). This study provided also information on genetic relations between wild *Secale* accessions and other rye germplasm types. Wild accessions turned out to be far more diverse then landraces and contemporary improved varieties, with the average GS values, respectively, 0.42, 0.60, and 0.72.

Furthermore, Bayesian clustering, PCO, and neighbor joining analyses performed on the combined data set of 379 rye accessions of different improvement status revealed that accessions of *S. sylvestre* and *S. strictum* were well separated and clearly divergent form the remaining accessions, whereas *S. vavilovii* and wild *S. cereale* accessions were relatively similar to landraces but distant from contemporary improved varieties.

Similar results regarding genetic relationships between wild *Secale cereale* taxa and cultivated ryes were obtained in an analysis of 114 *Secale cereale* ssp. accessions, mostly cultivars and landraces (Chikmawati et al. 2012). Wild *S. cereale* accessions were separated from cultivated accessions, as revealed by PCO and neighbor joining analysis, and again turned out to be more diverse—average genetic distance was 0.26 and 0.09 for wild and cultivated ryes, respectively (Chikmawati et al. 2012).

The consistency of the analyses of phylogenetic relationships of *Secale* taxa, obtained using different molecular markers and based on different accession sets indicate that they faithfully reflected the phylogenetic relationships between *Secale* species. On the other hand, the observed distinctness of wild ryes from improved varieties might imply that wild rye accessions have certain characteristics, not found in cultivated rye, which may be of advantage for rye and triticale improvement programs (Chikmawati et al. 2012).

5.3 Landraces—A Large but Poorly Characterized Resource for Broadening Genetic Diversity in Rye Breeding Programs

Landraces are geographically or ecologically distinctive populations, which have evolved under cultivation and become locally adapted to various environments. Landraces, contrary to modern varieties, have not been exposed to modern plant breeding procedures and are diverse in their genetic composition both within and between populations. They provide a broad representation of the natural variation that is present in a species and constitute the primary gene pool available to breeders for improvement of crop plants (Persson et al. 2001; Persson and von Bothmer 2002; McCouch 2004). It was demonstrated that landraces contain genomic segments that can enhance the performance of some of the world's most productive crop varieties (McCouch 2004).

Landraces constitute the largest germplasm subgroup maintained in rye genbanks, comprising ca. 6000 accessions—almost one third of all rye genebank accessions worldwide (FAO 2010), but have been not widely used in breeding programs, due to several reasons, such as heterozygosity, self-incompatibility, poor agricultural adaptation of the primitive accessions, and substantial difference in performance between elite and exotic germplasm for polygenic traits (Geiger and Miedaner 2009). Nevertheless, encouraging results were obtained in a study aimed

at improving baking quality, were a heterozygous Iranian primitive population was used as a donor (Falke et al. 2008).

Only few studies dealing with genetic diversity of rye landraces were published to date. Nine landraces, mostly from Central and Northern European, along with three advanced cultivars were characterized with RAPD markers (Persson et al. 2001). The study showed genetic differences between landraces and advanced cultivars, as demonstrated by their separation into different subclusters in the dendrogram. The advanced varieties clustered closely together and were similar to three of the landraces, while other landraces were separated from each other and from the other materials, which indicates not only the greater genetic diversity of the landraces, compared to advanced varieties but also complex genetic relationships within landraces. Similar results were observed in a larger, isoenzyme-based study involving 35 landraces and nine improved varieties from Northern Europe. Although some landraces were intermixed with improved varieties, which occupied a relatively small area in the resulting PCO plot, in general the landraces were much dispersed, with several of them being very distinct from the remaining accessions (Persson and von Bothmer 2002).

The DArT marker based study, including 153 landraces of various geographic origin among 379 accession analyzed in total, confirmed the fairly large diversity of rye landraces, with the average GS value obtained for this germplasm group being intermediate to those of wild accessions and improved varieties (Bolibok-Brągoszewska et al. 2014). The PCO analysis revealed that landraces occupied a considerable area of diversity space, and were clearly distant from contemporary improved varieties. However, some overlapping of landraces with improved varieties and cultivated materials from the collection of PAS BG was observed (Fig. 5.2). Nevertheless, in consistence with the results of the earlier studies, a

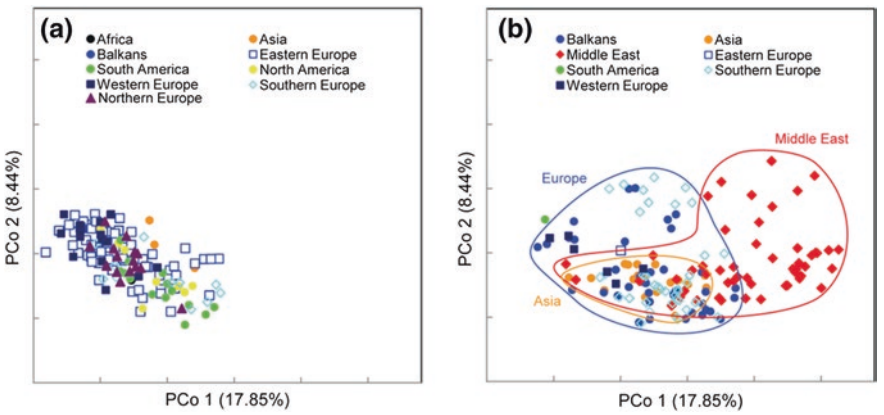

Fig. 5.2 Principal coordinate analysis of varieties, cultivated materials, and landraces from Polish Academy of Sciences Botanical Garden. Accessions were labeled according to the geographical origin. **a** Only cultivated materials and varieties are shown. **b** Only landraces are shown (Bolibok-Brągoszewska et al. 2014)

subset of landraces occupied a distinct diversity space, not overlapping with other accessions. Based on the PCO analysis landraces from South Europe, Balkans, and Middle East were the most diverse. Some correlation of clustering with geographic origin could also be observed—in the PCO plot a separation between European and Middle Eastern accessions and a cluster of Asian accessions could be seen, while in the neighbor joining tree Middle Eastern, Balkan, and Southern European accessions were placed in separate subclusters.

The vast disproportion between the number of landraces stored in germplasm collections and the number of landraces analyzed with molecular markers indicates the need to intensify efforts on characterization of genetic relationships between rye landraces and between landraces and advanced cultivars. The fact that the landraces distant from improved varieties were observed in all above mentioned studies demonstrates that there is a great potential for detection of unexplored alleles to broaden the genetic diversity in current breeding programs (Bolibok-Brągoszewska et al. 2014). Exploitation of landraces adapted to various local environments in breeding programs might be beneficial with respect to prospective increase of productivity and it could also facilitate the development of advanced varieties suitable for sustainable agriculture (Persson et al. 2001).

5.4 Genetic Diversity of Rye Varieties Is Relatively Narrow and Was Influenced by Germplasm Exchange

Improved varieties of crop species, the outcome of breeding practices imposed by humans, represent a subset of the variation found in their wild ancestors. Although the selection for unusual or extreme phenotypes, such as large seed size, stiff rachis, synchronous ripening, or inhibition of seed shattering may cause the varieties to appear to be very diverse, the domestication process usually represents a genetic bottleneck. Moreover, improved varieties are grown in relatively uniform agricultural environments, which tend to further narrow the gene pool. Therefore a high degree of phenotypic variation exhibited by improved varieties might not always be a good predictor of the extent of their genetic variation (McCouch 2004). The narrowing of the genetic pool of modern crop varieties has become a matter of concern also for rye breeders (Fischer et al. 2010).

Improved rye varieties were analyzed in several molecular marker based studies. Genetic diversity of 96 cultivated ryes representing diverse geographic regions was investigated by means of PCR-RFLP analysis of seven chloroplast and four mitochondrial coding and noncoding genome regions (Isik et al. 2007). Polymorphism level was low and the genotypes were not separated according to geographic location, with most genotypes from diverse locations grouped together in an UPGMA dendrogram and in a principal component analysis plot. Owing to observed similarity between ryes from Europe and Turkey and also from Europe and Russia, the obtained data seem to support the hypothesis that rye

was transferred to Europe both via Russia and Turkey. Also, close relationships between the American and European genotypes were discovered.

Interesting results concerning genetic relationships were obtained using RAPD markers in a study of 20 spring and 22 winter rye varieties from 14 countries (Ma et al. 2004). Cluster analysis clearly divided rye cultivars into spring and winter groups and genetic variation was higher in the case of spring rye cultivars. Within the spring and winter groups subclusters corresponded well with the geographical origins of varieties and their adaptation to ecogeographical conditions. Some evidence supporting the migration route of rye into Europe via Turkey was also observed. The results indicated that temporal and ecological isolations have influenced the genetic diversity of rye more than geographical isolation (Ma et al. 2004).

In the SSR marker based study involving 47 cultivated rye accessions from various countries no obvious differences in GS index between cultivated ryes from different continents were found (Shang et al. 2006). A correspondence between groupings of cultivated rye accessions in an UPGMA dendrogram and their geographical origin also could not be observed. Likewise no deductions regarding the domestication process of cultivated rye could be made.

Similarly, in the study of 114 cultivated rye accessions of various origins performed using AFLP markers a clustering of cultivated rye accessions into groups based strictly on geographical origin was also not found (Chikmawati et al. 2012). All cultivated ryes representing different origins were mixed and clustered together both in dendrogram and PCO plot. The lowest genetic diversity was displayed by European varieties, which was explained as a result of the large number of rye breeding programs in the region, which focus on uniformity of commercial cultivars.

These findings were further confirmed in the DArT markers based study, which revealed lack of correlation between clustering of 137 improved varieties from 29 countries and their geographic origin (Bolibok-Brągoszewska et al. 2014). A relatively limited diversity of improved rye accessions, both contemporary and historical, was also shown—the average GS values for contemporary varieties obtained from breeding companies and for varieties from the collection of PAS BG were very similar (0.72 and 0.71, respectively) and the highest among germplasm groups analyzed in the study, with the exception of breeding strains only. Moreover, both groups of varieties clustered tightly in PCO plot.

It is generally assumed that lack of correlation between clustering of cultivated ryes and their geographic origin, observed in the majority of studies on genetic relationships of advanced rye germplasm indicates common genetic background and is the consequence of germplasm exchange (Isik et al. 2007; Chikmawati et al. 2012; Bolibok-Brągoszewska et al. 2014). This hypothesis is in agreement with the available information on rye breeding history—for example, Eastern European OPVs were crossed with each other in the past and many of them include the variety Petkus in their ancestry. Additionally, introgressions of foreign material were common in Easter European breeding programs. In Germany, on the other hand, before the introduction of hybrid rye all leading OPVs belonged either to the

Petkus or to the Carsten gene pool (Persson and von Bothmer 2002; Fischer et al. 2010; Li et al. 2011). This supposition is further confirmed by the results of SSR analyses of single S_0 plants from five Eastern European OPVs. A close relationship between varieties was revealed, pointing to common ancestors and/or gene flow between the varieties (Fischer et al. 2010). The relatively narrow genetic diversity of rye varieties revealed by molecular analyses could also be caused by common breeding program selection criteria based on similar agronomic characteristics, such as, disease resistance and high and stable yield (Chikmawati et al. 2012).

Results of DNA marker based studies, demonstrating the limited genetic diversity of advanced rye varieties indicate the need for broadening of diversity of the breeding germplasm in order to avoid the usual consequences of the narrowing of genetic pool in breeding programs including a decrease in selection gain and an increase in susceptibility to biotic and abiotic stresses coupled with the threat of further genetic erosion (Fischer et al. 2010).

5.5 Inbred Lines—An Indispensable Resource in Rye Genetics—Display a Considerable Genetic Diversity

Cultivated rye (*S. cereale* ssp. *cereale*) is an obligate cross-pollinator with an efficient two-locus gametophytic self-incompatibility system (Lundqvist 1956), exhibiting strong inbreeding depression. Nevertheless, the development of inbred lines is possible in rye. It is facilitated by the fact that mutations at the self-incompatibility loci lead to self-fertility (Lundqvist 1960). Additionally several self-fertility genes were also identified (Voylokov et al. 1997). Inbred lines are an indispensable resource in rye genetics and breeding, since their primary uses involve development of mapping populations and hybrid breeding.

Owing to lack of an efficient method of obtaining numerous and fertile double haploids of rye (Tenhola-Roininen et al. 2011) the genetic linkage maps of rye were almost exclusively created based on analyses of segregating progenies developed from crosses between inbred lines and both F2 populations (Devos et al. 1993; Börner and Korzun 1998) and recombined inbred lines developed from F2 progenies using single seed descent method (Bolibok et al. 2007; Bolibok-Brągoszewska et al. 2009; Milczarski et al. 2011; Stojałowski et al. 2011; Myśków et al. 2012) were used.

The application of molecular markers for establishing of genetic relationships of inbred lines is extremely important in breeding of hybrid varieties, since the highest heterosis effect is expected when two genetically distant components are crossed. In the case of rye, estimations of genetic distance based solely on pedigree data may be often ineffective, due to extensive germplasm exchange between breeding programs and common genetic background of many varieties. Moreover, inbred lines derived from the same population variety may exhibit considerable differences in allelic composition (Myśków et al. 2001).

Despite the importance of inbred lines for rye research, not many studies on their genetic diversity were published so far. A moderate number (30) of rye

inbred lines, mostly from the collection of the Department of Plant Genetics, Breeding and Biotechnology, Warsaw University of Life Sciences (DPGBB WULS) was thoroughly characterized with various PCR-based DNA markers: SSRs, SAMPLs, ISSRs and SSAPs (Bolibok et al. 2005, Bolibok-Brągoszewska et al. 2012). While these studies revealed that the inbred lines analyzed were very diverse, with the average value of Jaccard's GS coefficient ranging from 0.29 (calculated from ISSR data) to 0.53 (obtained in the case of SAMPL assays), they indicated also that no ultimate conclusion about the genetic relationships of the accessions studied should be drawn based on cluster analysis performed with a single statistical method based on a moderate number of loci of one marker type. Although certain groupings were consistent, numerous differences in topology of the dendrograms obtained separately for each marker system were visible. Discrepancies were also observed when different clustering methods and different similarity coefficients were used to analyze the same data (Bolibok et al. 2005).

In another study 40 rye lines, developed mostly at the breeding company Danko for hybrid rye breeding program and representing different generations of inbreeding (2–24) were analyzed with RAPD markers (Myśków et al. 2001). Genetic distances between lines were highly variable and although lines of similar parentage were usually placed within the same cluster in the dendrogram, there were also several examples where lines having common parents were distributed in different clusters and displayed a higher genetic distances than could be predicted from the parentage data. This result clearly illustrates the necessity of application of molecular analyses for obtaining faithful reflection of genetic relationships of rye inbred lines (Myśków et al. 2001).

Unfortunately, to date there is only one report allowing for direct comparisons between genetic diversity of inbred lines and that of other rye accession types. In a study on development of DArT markers for rye and their utility for genetic diversity analyses and genetic mapping ten inbred lines characterized previously with different PCR-based markers were included alongside 16 populations varieties of various geographical origins form the collection of PAS BG (Bolibok-Brągoszewska et al. 2009). The PCO analysis done based on 1022 DArT markers revealed that the inbred lines were not only very diverse, as demonstrated by their dispersion throughout the plot area, but also clearly divergent from varieties, which in turn were clustered very tightly together (Fig. 5.3).

In a study on construction of an integrated linkage map of the rye genome (Milczarski et al. 2011) genetic diversity of nine inbred lines constituting parental components of population used for mapping was assessed based on 4048 DArT markers. It was shown that the parental lines differed from each other to a similar extent, with the Jaccard's GS values ranging from 0.35 to 0.46, the overall average was 0.43. Comparison of the average GS value for the nine inbred lines with GS values calculated for germplasm groups of different improvements status in the DArT based study of genetic diversity in rye (Bolibok-Brągoszewska et al. 2014) shows that diversity of the inbred lines was only slightly inferior to the diversity of wild rye accessions and much greater than in the case of cultivated accessions, or even landraces.

The recent developments in the next-generation sequencing technologies resulted, among others, in the establishment of new genotyping methods, such as

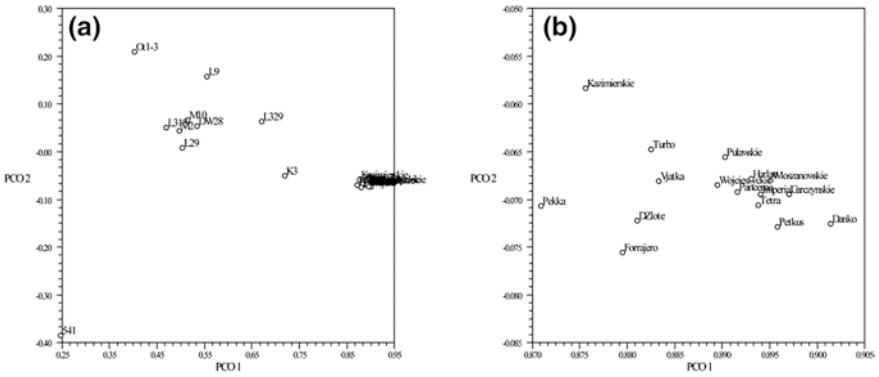

Fig. 5.3 Principal correspondence plot based on DArT data. **a** Patterns of relationships among 26 rye genotypes—10 inbred lines and 16 varieties. **b** Close up of the PCO plot section containing rye varieties. The first and the second principal axis explain 25.9 and 11.2 % of the variation, respectively (Bolibok-Brągoszewska et al. 2009)

genotyping by sequencing (GBS) (Davey et al. 2011). One of GBS variants is the DArTseq method (Cruz et al. 2013), which allows for simultaneous detection of several tens of thousands of sequence tagged markers, located preferentially in low-copy genomic regions, with the availability of a reference genome not being a prerequisite. Recently, this method was successfully applied in DPBGB-WULS for assessing genetic diversity in a broad population consisting of 146 diverse inbred lines (DIL) of rye (Targońska et al. 2014). The PCO analysis of genotyping results divided DILs into two groups—the first comprised lines bred in the Wrocław University of Environmental and Life Sciences and the second involved DILs bred independently in different institutions. Interestingly, the lines from two main Polish cereal breeding companies formed an almost homogenous group. This observation clearly points out the narrowing of genetic diversity among rye inbred lines used in current breeding programs, which is probably caused by the use of interrelated starting materials displaying similar features and by the application of the same selection criteria (Targońska et al. 2014).

5.6 Observed Genetic Diversity Patterns Reflect the Source and the Improvements Status of Accessions and Are Likely Influenced by Germplasm Reproduction Methods

The DArT markers based study of genetic diversity revealed that the source and the improvement status of the accessions contributed significantly to the structure observed in the analyzed set of *Secale* germplasm (Bolibok-Brągoszewska et al. 2014). In this study 379 accessions from three sources were analyzed: (a) varieties

currently registered and marketed in Europe and breeding strains obtained directly from breeding companies, (b) varieties, cultivated materials and landraces from PAS BG, representing a broad range of historic rye germplasm and geographic regions, and (c) accessions from A. Lukaszewski's collection. The source of the accessions was well reflected in the neighbor joining tree, which consisted of three major clusters I, II, and III. Cluster I, containing the majority of the accessions could be further subdivided into three clusters a, b, and c. The accessions from breeding companies were placed almost exclusively in cluster II, the varieties from PAS BG mostly in cluster Ic, cultivated materials and landraces from PAS BG mostly in clusters Ia, Ib, Ic, and the accessions from A. Lukaszewski's collection in cluster III. Similarly, in the PCO analysis, most materials supplied by breeding companies occupied a distinct area of diversity space, well separated from the accessions from other sources. Varieties and cultivated materials from PAS BG occupied a relatively narrow space, while landraces were more dispersed. Accessions from A. Lukaszewski's collection were placed between materials from other subgroups. This observations were confirmed by AMOVA analyses, which demonstrated high genetic differentiation between accessions from breeding companies and the following four germplasm groups: landraces, cultivated materials, varieties from PAS BG and accessions from A. Lukaszewski's collection (PhiPT values ranging from 0.15 to 0.20) (Bolibok-Brągoszewska et al. 2014). Since varieties from breeding companies used in the study were destined for cultivation in Central and Northern Europe, their clear differentiation from the remaining accessions could reflect the adaptation to local conditions of Central and Northern Europe and to requirements of modern agriculture. However, the influence of the accessions source on the observed diversity patterns is, very likely, attributable to different reproduction methods and to the accuracy of the procedures used by breeders and during genebank maintenance. This supposition is supported by the result obtained in this study for two independent samples of Dankowskie Nowe variety originating from different sources, the breeding company Danko and PAS BG: a relatively low GS value and assignment to different clusters in PCO and neighbor joining analysis (Bolibok-Brągoszewska et al. 2014).

When reproduction of accessions becomes necessary during genebank maintenance, seeds are sown in 1 m^2 field plots and before flowering, the whole plot area is covered with a 2 m high metal frame covered with pollen-proof tissue (Schlegel 2013). Rye accessions reproduced by breeders, however, are subjected to conservative breeding. These different reproduction methods can cause changes in allelic composition of accessions and in result the allelic composition of a variety maintained by breeders may be not equivalent to the allelic composition of the same variety maintained at a genebank (Bolibok-Brągoszewska et al. 2014).

The issue of the change in allele frequencies during genebank maintenance was addressed by Chebotar et al. (2003) in a study where ten SSR markers were deployed to analyze samples of six randomly chosen rye accessions form the collection from the genebank of the IPK Gatersleben. For each accession individual grains from the first multiplication (herbarium collection, 30- to 50-year-old) and the most recent multiplication (cold store) were used for DNA extraction.

The accessions chosen for the study were regenerated from 2 up to 14 times during genebank maintenance. It turned out that almost half of the alleles discovered in the original samples were not observed in the material from the cold store. On the other hand, the samples from the cold storage contained alleles that were not found in the original samples. Moreover, highly significant differences in allele frequencies were found, resulting most probably from selection pressure that occurred during the regeneration cycles. Therefore, the authors stressed that extended efforts should be made to sustain the genetic identity of open-pollinating rye accessions during ex situ maintenance, involving the use of plots large enough for growing a number of plants sufficient to cover the whole diversity of the populations, omitting harvest when a large proportion of plants is lost during a regeneration cycle and dividing the resources into base and active collections (Chebotar et al. 2003).

5.7 Genome-Wide Analyses Indicate Genome Regions Targeted by Domestication and Selection

Genome-wide, large scale genetic diversity analyses allow us to gain insight into evolution of crop plants and their genomes, since the genomic regions that were subjected to selection during crop evolution are expected to exhibit characteristic changes in levels of polymorphism (Mandel et al. 2013). The development of DArT genotyping panel for rye (Bolibok-Brągoszewska et al. 2009) opened the possibility for affordable genome-wide characterizations of large germplasm collections for this species. Recently, 1054 DArT markers with defined chromosomal location, (number of DArTs per chromosome ranged from 112 for 1R to 231 for 4R) were used to analyze 379 rye accessions of different improvement status and to compare the distribution of DNA polymorphisms among rye chromosomes (Bolibok-Brągoszewska et al. 2014). It was found that the average GS values for individual chromosomes were significantly different ($p = 0.01$), except for the average GS for chromosomes 5R and 7R. Calculation of chromosome specific average GS for seven germplasm subgroups created according to the accessions' source and type revealed a moderately consistent pattern of differences (Fig. 5.4). The highest average chromosome specific GS values were observed for chromosome 6R for all subgroups, with exception of wild accessions, while the lowest average GS for accession groups occurred in the case of chromosome 1R, except for varieties supplied by breeding companies. In the case of varieties from breeding companies noteworthy was also a markedly higher average GS value for chromosome 4R. The high average GS value on 6R that occurred in all subgroups of cultivated germplasm, but was not observed in wild accessions may indicate that genome regions that were subjected to strong selection pressure during

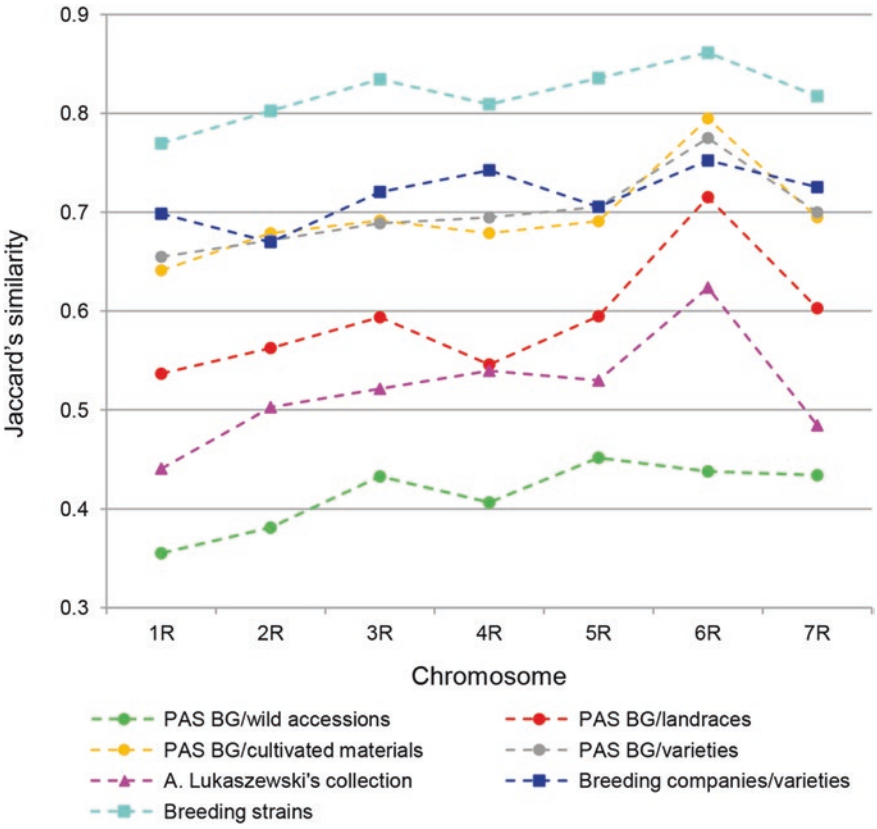

Fig. 5.4 Average GS values for groups of accessions by chromosome, based on 1054 DArT markers (Bolibok-Brągoszewska et al. 2014)

domestication are located in this chromosome. Likewise, the relatively high GS averages on 1R and 4R in contemporary varieties might indicate that these chromosome regions are targeted by selection in current breeding program and therefore contain regions with limited polymorphism. The available information on chromosomal location of some genes relevant for agricultural cultivation of rye supports this supposition, as chromosome 1R contains genes controlling resistance to diseases and insects, improving adaptation and increasing yield (Simkova et al. 2008), whereas chromosome 4R harbors QTLs for alpha-amylase activity, preharvest sprouting, kernel thickness, heading time, chlorophyll content in leaves, and flag leaf length (Schlegel and Korzun 2013). Furthermore, the relatively high GS averages on 1R and 4R that occurred in varieties supplied by breeding companies could also reflect the locations of self-fertility mutations and fertility restoration genes deployed in hybrid rye breeding (Bolibok-Brągoszewska et al. 2014).

5.8 Conclusions and Future Implications

Various molecular markers were used so far to test genetic relationships in different sets of rye germplasm of different origin and improvement status. The congruence of the results of multiple independent studies involving different molecular methods and accession sets, such as in the case of phylogenetic analyses of *Secale* species, indicate that DNA markers faithfully reflect actual genetic relationships between rye accessions. Molecular markers studies delivered important and sometimes surprising information on the extent and the structure of genetic variation, which can be crucial for the preservation of the genetic diversity of rye germplasm and its efficient use in rye improvement. Nevertheless, the accessions characterized to date with molecular markers constitute a very small fraction of a worldwide genetic resources and further research is needed. Follow-up and comparative studies, involving accessions from different genebanks, as well as contemporary advanced varieties from different geographic areas would be desirable to verify the findings pointing out, respectively, to the influence of reproduction methods on population structure and to the narrowing of genetic diversity in rye breeding programs. A wider application of molecular markers in characterization of rye accessions in genebanks worldwide is necessary to obtain a fuller picture of genetic diversity and population structure, and, subsequently, to create a core collection, where the whole sampled genetic diversity would be concentrated in a smaller, manageable number of accessions. It would facilitate not only the conservation issues but also the practical use of unimproved germplasm in rye breeding, since a detailed characterization of the genetic diversity structure of less-adapted germplasm based on numerous marker loci distributed throughout the genome would provide a good foundation for targeted broadening of diversity of the breeding germplasm in defined genome regions (Alheit et al. 2012). The core collection concept has been already applied in a range of crops (Upadhyaya et al. 2008; Lv et al. 2012; Muñoz-Amatriaín et al. 2014), however this approach has not yet been attempted in rye. The next-generation sequencing-based genotyping, offering affordable detection of several tens of thousands DNA polymorphism dispersed throughout a genome and allowing for discrimination between homo-and heterozygous loci, as well as estimation of allele frequencies (Davey et al. 2011), could be a method of choice for the characterization of numerous heterozygous and heterogenous rye accessions.

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Chapter 6
Using Molecular Techniques to Dissect Plant Genetic Diversity

Linda Mondini and Mario A. Pagnotta

Abstract During the past few decades, the employment of molecular markers to discover polymorphisms in DNA has been playing an increasing role in conservation strategies and use of plant genetic resources (PGR). Molecular markers are indispensable tools for determining the genetic variation and biodiversity with high levels of accuracy and reproducibility in short times. Different typologies of molecular markers exist, specific for the different applications in molecular genetic methods. Molecular tools have been successfully applied in the analysis of specific genes and gene pathways, as well as to increase understanding of gene action, to generate genetic maps and assist in the development of gene transfer technologies. Molecular markers have also had a critical role in studies of phylogeny and species evolution, and have been applied to increase our understanding of the distribution and extent of genetic variation within and between species. The main two groups of molecular markers can be classified on the basis of the analysis method used: polymerase chain reaction (PCR) and non-PCR-based. Recently, a new class of advanced techniques has emerged, primarily derived from a combination of earlier, more basic techniques. Advanced marker techniques tend to amalgamate advantageous features of several basic techniques, in order to increase the sensitivity and resolution to detect genetic discontinuity and distinctiveness. The past several years have seen revolutionary advances in DNA sequencing technologies with the advent of next-generation sequencing (NGS) techniques. NGS methods now allow millions of bases to be sequenced in one round, at moderate prices and in very short times. This paper is an overview of the diverse, predominantly molecular techniques, used in assessing plant genetic diversity, discussing

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about the most important and recent advances made in molecular marker techniques, their applications, advantages, and limitations applied to plant sciences in order to provide base platform information to the researchers working in the area.

Keywords Genetic variation · Biodiversity · Molecular markers · Molecular techniques

6.1 Introduction

Unraveling the molecular basis of the essential biological phenomena in plants is crucial for effective and sustainable conservation, management, and efficient utilization of plant genetic resources (PGR). An adequate understanding of existing genetic diversity and how to best utilize it, is of fundamental interest for basic science and applied aspects like the efficient management of PGR. In particular, the improvement of crop genetic resources strictly depends on the continuous introduction of wild relatives, traditional varieties, and the use of modern breeding techniques, requiring an assessment of diversity at some levels in order to select promising varieties.

6.1.1 The Assessment of Genetic Variation

When studying and measuring diversity, it is imperative to understand what to conserve and/or what is being lost. For the conservation and utilization of PGR, genetic relationships are more important than the taxonomy per se. The gene pools concept, as proposed by Harlan and de Wet (1971), focuses neatly on the relationships between individuals and populations and it is of particular relevance to plant breeders to improve crops (Greene and Morris 2001). The concept is based on the division of the genetic resources into three gene pools: (i) primary gene pool (gene pool 1 or GP 1) to which the crop species, crop wild relatives, and related weedy species belong with crosses yielding fertile hybrids; (ii) secondary gene pool (GP 2) comprises related taxa which are able to hybridize with the crop species but the gene transfer is poor and the progeny are often sterile and not viable; and (iii) tertiary gene pool (GP 3) includes distantly related taxa which do not cross readily in the wild and require anthropogenic assistance in gene transfer and hybridizing through sophisticated techniques, such as embryo culture, grafting, chromosome doubling, and the use of bridging species. We can argue that plant breeding now requires the addition of a quaternary gene pool (GP 4) where gene transfer could take place but only through genetic engineering. Diversity can be measured at the morphological, biochemical, and/or molecular level.

6.1.2 Morphological Characterization

Morphological characterization is based on assessing the phenotype, which is the result of genetic and environment interactions, and can be modified to diverse extents by different environmental factors. The capability to respond to environmental pressures without the implication of mutations, known as phenotypic plasticity, can be divided into two main categories: (i) *development flexibility*, which produces the development of different genotypes in different environmental conditions and (ii) *behavioral flexibility*, which comprises all the behavioral elements which allow a temporary adaptation to a particular environmental condition. Genetic variation has been found to contribute significantly to phenotypic variation and produces two main types of characters: (a) *quantitative* characters which are measurable characters and give rise to continuous variability (defined by a Gaussian curve) and (b) *qualitative* characters which are alternative, discontinuous, not defined by a Gaussian curve and producing a type of variability, so-called "discontinuous". The study of morphological variability is the classical way of assessing genetic diversity. For many species, especially minor crops, it is still the only approach used. Nevertheless, morphological characterization, even if does not require expensive analysis tools, requires large tracts of land for the experiments, making it even more expensive than molecular detection. Moreover, the traits analyzed are often susceptible to phenotypic plasticity; conversely, this allows assessment of diversity in the presence of environmental variation. However, an analysis of genetic diversity based only on agronomic and morphological traits might be erroneous considering that distinct morph types can result from a few mutations.

6.2 Cytological Characterization

Cytological markers have been deeply used for the assessment of PGR based on the numbers and morphology of plant chromosomes. Cytological markers include chromosome karyotypes, bandings, repeats, deletions, translocations, and inversions. Mitotic chromosomes permit to analyze the nuclear genome by microscopic means, allowing the observation of its components individually, as well as globally (the karyotype). Karyotypes offer a phenotypic view of the genotype and prior to the application of chromosome banding, distance analysis was done using various numerical and metric values that described the karyotype such as diploid number (number of chromosomes or $2n$) and fundamental number (number of chromosomal arms or Nfa).

Ploidy levels are sometimes used to compare species, mainly in plants (Saideswara et al. 1989). Polyploidization, widely spread in plant genomes, can result from genome duplication (autopolyploidization) or by hybridization (allopolyploidization). Nevertheless, even if these changes are considered as rare,

convergence in ploidy may not be that uncommon and tending sometimes to revert to the diploid level complicating the understanding of polyploidization patterns. However, a recently developed technique based on genomic in situ hybridization (GISH) represents a powerful tool for investigating the evolution of polyploidy organisms (D'Hont et al. 2002). Chromosome banding is a powerful and routinely used tool to investigate chromosomal homology and comprise differential staining techniques that reveal a succession of bands along the length of a chromosome that vary in width and staining intensity. These bands reflect intrinsic properties of the genome (Sumner 1990) allowing access to information involving both structural (GTG-, RHG-, and CBG-banding) and functional patterns (replication RBG-banding) of chromosomes (Viegas-P´equignot and Dutrillaux 1978). Moreover, the development of in situ hybridization, and in particular fluorescent in situ hybridization (FISH) using chromosome painting probes (Ferguson-Smith 1997), has confirmed the evidence that homology in banding patterns is significantly related to homology in gene content and synteny conservation. The development of powerful molecular cytogenetic and genomic strategies such as FISH, flow-cytometry, and chromosome painting jointly to gene mapping, allows to overcome the limitations of conventional banding analysis (Ferguson-Smith 1997). Based on the hybridization between labeled DNA probes and genomic DNA, in situ hybridization techniques permit the unequivocal confirmation of homology among chromosomes. Therefore, molecular cytogenetics makes it possible to assess homologies between distantly related taxa and this creates new opportunities for determining chromosomal relationships at higher taxonomic levels (Yang et al. 2003).

6.3 Biochemical Characterization

Biochemical characterization includes the assessment of seed storage proteins and *allozymes/isozymes*. These techniques use enzymatic functions and are comparatively inexpensive while being powerful methods of measuring allele frequencies for specific genes. However, because there are only a few allozyme systems per species (not more than 30), there are correspondingly few markers. Analyses of allozymes provide an estimate of gene and genotypic frequencies within and between populations. Such data can be used to measure population subdivision, genetic diversity, gene flow, genetic structure of species, and comparisons among species (Spooner et al. 2005). The first experiences in analysis of isoenzymatic polymorphism in natural populations date up to Zouros and Foltz (1987). Since then, isozymes have been heavily employed also in plant studies and particularly for population genetics studies (Brown 1979). Therefore, allozymes have been used in studying out-crossing rates, population structure, and population divergence, such as in the case of crop wild relatives (Hamrick and Godt 1997; Guarino 1999; Volis et al. 2001; Gonzalez et al. 2005).

Among the major advantages of these types of markers are co-dominance, absence of epistatic and pleiotropic effects, ease of use, and low costs even if at

the same time they present some important limitations such as the limited number of polymorphic enzymatic systems available, the fact that enzymatic loci represent the expressed part of the genome which is only a small and not random portion; they are affected by the phenological phase of the plant, and finally that the observed variability may be not representative of the entire genome. Moreover, although these markers permit a high processivity, a comparison of samples from different species, loci, and laboratories is problematic being affected by extraction methodology, plant tissue, and plant stage.

6.4 Molecular Characterization

Analyses of genetic diversity are usually based on either allozymes or molecular markers, which tend to be selectively neutral. It has been argued that the rate of diversity loss of these neutral markers will be higher than those that are associated with fitness. In order to verify this, Reed and Frankham (2003) conducted a meta-analysis of fitness components in three populations and in which heterozygosity, and/or heritability, and/or population size were measured. Their findings, based on 34 datasets, concluded that heterozygosity, population size, and quantitative genetic variation, which are all used as indicators of fitness, were all significantly positively correlated with population fitness.

Genetic variability within a population can be assessed through:

1. The number (and percentage) of polymorphic genes in the population.
2. The number of alleles for each polymorphic gene.
3. The proportion of heterozygous loci per individual (Primack 1993).

Protein methods, such as allozyme electrophoresis, and molecular methods, such as DNA analysis, directly measure genetic variation, giving a clear indication of the levels of genetic variation present in a species and/or population (Karp et al. 1996) without direct interference from environmental factors. However, they have the disadvantage of being relatively expensive, time-consuming, and require high levels of expertise and materials in analysis.

The concept of genetic markers is not a new one; in the nineteenth century, Gregor Mendel employed phenotype-based genetic markers in his experiments. Later, phenotype-based genetic markers for *Drosophila melanogaster* led to the founding of the theory of genetic linkage, occurring when particular genetic loci or alleles for genes are inherited jointly. The limitations of phenotype-based genetic markers led to the development of DNA-based markers, i.e., molecular markers. A molecular marker can be defined as a genomic locus, detected through probe or specific starters (primer) which, in virtue of its presence, distinguishes unequivocally the chromosomic trait which it represents as well as the flanking regions at the 3' or 5' extremity (Barcaccia et al. 2000).

Molecular markers may or may not correlate with phenotypic expression of a genomic trait. They offer numerous advantages over conventional, phenotype-based

alternatives as they are stable and detectable in all tissues regardless of growth, differentiation, development, or defense status of the cell. Additionally, they are not confounded by environmental, pleiotropic, and epistatic effects. Molecular characterization is more expensive, but many markers are now known, thus enabling the study of a much larger number of genes that code for plant expression, as well as for other noncoding segments of the chromosomes. Analysis is based on extracting DNA, amplifying it (more often than not, through polymerase chain reaction procedures) and analyzing the resulting gene frequencies and DNA sequences. A molecular marker detects gene sequences at a known location of a chromosome. These markers do not refer to the activity of specific genes, but are directly based on highlighting differences (polymorphisms) within a nucleic sequence in different individuals, as a result of insertion, deletions, translocations, duplications, point mutations, etc.

The seemingly bewildering array of possible approaches is among the first problem faced by newcomers considering the application of these techniques to their own system. A starting point for discerning the different classes of molecular markers can be to consider the different techniques employed. These are based either on restriction-hybridization of nucleic acid or techniques based on polymerase chain reaction (PCR), or both. A further distinction can be obtained through the selection of either multi-locus or single-locus markers.

Multi-locus markers allow simultaneous analyses of several genomic loci, which are based on the amplification of casual chromosomic traits through oligonucleic primers with arbitrary sequences. These types of markers are also defined as *dominant* since it is possible to observe the presence or the absence of a band for any locus, but it is not possible to distinguish between heterozygote (a/–) condition and homozygote for the same allele (a/a) and attribute different allelic variants at the same locus. By contrast, single-locus markers employ probes or primers specific to genomic loci, and are able to hybridize or amplify chromosome traits with well-known sequences. They are defined as co-dominant since they allow discrimination between homozygote and heterozygote loci.

Advances in the development of molecular marker techniques, powerful tools have been developed so that genetic resources can be accurately assessed and characterized (Table 6.1). Most of these techniques, based on the analysis of information-rich nucleic molecules, provide a reliable estimation of relatedness, phylogeny, and inheritance of genetic characteristics (Caetano-Anolles et al. 1991). Through molecular markers and maps, it is possible to obtain an overall vision on the genes controlling agronomic, morphological, and biochemical traits in plants. Additionally, they become essential for explaining whether existing genetic variability, which is assessed by measuring biochemical factors and morphological traits, is related to genetic diversity analyzed measuring allelic frequencies detected with molecular markers. Through this information it is possible to construct a core collection, which can represent a base for future breeding programs. Hence, in the current scenario, molecular markers become the marker of choice for the study of crop genetic diversity revolutionizing the plant biotechnology.

Table 6.1 Molecular markers classification

Non-PCR-based techniques	
Restriction-hybridization techniques	
RFLP	Restriction Fragment Length Polymorphism
VNTR	Variable Number Tandem Repeats
REF	Restriction Endonuclease Fingerprinting
PCR derived	
Multiple Arbitrary Amplicon Profiling (*MAAP*)	
RAPD	Randomly Amplified Polymorphic DNA
DAF	DNA Amplification Fingerprint
AP-PCR	Arbitrarily primed PCR
AFLP	Amplified Fragment Length Polymorphism
SAMPL	Selective Amplification of Polymorphic Loci
ISSR	Inter-Simple Sequence Repeats
SPAR	Single Primer Amplification Reaction
DAMD	Directed Amplification of Minisatellites DNA
Targeted PCR	
Sequence Tagged Sites (*STS*)	
ARMS	Amplification Refractory Mutation System
ASAP	Arbitrary Signatures from Amplification
ASH	Allele-Specific Hybridization
ASLP	Amplified Sequence Length Polymorphism
ISTR	Inverse Sequence-Tagged Repeats
SSCP	Single Strand Conformation Polymorphism
SPLAT	Single Polymorphic Amplification Test
TGGE	Thermal Gradient Gel Electrophoresis
DGGE	Denaturing Gradient Gel Electrophoresis
Markers based on microsatellite sequences	
SSR	Simple Sequence Repeats
RAHM	Randomly Amplified Hybridizing Microsatellites
RAMPs	Randomly Amplified Microsatellite Polymorphisms
STMS	Sequence Tagged Microsatellite Site
SSLP	Single Sequence Length Polymorphism
MP-PCR	Microsatellite-Primed PCR
RAMS	Randomly Amplified Microsatellites
CAPS	Cleaved Amplification Polymorphic Sequence
SCAR	Sequence Characterized Amplification Regions
SNP	Single Nucleotide Polymorphism
EST-SSR	Expressed Sequence Tags-SSR
Markers based on DNA sequencing	
DART	Diversity Arrays Technology
ASO	Allele Specific Oligonucleotide
CAS	Coupled Amplification and Sequencing

(continued)

Table 6.1 (continued)

GBA	Genetic Bit Analysis
OLA	Oligonucleotide Ligation Assay
RNA-Based Molecular Markers (*RBMs*)	
iSNAP	Inter Small RNA Polymorphism
RAP-PCR	RNA Arbitrarily Primed PCR
cDNA-SSCP	cDNA-Single Strand Conformation Polymorphism
cDNA-AFLP	cDNA-Amplified Fragment Length Polymorphism
cDNA-RFLP	cDNA-Restriction Fragment Length Polymorphism
Transposable elements-based molecular markers	
REMAP	Retrotrasposon-Microsatellite Amplified Polymorphism
RBIP	Retrotrasposon-Based Insertion Polymorphism
IRAO	Inter-Retrotrasposon Amplified Polymorphism
IRAP	Inter-Retrotransposon Amplified Polymorphism
S-SAP	Sequence-Specific Amplification Polymorphisms

6.5 The Choice of the "Perfect" Molecular Marker

Due to the rapid developments in the field of molecular genetics and thanks to the novel findings in next-generation sequencing (NGS), a large amount of different techniques have emerged to analyze genetic variation in the recent years.

Unfortunately, there is no single molecular approach for many of the problems facing gene bank managers, and many techniques complement each other, hence the choice of marker typology that "suits me" becomes very difficult. However, some techniques are clearly more appropriate than others for some specific applications like crop diversity and taxonomy studies. In this perspective, the understanding of all features that characterize a molecular marker class is crucial.

Genetic markers can differ with respect to important features such as

- level of polymorphism detected;
- locus specificity,
- genomic abundance,
- reproducibility,
- technical requirements and highly qualified personnel,
- costs, and
- time constraints.

No marker is superior to all others for a wide range of applications and the most appropriate genetic marker strictly depends on the application (Table 6.2). An ideal molecular marker should possess the following features:

1. Be highly polymorphic: necessary condition to assess genetic variability;
2. Co-dominant: able to discriminate between homozygous and heterozygous states in diploid organisms;

3. Frequent occurrence in genome;
4. Provide adequate resolution of genetic differences;
5. Detect multiple, independent, and reliable loci;
6. Selective neutral behaviors: the DNA sequences of any organism are neutral to environmental conditions or management practices, this permits to confer the variation only to a genetic origin;
7. Easy access and fast assay: it must be simple, quick, and inexpensive;
8. High reproducibility: to guarantee robust results among different laboratory and equips;
9. Requiring small amounts of tissue and DNA samples;
10. Link to distinct phenotypes;
11. Require no prior information about the genome of an organism.

However, it is practically impossible to define a molecular marker which would meet all the above criteria. Hence, the choice of the right marker is based on the capability to associate the different features to the specific application to be undertaken (Weising et al. 1995) (Fig. 6.1). At first, molecular markers can be classified as hybridization-based markers and PCR-based markers. In the former, DNA profiles are visualized by hybridizing the restriction enzyme-digested DNA, to a labeled probe, which is a DNA fragment of known origin or sequence. A PCR-based marker involves in vitro amplification of DNA sequences or loci, using specifically or arbitrarily chosen oligonucleotide fragments (primers) and a thermostable DNA polymerase enzyme (*Taq* polymerase). The amplified fragments are separated electrophoretically and banding patterns are detected by different methods such as staining, autoradiography, or directly sequenced. The primer sequences are chosen to allow base-specific binding to the template in reverse orientation. PCR is extremely sensitive, fast, and reliable. Its application for diverse purposes has opened up a multitude of new possibilities in the field of molecular biology and genetics.

Recently, a new class of advanced techniques has emerged, primarily derived from a combination of the earlier, more basic techniques. These advanced marker techniques combine advantageous aspects of several basic techniques. In particular, the newer methods incorporate modifications in the basic techniques, thereby increasing the sensitivity and resolution in detecting genetic discontinuity and distinctiveness. The advanced marker techniques also utilize newer classes of DNA elements such as retrotransposons, mitochondrial, and chloroplast-based microsatellites, allowing increased genome coverage. Techniques such as Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) are also being applied to cDNA-based templates (i.e., sequences of complementary DNA obtained by mRNA retrotranscription) to study patterns of gene expression and uncover the genetic basis of biological responses. With the advent of NGS technologies it is presently possible to analyze high numbers of samples over smaller periods of time.

Table 6.2 Comparison among the most widely used molecular markers in plants

Marker tipology	PD	LS	I	QD	R	SI	A	C	TE	AD	DS
RFLP	M	Y	C	H	H	Y	N	H	M	-High genomic abundance and coverage -Possibility to use filters many times	-Large amount of gDNA required -Laborious and time-consuming -Radioactive labeling -Cloning/characterization of probe
AFLP	H	N	D	M	H	N	Y	M	M	-High genomic abundance -Used across species	-Dominant markers -Laborious interpretation of patterns -Laborious and time-consuming
RAPD	M	N	D	L	L	N	Y	L	L	-High genomic abundance and coverage -Easy and fast	-Dominant markers -Not reproducible -Not across species
SSR	H	N	C	L	M	N	Y	L	L	-High genomic abundance and coverage -Amenability to multiplexing and automation -Presence of multiple alleles	-Medium reproducibility -Not across species -Preliminary sequence information
SNP	H	Y	C	L	H	Y	Y	M	M	-Amenability to automation -Highly polymorphic -Allow cultivar discrimination	-Preliminary sequence information -High costs
DART	H	Y	D	L	H	N	Y	L	H	-Any sequence information required -High-throughput data production -Low costs	-Complex and expensive technical requirement -Primarily dominant markers -Currently used in few species
CAPS	L	Y	C	L	H	Y	Y	H	M	-High reproducibility -Co-dominant markers -Easy and fast respect to RFLP and CAPS	-Low polymorphism detected -Preliminary sequence information
RAMP	M	Y	D	L	M	N	Y	M	M	-High degree of allelic polymorphism respect to RAPD	-Labor intensive technique -Use of radiolabeled primer

(continued)

Table 6.2 (continued)

Marker tipology	PD	LS	I	QD	R	SI	A	C	TE	AD	DS
SSCP	L	Y	C	L	M	Y	N	H	M	-Co-dominant markers -Low quantity of DNA required	-Need of highly standardized protocols -Inability to detect all the mutations present -Low results robustness
IRAP/ REMAP/SSAP	H	Y	D	L	H	Y	Y	L	M	-Great for phylogenetic studies -High genomic coverage -Amenability to multiplexing	-Preliminary sequence information required for flanking regions -Dominant markers
ISSR	H	N	D	L	L	N	Y	L	L	-Easier interpretation of patterns respect to AFLP	-Low reproducibility -Multi-locus markers
SCAR	M	Y	C	L	H	Y	Y	L	L	-Quick and easy to use -High reproducibility -Locus specificity	-Preliminary sequence information required
VNTR	H	Y	D	H	H	Y	N	H	M	-High level of polymorphism -High reproducibility	-Large amount of gDNA required -Laborious and time-consuming -Cloning/characterization of probe

PD polymorphism degree, *LS* locus specificity, *I* inheritance [dominant (*D*)/co-dominant(*C*)], *QD* quantity of DNA required, *R* reproducibility, *SI* sequence information required, *A* amenability to automation, *C* costs, *TE* technical equipment, *H* high, *L* low, *M* medium, *Y* yes, *N* no

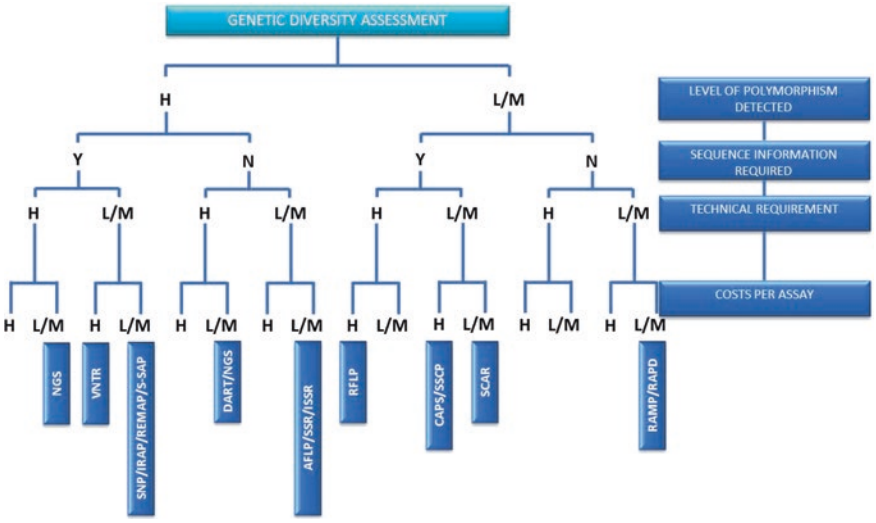

Fig. 6.1 A rational scheme for choosing the most appropriate molecular genetics analysis strategy. *H* high, *L* low, *M* medium, *Y* yes, *N* no

6.6 Non-PCR-Based Techniques

6.6.1 Restriction-Hybridization Techniques

Molecular markers based on restriction-hybridization techniques were employed relatively early in the field of plant studies and combined the use of restriction endonucleases and the hybridization method (Southern 1975). Restriction endonucleases are bacterial enzymes able to cut DNA, identifying specific palindrome sequences and producing polynucleotidic fragments with variable dimensions. Any changes within sequences (i.e., point mutations), mutations between two sites (i.e., deletions and translocations), or mutations within the enzyme site, can generate variations in the length of restriction fragment obtained after enzymatic digestion.

Restriction Fragment Length Polymorphisms (RFLPs) was the first technology developed which enabled the detection of polymorphisms at the sequence level. The approach comprises the digestion of genomic DNA with restriction enzymes, separations of the resultant DNA fragments by gel electrophoresis, blotting of the fragments to a filter followed by the hybridization with a chemically-labeled DNA probe to a Southern blot resulting in differential DNA fragment profile. The sequences of the probes may be known (e.g., from a cloned gene) or unknown (e.g., genomic or cDNA random cloned fragments) (Fig. 6.2). The combination of specific systems probes/enzymes produces highly reproducible patterns for a given individual and the variation in the restriction profiles between two different individuals occurs when mutations in the DNA sequences change the restriction sites which cannot be recognized by the restriction enzymes. RFLP technique was widely exploited to construct genetic maps and has been successfully applied

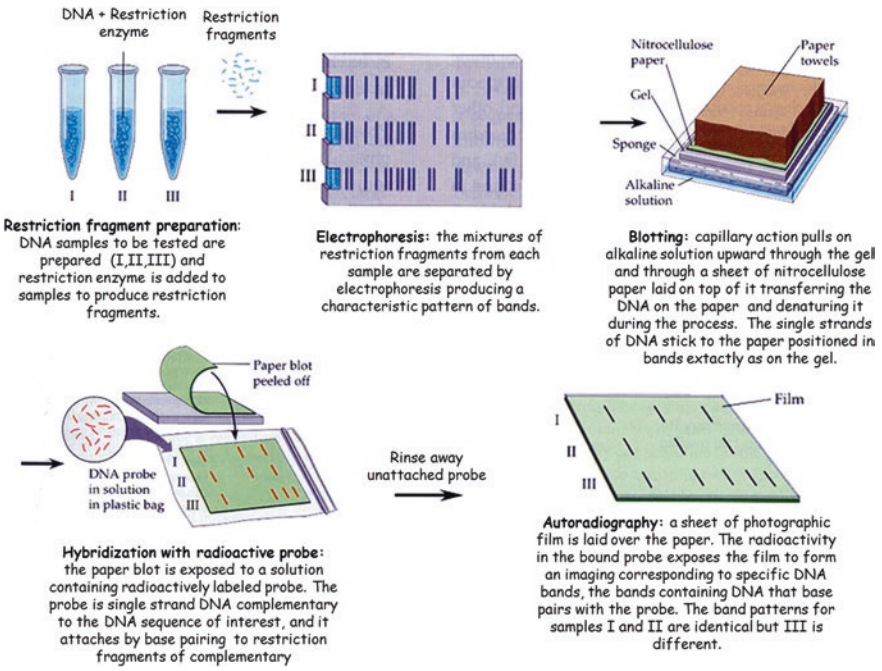

Fig. 6.2 Different steps of restriction fragment length polymorphism technique

to genetic diversity assessment, particularly in cultivated plants (Castagna et al. 1994; Deu et al. 1994) as well as in populations and wild relatives (Besse et al. 1994; Laurent et al. 1994; Bark and Havey 1995).

The RFLP markers are relatively highly polymorphic, co-dominantly inherited, highly replicable, and allow the simultaneous screening of numerous samples. DNA blots can be analyzed repeatedly by stripping and reprobing (usually from eight to ten times) with different RFLP probes. Nevertheless, this technique is not very widely used as it is time-consuming, involves expensive and radioactive/toxic reagents, and requires large quantities of high quality genomic DNA (e.g. 10 μg per digestion). Moreover, the prerequisite of prior sequence information for probe construction contributes to the complexity of the methodology. However, the main problem faced is simply that insufficient level of polymorphism is detectable at the below species level. Nevertheless, RFLPs have been widely used to investigate relationships of closely related taxa (Miller and Tanksley 1990; Lanner et al. 1996), for studies on hybridization and introgression (in particular studies concerning the gene flow between crops and weeds) (Brubaker and Wendel 1994; Clausen and Spooner 1998), for diversity studies (Dubreuil et al. 1996), and as fingerprinting tools (Fang et al. 1997). They have also been successfully employed in gene mapping studies due to their high genomic abundance and random distribution throughout the genome (Neale and Williams 1991). Moreover, RFLP markers were used for the first time in the construction of genetic maps by Botstein et al. (1980). Nevertheless,

a class of molecular markers able to overcome this inconvenience exists. These markers are developed on a particular class of highly variable regions interspersed along the genome and constituted by repeats of short simple sequences. These are known as "microsatellites" and are formed by basic repeat units from 2 to 8 base pairs in length (microsatellites or SSR) up to longer repeats of 16–100 base pairs called "minisatellites". Being highly hypervariable, RFLP analysis using probes for mini-microsatellites produces multi-locus patterns able to discriminate at the level of populations and individuals. The variation produced derives from changes in the copy number of the basic repeat and the marker class based on this kind of variation is specifically called Variable Numbers of Tandem Repeats (VNTRs). Being highly polymorphic VNTRs have been widely applied for studying within and between population variation, for estimating genetic distances, and for ecological applications (Lynch 1990; Alberte et al. 1994; Antonious and Nybom 1994).

However, like the RFLP approach VNTRs show the same limitations that led to the development of a new set of less technically complex methods known as PCR-based techniques. Nevertheless, when combined with PCR amplification of a specific locus both RFLPs and VNTRs probes have much to offer.

6.7 Markers Based on Amplification Techniques (PCR-Derived)

With the advent of PCR analysis, an increasing number of techniques became available to screen the genetic diversity. In fact, the use of this kind of marker has been exponential, following the development by Mullis et al. (1986) of PCR assay consisting in the amplification of several discrete DNA products, deriving from regions of DNA which are flanked by regions of high homology with the primers. These regions must be close enough to one another to permit the elongation phase producing several discrete DNA products.

The use of random primers overcame the limitation of prior sequence knowledge for PCR analysis, and being applicable to all organisms facilitated the development of genetic markers for a variety of purposes. PCR-based techniques can further be subdivided into two subcategories: (1) arbitrarily primed PCR-based techniques or sequence nonspecific techniques and (2) sequence targeted PCR-based techniques. Based on the first category, two different types of molecular markers have been developed: RAPD and AFLP.

6.7.1 PCR Arbitrary Priming Techniques

In the first category a number of closely related techniques have been developed and jointly referred to as Multiple Arbitrary Amplicon Profiling (MAAP) (Caetano-Anolles 1994). Even if, among these, RAPD is the most commonly

used, other techniques can be included such as Arbitrary Primed PCR (AP-PCR) (Welsh and McClelland 1990) and DNA Amplification Fingerprinting (DAF) (Caetano-Anolles et al. 1991) differing from RAPDs for primer length, stringency of the conditions, and the method of separation and detection.

6.7.2 *Random Amplified Polymorphic DNA (RAPD)*

RAPDs have been deeply applied thanks to the fact that these kinds of markers do not require DNA probes or any types of sequence information for the design of the specific markers.

RAPDs were the first PCR-based molecular markers to be employed in genetic variation analyses (Welsh and McClelland 1990; Williams et al. 1991). RAPD markers consist of random amplification of genomic DNA using short primers (decamers) and separation of the obtained fragments. The use of short primers is necessary to increase the probability that, although the sequences are random, they are able to find homologous sequences suitable for annealing (Fig. 6.3). Thence, DNA polymorphisms are generated by rearrangements or deletions occurring at or between oligonucleotide primer binding sites along the genome. RAPD–PCR fingerprint has been successfully applied in dissecting genetic diversity among different species. RAPD markers show several advantages: (i) no prior sequence information is needed for designing the primers that can be used for different

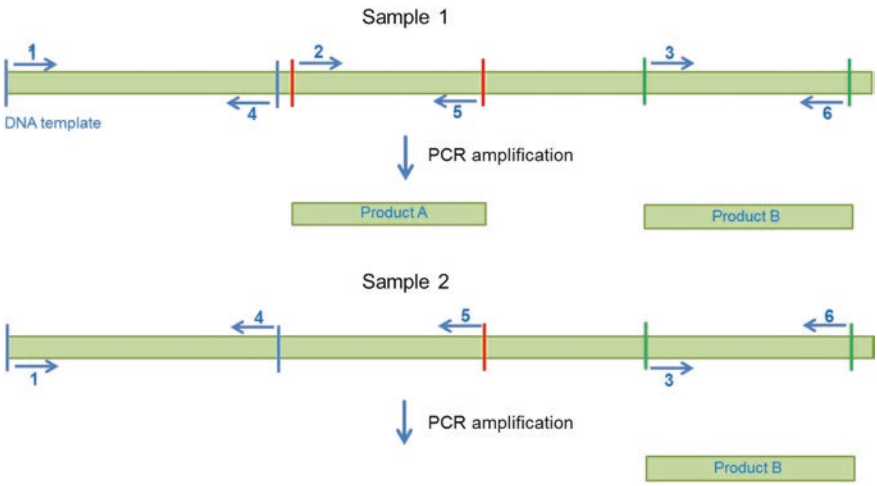

Fig. 6.3 Schematic representation of a Random Amplified Polymorphic DNA (*RAPD*) reaction. In order to obtain an amplification product, the primers must anneal in the right orientation, pointing toward each other and at a reasonable distance. The *arrows* represent the single primers and the direction indicates the direction in which DNA synthesis will occur. The *numbers* represent primer annealing sites on the target DNA

templates; (ii) RAPDs are simple, quick, and cost-effective especially if compared to RFLP (Williams et al. 1991; Bardakci 2001); (iii) the quantity of DNA to be used is very small being amplified by PCR. At the same time, RAPDs present some not insignificant disadvantages that include: (i) the very low repeatability and reliability of RAPD polymorphic profiles (Vos et al. 1995); (ii) RAPDs, being dominant, cannot be used to distinguish homozygote from heterozygote genotypes in F2 populations; (iii) nonspecific and therefore non-reproducible binding of primers occurring, insomuch as even a small difference in annealing temperature is sufficient to produce different patterns.

Some variants of RAPD markers have been independently developed named AP-PCR and DAF. They differ from RAPDs essentially in primer length, the stringency conditions, and the method of separation/detection of the fragments. With AP–PCR (Welsh and McClelland 1990), a single primer 10–15 nucleotides long is employed with an initial amplification of two PCR cycles at low stringency. Thereafter, the remaining cycles are carried out at higher stringency by increasing the annealing temperatures.

RAPDs have been used for many purposes, ranging from studies at the individual level (e.g., genetic identity) to studies involving closely related species. RAPDs have also been applied in gene mapping studies to fill gaps not covered by other markers (Williams et al. 1990; Hadrys et al. 1992).

Moreover, thanks to the speed and efficiency of RAPD analysis, high-density genetic mapping in many plant species such as faba bean (Torres et al. 1993), alfalfa (Kiss et al. 1993), and apple (Hemmat et al. 1994) were developed in a relatively short times. The RAPD analysis of non-isogenic lines (NILs) has been successfully employed in identifying markers linked to disease resistance genes in common bean (*Phaseolus vulgaris*) (Adam-Blondon et al. 1994), tomato (Lycopersicon sp.) (Martin et al. 1991), and lettuce (Lactuca sp.) (Paran et al. 1991).

6.7.3 Amplified Fragment Length Polymorphism (AFLP)

Considered an intermediate between RFLPs and RAPDs methodologies, AFLP technique, developed by the Dutch company, Keygene (Zabeau and Vos 1992) combines the power of RFLP with the flexibility of PCR-based technology. AFLP analysis is based on the combination of the main analysis techniques: DNA digestion using restriction endonuclease enzymes and PCR technology. The AFLP protocol consists of DNA digestion using two different restriction enzymes (typically EcoRI and MseI) (Fig. 6.4), ligation of adapters to the extremity of the restriction fragments, DNA preamplification of ligated product using primers complementary to the adapter and restriction site sequences, DNA amplification of a subset of restriction fragments using selective AFLP primers, and separation and detection of the produced patterns, scoring fragments as either presence or absence among samples. The primer pairs used for AFLP usually produce 50–100 bands per assay. The number of amplicons per AFLP assay is a function of the number of selective

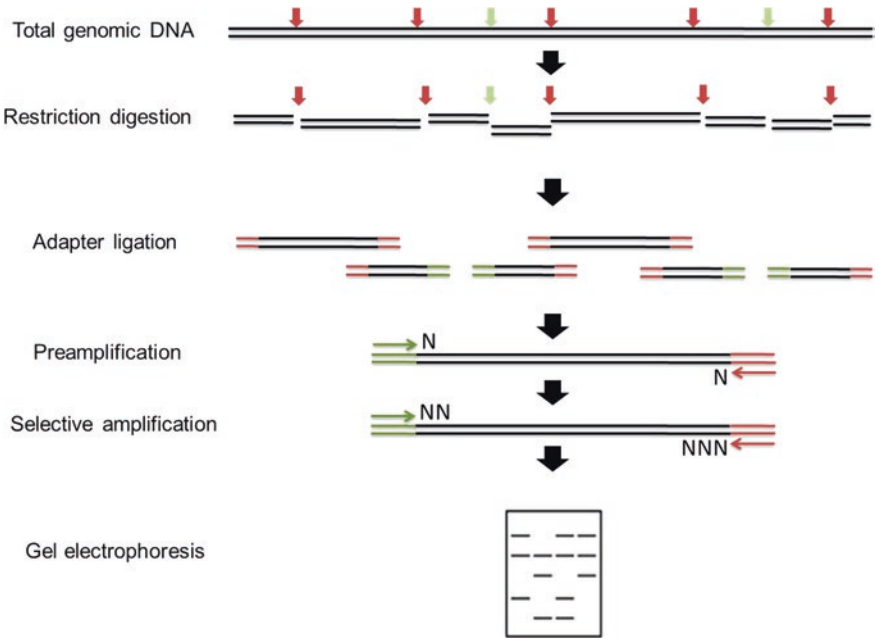

Fig. 6.4 Different steps of Amplified Fragment Length Polymorphism (*AFLP*). Genomic DNA is digested with two restriction enzymes and adaptors are ligated to these ends. The first PCR (preamplification) is performed with a single-bp extension, followed by a more selective primer with up to a 3-bp extension. *N* nucleotide

nucleotides in the AFLP primer combination, the selective nucleotide motif, GC content, and physical genome size and complexity. In particular, AFLP polymorphisms can be produced in different ways: (i) insertions, duplications, or deletions inside amplification fragments; (ii) mutations of sequences flanking the restriction sites and complementary to the extension sites of the selective primers enabling possible primer annealing; (iii) mutations in the restriction site able to create or delete it. All these mutations can bring to an appearance/disappearance of a particular fragment or to the modifications (increase or decrease) of an amplified-restricted fragment.

AFLP generates fingerprints of any DNA regardless of its source, and without any prior knowledge of DNA sequence. Most AFLP fragments correspond to unique positions on the genome and hence can be exploited as landmarks in genetic and physical mapping. The technique can be used to distinguish closely related individuals at the subspecies level (Althoff et al. 2007) and can also map genes.

This technique, being PCR based requires no probe or previous sequence information as needed by RFLP. It is sufficiently reliable because of high stringent PCR in contrast to RAPD's problem of low reproducibility. However, the major advantage of AFLPs is the large number of polymorphisms scored. In fact, AFLP seems

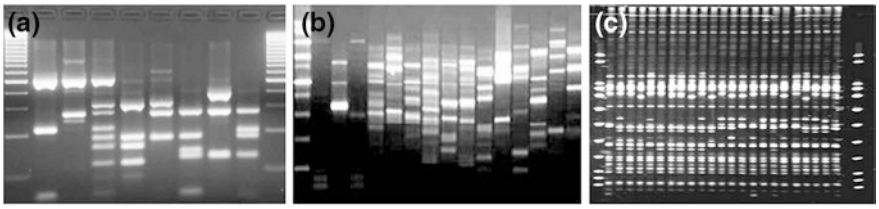

Fig. 6.5 Comparison among different amplification profiles obtained after PCR reactions and staining on ethidium bromide agarose gel: **a** RFLP profile; **b** RAPD profile, and **c** AFLP profile

to be much more efficient than the microsatellite loci in discriminating the source of an individual among putative populations. Similar to RAPD, AFLP analysis allows screening of many loci within the genome in a relatively short time and in an inexpensive way. The weak points of this technique are that this methodology is difficult to analyze due to the large number of unrelated fragments produced and that they are dominant markers.

Nevertheless, their high genomic abundance and generally random distribution throughout the genome make AFLPs a widely valued technology which has been successfully employed for DNA fingerprinting in barley (Becker et al. 1995; Simons et al. 1997), rice (Waugh et al. 1997), in einkorn wheat (Heun et al. 1997), for gene mapping studies (Mackill et al. 1996; Vos et al. 1995; Qi et al. 1998), and for QTL analysis (Powell et al. 1996; Nandi et al. 1997). AFLP markers have been successfully also used for analyzing genetic diversity in some other plant species such as peanut (Herselman 2003), soybean (Ude et al. 2003), and maize (Lübberstedt et al. 2000) (Fig. 6.5).

6.8 Sequence-Specific PCR-Based Markers

The alternative approach to arbitrary PCR amplification consists in the amplification of target regions of the genome using specific primers. In particular, with the advent of high-throughput sequencing technologies, abundant information on DNA sequences of many plant species is now available (Goff et al. 2002; Yu et al. 2002; Arabidopsis Genome Initiative 2000).

6.8.1 Expressed Sequence Tags (EST)–SSR

Expressed Sequence Tags (ESTs) are single-read sequences produced from partial sequencing of a bulk mRNA pool that has been reverse transcribed into cDNA (Putney et al. 1983). High-throughput sequencing produces information on thousands of ESTs and the new sequences are promptly accessible in the different

databases, increasing the growing information on gene expression. EST libraries provide a snapshot of the genes expressed in the tissue at the time of, and under the conditions in which, they were sampled (Bouck and Vision 2007). Despite the several advantages that these kinds of markers show, however, EST–SSRs are not without weak points. At first, the possibility to have null alleles, which compromise the amplification due to primer site variation, resulting in the lacking of visible amplicons. The second that being cDNA lacking of introns, unrecognized intron splice sites could disrupt primer annealing sites making impossible the amplification. Lastly, being EST–SSRs placed within genes and thus more conserved across species, they may be less polymorphic than anonymous SSRs. However, on the contrary, many advantages derive from the fact that ESTs are an inexpensive source for identifying gene-linked markers with higher levels of polymorphism, which can also be applied to closely related species in many cases (Cordeiro et al. 2001; Vasemagi 2005; Karaiskou 2008).

6.8.2 Microsatellite-Based Marker Technique

Microsatellites or Simple Sequence Repeats (SSR) are sequences constituted by sets of repeated motifs found within eukaryotic genomes (Dietrich et al. 1992; Bell and Ecker 1994; Morgante and Olivieri 1993). These sequences comprise basic short motifs (generally between 2 and 6 base pairs long) tandemly repeated several times. Thence, the polymorphisms associated with a specific locus are due to the variation in length of the microsatellite sequence depending on the number of repetitions of the basic motif. The flanking regions of the repeated sequences are mostly conservative and the repetition motifs are highly variable between different species and even different individuals of the same species. In fact, microsatellite assays permit to identify extensive interindividual length polymorphisms during PCR analysis of unique loci using discriminatory primers sets.

Variations in the number of tandemly repeated units are mainly due to polymerase strand slippage occurring during DNA replication where the repeats allow matching, via excision or addition, of repeats (Schlotterer and Tautz 1992). Being the polymerase slippage more probable with respect to point mutations, microsatellite loci tend to be hypervariable.

Microsatellites are among the most used genetic markers for different advantages: (i) they show co-dominant inheritance, (ii) are highly widespread into the genome, (iii) are highly sensible to detect an enormous extent of allelic diversity, (iv) are easy to use and highly reproducible, and (v) different microsatellites can be multiplexed in PCR and automation is possible. However, the development of microsatellites requires preventive and extensive knowledge of DNA sequences. Moreover, sometimes they tend to underestimate genetic structure measurements, hence they have been developed primarily for agricultural species, rather than wild species. Nevertheless, they are not free from disadvantages because: (i) they are time-consuming and expensive to develop; (ii) the heterozygotes may be misclassified as

homozygotes when null alleles occur because of mutations in the primer annealing sites; (ii) stutter bands may complicate accurate scoring of polymorphisms, and even if microsatellite markers are able to identify neutral biodiversity, nevertheless do not provide information about functional trait biodiversity.

The main molecular markers based on assessment of variability generated by microsatellites sequences are: Sequence Tagged Microsatellite Site (STMSs), Simple Sequence Length Polymorphism (SSLPs), Single-Nucleotide Polymorphisms (SNPs), Sequence Characterized Amplified Region (SCARs), and Cleaved Amplified Polymorphic Sequences (CAPS). Moreover, some new markers have recently emerged and are being used in the evaluation of PGR; these include high-density SNP arrays, whole-genome sequencing, and DNA barcoding.

In the main, microsatellite markers detect a high level of polymorphism and being very informative are currently used for population genetics studies due to the capability to be suitable both for the individual level and for closely related species. Microsatellite markers have proven useful for assessment of genetic variation in germplasm collections (Mohammadi and Prasanna 2003). The trend analysis of SSR repeats in genes of known function has permitted to use these markers' typology for association studies with phenotypic variation and biological function (Ayers et al. 1997). Several studies have demonstrated the usefulness of SSRs for estimating genetic relationship and for the detection of functional diversity in relation to adaptive variation (Eujayl et al. 2001; Russell et al. 2004). Microsatellites have been successfully applied also in gene mapping studies (Hearne et al. 1992; Morgante and Olivieri 1993; Jarne and Lagoda 1996).

6.8.3 Single Nucleotide Polymorphisms (SNPs)

The complications found to fully automate microsatellite genotyping and the advent of NGS has renewed the interest of the scientific community in a new type of marker named SNPs. SNPs are the most abundant molecular markers in the genome and consist of single nucleotide variations in genome sequence. SNPs polymorphisms derive from single nucleotide substitutions (transitions/transversions) or single nucleotide insertions/deletions. They are widely dispersed throughout the genomes with a variable distribution among species and are usually more prevalent in the noncoding regions of the genome where their effects are neutral. Nevertheless, when an SNP occurs within the coding regions, it can generate either synonymous mutations that do not alter the amino acid sequence but also non-synonymous mutations resulting in an amino acid sequence changing (Sunyaev et al. 1999). Synonymous changes can modify mRNA splicing generating phenotypic differences (Richard and Beckman 1995). Moreover, a group of associated SNP loci located on a certain region of the chromosome can form one SNP haplotype. SNPs, distributed in both coding and noncoding regions of genomes, represent key players in the process of population genetic variations and species evolution (Syvänen 2001).

The majority of SNP genotyping analyses are based on: allele-specific hybridization, oligonucleotide ligation, primer extension, or invasive cleavage (Sobrino et al. 2005). These kinds of markers can be easily detected using traditional PCR and sequencing, High Resolution Melting (HRM) technology, microchip arrays, and fluorescence technology. These genotyping methods are particularly attractive for their high data throughput and for their suitability for automation.

SNPs can be considered as the third-generation molecular markers coming after RFLPs and SSRs (Peter 2001). To date, SNP markers are not yet routinely applied in gene banks activity, in particular because of the high costs involved, even if they have been successfully applied to investigate genetic variation among different species (Brooks et al. 2010; Amaral et al. 2008). On the contrary, SNP analysis has revealed to be particularly useful for cultivar discrimination in crops where it is difficult to find polymorphisms. SNPs may also be used for a wide range of purposes, including population structure, genetic differentiation, and construction of ultra high-density genetic maps to saturate linkage maps in order to locate relevant traits in the genome. For instance, a high-density linkage map developed in *Arabidopsis thaliana* was completed only after SNP markers development (Cho et al. 1999). Moreover, linkage disequilibrium (LD) among different SNPs can be utilized for association analysis. Furthermore, SNPs can produce information concerning population diversity and evolution (origins, differentiation, and migrations) via SNP haplotypes among different populations. Compared with previous markers, SNPs show the following advantages because they are:

– abundant and widely distributed throughout the entire genome;
– characterized by a high genetic stability, excellent repeatability, and high accuracy;
– they lend to automation and fast high-throughput genotyping;
– being co-dominant are able to distinguish heterozygote from homozygote alleles.

6.8.4 SNP Markers and Whole-Genome Sequencing

One disadvantage of SNP markers consist in the low level information obtained respect to the highly polymorphic microsatellite markers. Nevertheless, this inconvenience can be compensated employing a higher numbers of markers (SNP chips) and whole-genome sequencing (Werner et al. 2002, 2004). Thanks to the improvement of sequencing technology with the advent of high-throughput sequencing, whole-genome/gene sequencing has permitted the detection and characterization of genetic diversity among individuals. Nowadays, it can be considered the most straightforward method producing more complete information on the genetic variation among different populations going to detect all the variations within the genome. However, even if a problem with whole-genome sequencing consists in the development of a high-throughput data analysis platform, the in-depth analysis of NGS data, extensively produced by genetics and genomics studies, has strongly increased the accurate calling of SNPs and genotypes thanks also to the development of recent statistical methods able to improve and quantify the

considerable uncertainty associated with genotype calling. Before the advent of NGS, SSR markers were developed using the time-consuming and laborious construction of genomic libraries, starting from recombinant DNA with the consequent isolation and sequencing of clones containing the SSRs. Zalapa et al. (2012) have demonstrated the power of NGS for developing SSRs in plants in a review focusing on their work on cranberry and several other studies where SSRs were developed using Sanger, 454, and Illumina platforms.

6.9 Markers Based on Other DNA Typology

Ribosomal RNA (rRNA) represents another kind of nuclear genome and, due to the fact that some regions of rRNA are well preserved in eukaryotes, has been extensively employed to study genetic diversity. rRNA genes are placed on the specific chromosomal loci Nor, and organized in tandem repeats which can be repeated up to thousands of times. A particular feature of rRNA, which could explain its wide application, consists in the contemporary presence of regions that are highly conserved throughout eukaryotic evolution providing very useful genetic tools and other regions called "Internal Transcriber Spacers" (ITS) that are highly variable and hence can be used to detect polymorphisms at intraspecific level.

Other highly informative approaches exist, based on organelle microsatellite sequences detection. Due to their uniparental mode of transmission, chloroplast (cpDNA) and mitochondrial genomes (mtDNA) allow to detect different patterns of genetic differentiation with respect to nuclear alleles (Provan et al. 1999a, b). Consequently, in addition to nuclear markers, other marker typologies based on chloroplast and mitochondrial microsatellites have also been developed. The cpDNA, which is maternally inherited in most plants, can be considered an additional tool for within-species genetic variation analysis (Ali et al. 1991; McCauley 1994) and has proved to be a powerful tool for phylogenetic studies. Thanks to its good level of conservation within the genome, CpDNA has been employed widely for studying plant populations through the use of PCR–RFLP and PCR sequencing approaches (McCauley 1994), in the detection of hybridization/introgression (Bucci et al. 1998), in the analysis of genetic diversity (Clark et al. 2000), and in obtaining the phylogeography of plant populations (Parducci et al. 2001; Shaw et al. 2005). On the contrary, mitochondrial DNA in plants, being quantitatively scarce, is unsuitable for studying phylogenesis and genetic diversity.

6.9.1 RNA-Based Molecular Markers (RBMs)

Biological responses and the developmental programming in organisms are crucial phenomena, thence the analysis of mechanisms which control their genetic

expression are essential. This has led to the development of markers derived from transcribed/expressed regions of genomes. The greatest advantage of RBMs is that, being derived from the expressed regions of the genome, the generated fragments can easily be associated with phenotypic traits becoming a key tool for genetic mapping studies of Quantitative Traits Loci (QTLs). On the contrary, these markers should be used with caution in such studies aiming to detect genetic variation in natural populations because they may be under selection. RNA-based markers, designed on coding regions of the genome characterized by a good level of conservation, are also expected to be transferable between related species and genera. Among PCR-based marker techniques, inter small RNA polymorphism (iSNAP) is the most recent and is based on endogenous noncoding small RNAs consisting of 20–24 nucleotides that are ubiquitous in eukaryotic genomes where they play important regulatory roles, representing an excellent source for molecular marker development. This technique is highly reproducible and feasible for automation and it has been successfully applied for genome mapping and for genotyping. Nevertheless, a negative point is that being based on the expressed portion of the genome, it could be also affected by phenological plant stage and environmental conditions. Other techniques such as cDNA–SSCP, cDNA–AFLP, cDNA–RFLP, and RAP–PCR are used for differential RNA studies, using selective amplification of cDNA. These techniques are efficient for the identification of common and rare transcripts and for studying genome-wide gene expression (Xiao et al. 2009) and can also be used to identify differences in the expression of different genes under various stress conditions (Song et al. 2012). Moreover, another RBM technique exists consisting in EST–SSR markers where thanks to the recent increase in the availability of EST data, have been developed in a number of plant species groups (La Rota and Sorrells 2004). Technically, EST–SSR is identical to common genomic (gSSR) microsatellites in terms of amplification and detection but differs in primer development and the locations of the primers being generated from the transcribed portion of the genome.

6.9.2 Transposable Elements-Based Molecular Markers

Transposable elements (TE) are mobile DNA sequences which can change their positions in the genome. Based on their excision mechanism, TEs can be divided into Class I (retrotransposons), commonly called ‘copy-and-paste’ elements, and Class II (DNA transposons), or ‘cut-and-paste’ elements (Finnegan 1989). In particular, LTR retrotransposons are elements surrounded by long terminal repeats (LTRs) that do not code for any protein and contain the promoters and terminators for transcription. These regions provide the basis for primer binding sites in many techniques. Retrotransposons represent an excellent basis for the development of markers due to their dispersion (Katsiotis et al. 1996; Suoniemi et al. 1996), ubiquity (Flavell et al. 1992; Voytas et al. 1992), and prevalence in plant genomes; for this reason most TE-based markers utilize Class I retrotransposons.

Even if transposon insertions can be deleterious for host genomes, transposons are actually considered crucial for adaptative evolution favoring the rearrangement of the genomes and the acquiring of novel traits (Miller et al. 1997; Agrawal et al. 1998; May and Dellaporta 1998; Girard and Freeling 1999; Gray 2000). Despite their great contribution to the genome structure, size, and variation, only recently retrotransposons have received attention for the assessment of genetic diversity (Gynheung et al. 2005) where retrotransposons can be used alone or in combination with other markers, such as AFLPs and SSRs. Retrotransposon-based molecular analysis relies on amplification using a primer corresponding to the retrotransposon and a primer matching a section of the neighboring genome. To this type of class of molecular markers belong: Sequence-Specific Amplified Polymorphism (S-SAP), Inter-Retrotransposon Amplified Polymorphism (IRAP), Retrotransposon-Microsatellite Amplified Polymorphism (REMAP), Retrotransposon-Based Amplified Polymorphism (RBIP), and finally, Transposable Display (TD).

6.10 Optimization of Molecular Marker-Based Analysis: Multiplex PCR

Through multiplex PCR system it is possible to contemporarily detect multiple target sequences using simultaneous amplification reactions (James et al. 2003). Multiplex PCR presents many advantages being more sensitive, fast, and easy to perform. The multiplex-ready PCR technology provides several enabling advances in marker genotyping reducing assay costs, increasing information throughput and permitting automation. It requires limited sample concentration, makes it possible to obtain more information per unit of time and using standardized protocols, economizes on reagents, enzyme, buffers and labor, streamlines data analysis, and has a high tolerance to variation in the concentration and quality of DNA samples. Moreover, multiple-tube amplification permits to avoid allelic dropout consisting in an erroneous classification of one locus as homozygous due to the chance amplification of only one of the two heterozygous alleles, and false alleles due to reaction contaminations, PCR slippage artifacts, or other causes (Taberlet et al. 1996, 1999; Broquet and Petit 2004). However, multiplex PCR reactions require several devices such as uniformity in product abundance, especially for simultaneous SSRs and SNPs genotyping, and differential sizes of the amplification fragments obtained in order to connect a specific allele to the marker that characterizes it. In particular, multiplex amplifications using fluorescence detection show high power of discrimination in a single test and permit to jointly analyze up to 10 different genomic loci. This technique has been successfully applied in high-throughput SNP genotyping, gene deletion, mutation, and linkage analysis.

6.11 DNA Barcoding Markers

With the advent of practical computer technologies applied to genetic studies, such new identification technologies have been developed to facilitate the analysis in the presence of an increasing number of samples. Among these, barcoding system is an automatic scanning identification tool that has been applied by biological taxonomists to species classification, referring to a DNA barcode. In particular, a DNA barcode is a short DNA sequence deriving from a standardized region of the genome used for identifying species. DNA barcoding permits using a large-scale screening of one or more reference genes, to assign an unknown individual to an exact specie, and enhance discovery of new species (Hebert et al. 2003; Stoecklem 2003). In this perspective, public libraries of DNA barcodes linked to named specimens are available (Tautz et al. 2002; Hebert et al. 2004). Compared with time-consuming and inefficient traditional morphological classification (Huang et al. 2007), DNA barcoding presents several advantages being very fast and having a high accuracy of 97.9 % (Hajibabaei et al. 2006). On the contrary, in DNA barcoding technique, the genome fragments are difficult to obtain and being relatively conserved have no enough variations.

6.12 Diversity Arrays Technology (DArT)

DArT is a genotyping technology developed to overcome some of the limitations of other molecular marker technologies such as RFLP, AFLP, and SSR (Akbari et al. 2006). DArT represents a fast and cost-effective alternative method to time-consuming hybridization-based techniques, characterizing simultaneously several thousand loci in a single assay. DArT has been successfully applied to genotyping polyploid species with large genomes, such as wheat. This technology generates whole-genome fingerprints by scoring the presence/absence of DNA fragments in genomic representations and acts by reducing the complexity of a DNA sample to obtain a "representation" of that sample. DArT technology consists of several steps: (i) library creation, (ii) microarray of libraries onto glass slides, (iii) hybridization of fluoro-labeled DNA onto slides, (iv) scanning of slides for hybridisation signal, and (v) data analysis (Fig. 6.6). Among the methods used for DNA complexity reduction, the main method consists of a combination of restriction enzyme digestion and adapter ligation, followed by amplification even if an infinite range of alternative methods can be used. DArT markers for new specie are produced by screening a library deriving from a genomic representation prepared starting from a pool of DNA samples that embrace the diversity of the specie. Thanks to the use of the microarray platform, the discovery process results as more efficient being all markers scored simultaneously, and for each reduction method an independent collection of DArT markers can be assembled on a separate DArT array. The number of markers to use for the analysis of a given species

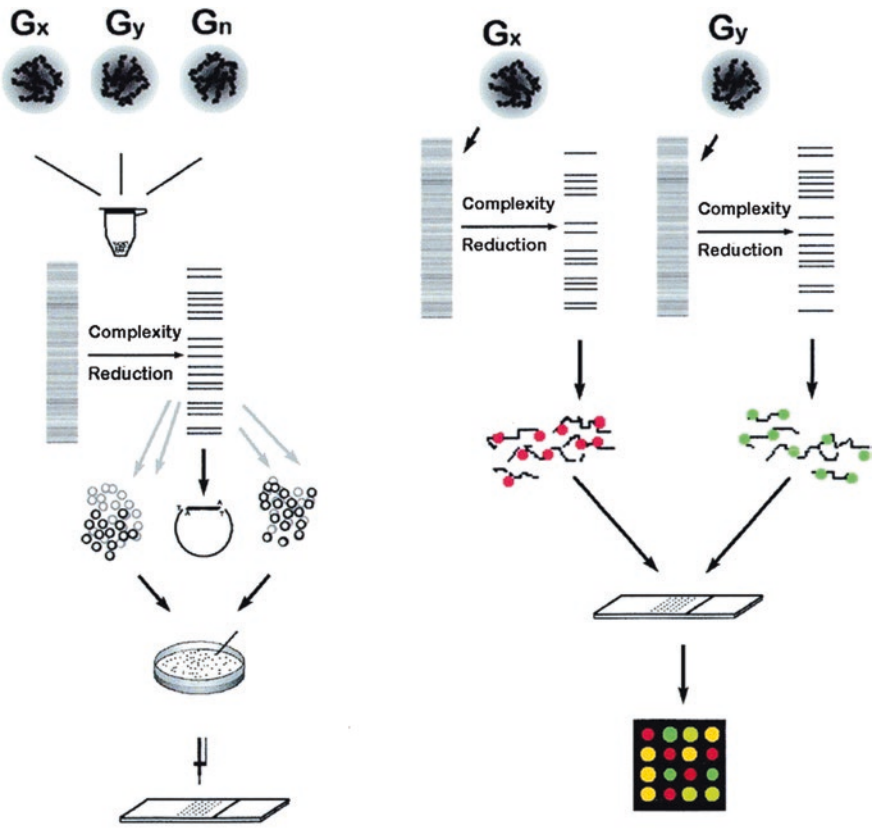

Fig. 6.6 Schematic drawing of DArT pipeline. Gx, Gy, and Gn represent DNA from three different individuals in the reduction step to obtain single genomic DNA

is only dependent on the level of genetic variation within the species (or gene pool) and the number of complexity reduction methods screened. DArT technology was originally developed in rice due to its small genome (430 Mbp) (Jaccoud et al. 2001) and subsequently applied to several other crops. To date, DArT has been successfully applied for genetic mapping and genetic diversity analysis, also to species characterized by large genomes such as wheat and barley, (Mochida et al. 2004; Wenzl et al. 2004) up to the 16,000 Mbp of the hexaploid genome of bread wheat (Akbari et al. 2006).

6.13 Next-Generation Sequencing Technologies

In the past decade, the emergence of NGS technologies has deeply changed all the genetics disciplines that depend on DNA sequence data. NGS technologies have revolutionized and increased the capabilities of traditional Sanger sequencing

method (Sanger et al. 1977), allowing millions of bases to be sequenced in one round at a fraction of the cost. NGS techniques can be distinguished into three main types: sequencing by synthesis, sequencing by ligation, and single-molecule sequencing.

6.13.1 Sequencing by Synthesis

Like Sanger sequencing, NGS techniques use the emission of chemiluminescence created by nucleotide incorporation during synthesis of the complementary DNA strand by DNA polymerase, to determine base composition. In sequencing by synthesis, DNA is fragmented to obtain the appropriate size, ligated to adaptor sequences, and then amplified to enhance the fluorescent or chemical signal. Templates are then separated and immobilized in preparation for flow-cell cycles. Among the techniques available for sequencing by synthesis the most used are *Illumina* (http://www.illumina.com), *Roche 454 pyrosequencing* (http://www.my454.com), and *Ion torrent* (http://www.iontorrent.com), which differ by read length and in how templates are amplified and immobilized.

6.13.2 Sequencing by Ligation

This method is based on the use of oligonucleotide probes which differ in lengths and labeled with fluorescent tags depending on the nucleotide types to be determined (Landegren et al. 1988). The DNA template is fragmented and primed with a short, known anchor sequence favoring the probe hybridization and consequently DNA ligase is added. The fluorescent emission is analyzed to determine which probe was incorporated. This process is repeated with different sets of probes to query the DNA template and assess the sequence of nucleotides. Among the methods based on this technique the most used are *SOLiD* (http://www.appliedbiosystems.com) and *Polonator G.007 system* (http://www.azcobiotech.com/instruments/polonator.php).

6.13.3 Single-Molecule Sequencing

Single-molecule sequencing (SMS) technique, also called "third-generation sequencing," is based on the detection of a chemiluminescent signal produced by nucleotide incorporation occurring during DNA sequencing from a single nucleic acid molecule. This method offers several advantages with respect to other NGS methods because it can make use of degraded or low concentrations of starting material and escape from PCR errors due to template amplification.

Presently, the main techniques based on this method are *Helicos Genetic Analysis System* (http://www.helicosbio.com) and PacBio RS SMS platform (http://www.pacifi cbiosciences.com).

6.14 Conclusion and Prospects

The idea of using gene markers for a variety of purposes in applied genetics, conservation strategies, and genetic diversity assessment is not new. However, until the advent of molecular markers, many of the proposals were technically unfeasible. Molecular analysis of plants has found many applications in plant improvement, in the management of plant production, and in conservation of plant resources. Molecular tools have become key contributors to the management of wild plant populations helping to conserve biodiversity.

Recent dramatic advances in DNA sequencing are now providing cost-effective options for the discovery of very large numbers of markers for any plant species. These developments significantly change the approach to marker discovery and analysis in plants and greatly expand the potential range of application. Advances in biotechnology have resulted in a large variety of molecular marker systems and enhanced opportunities for automation of the majority of the techniques, resulting in a wealth of information. Moreover, due to the developments in the detection techniques, molecular markers are particularly useful in diagnostic applications, such as the screening of samples for the presence or incorporation of favorable traits, the detection of pathogens and diseases in plants, and the screening of plant material for the presence of transgenic elements and jointly with the concept of marker-assisted selection provide new solutions for selecting and maintaining desirable genotypes.

Hence, molecular markers make the prospect excellent for a rapid development of new methodologies for plant genetic diversity dissection that take advantage of the modern techniques.

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Chapter 7
Erosion and Prevention of Crop Genetic Diversity Landraces of Georgia (South Caucasus)

Maia Akhalkatsi

Abstract Georgia (South Caucasus) has many ancient crop varieties used with very old farming traditions and owns linguistics of old civilization coinciding with early Neolithic epoch. The traditional landraces used by local people for thousands of years affected the health and human longevity of individuals in the Georgian population predicting adaptation to healthy food. Crop domestication is associated to existence of crop wild relatives (CWRs) on the territory of Georgia. Molecular studies confirmed domestication of grapevine (*Vitis vinifera*) from wild species (*V. vinifera* subsp. *sylvestris*) and pear varieties from wild Caucasian pear (*Pyrus caucasica*). Many fruits are associated to wild tree species distributed in the refugium territory of the western Georgia. Some crops: wheat, barley, ray, oats, lentil, pea, chickpea, etc., are genetically related with wild species. Therefore, the most important challenges in the near future are certainly the molecular characterization of germplasm collections for preserving them from genetic erosion and the identification of phenotypic variants potentially useful for breeding new varieties. Georgian ancient crop varieties reveal a high level of adaptation to local climatic conditions, and often have high resistance to diseases. The loss of landraces and ancient crop varieties should be considered as main threat to agrobiodiversity in Georgia. Besides the diminishing of the amount of agricultural products, the main threat to agrobiodiversity is the loss of the territory of Georgia. Additionally, there are several reasons for the genetic erosion of the ancient cultivars and the wide distribution of new varieties of introduced crops. Germplasm of the landraces extinct in the local farms are stored only in the gene banks and in the living collections of Georgia and foreign countries. One of the problems is the deficit of information about the current state of ancient crops and recommendations for their conservation are inadequate. Therefore, it is necessary to assess research needs and implications for protection of genetic resources and to formulate recommendations for the conservation and on-farm maintenance of Georgian landraces.

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7.1 Introduction

Georgia is located in the South Caucasus and owns very old agricultural traditions that have preserved to our time. Georgia officially covers a territory of 69,700 km^2, and its population is almost 4.6 million. The name of the country is "Sakartvelo" in the Georgian language but it is common name "Georgia" is semantically linked to Greek (γεωργία, transliterated geōrgía) and Latin (georgicus) roots meaning "agriculture" (Javakhishvili 1930). Archeological data clearly show that Georgian nation was settled in the Caucasus and Asia Minor areas from prehistoric time and the origin of ancient crop varieties and landraces in Georgia coincides with early Neolithic epoch. According to Vavilov (1992), the primary domestication in the fourth center of crop origin and diversity named as the Near East included the South Caucasus, Asia Minor, Iran, and the Fertile Crescent. Many local varieties and endemic species of Georgian ancient crops are known in this domesticated center. Especially, they are characterized by the introduction of the varieties of wheat, rye, oats, seed and forage legumes, herbs, fruits, and grapes for winemaking; 83 species all tolled (Zhukovskij 1962). The ownership of the local cultivars for Georgians living on this territory is confirmed by concrete names of prehistorically Georgian language, fonts, and traditions (Ketskhoveli 1957). The language of the Georgian people is not part of the Indo-European language and belong to the proto-Georgian language group known as Kartveluri (Melikishvili 1970). Moreover, the traditional landraces used by local people for thousands of years affected the health and human longevity of individuals in the Georgian population predicting adaptation to healthy food (Fox 2004). Georgian centenarians were reputed to have been over age 120 in 1959 and the percentage of males over age 70 was 0.9 % in 1959 and 1.07 % of women were over 70 (Garson 1991). This percentage of human longevity is diminished last time, when local population replaced landraces and agriculture is generally oriented on introduced cultivars from different countries.

The loss of landraces and ancient crop varieties should be considered to be the main threat to agrobiodiversity in Georgia. These varieties reveal a high level of adaptation to local climatic conditions, and often have high resistance to diseases. Colchis forest is refugium in the Western Georgia of Tertiary geologic period from 66 to 2.588 million years ago (Nakhutsrishvili 2013). The relict trees of the Colchis forest are remained from Tertiary period till recently and represent the ancestral species. According to palaeontological and palynological data, European territory contained a mixed forest of Colchis type dominated by fir-trees including *Abies nordmanniana* (Steven) Spach and pine-trees, together with the broad-leaved trees *Zelkova* Spach, *Quercus* L., *Ulmus* L., *Tilia* L., *Carpinus* L., *Corylus*

L., *Fagus* L., *Betula* L., and *Castanea* Mill (Paganelli 1996). European beech tree (*Fagus sylvatica*) contains genetic relationships with Caucasian relict *F. orientalis* throughout the Tertiary and Quaternary period, and divergence with relict is determined during the last interglacial period which started around 130,000 years ago (Peffetti et al. 2007). The relict tree species locations in forest vegetation are modeling by GIS program, which potentially existed in six regions of western Asia: Colchis forest of Georgia, western Anatolia, western Taurus, the upper reaches of the Tigris River, Levant, and the southern Caspian basin (Tarkhnishvili et al. 2012). Nowadays, the real existence of relict species is in Colchis forest and the southern Caspian basin. Relict trees are reforested in other modeling regions in the unknown period. Therefore, the Colchis forest tree species might be determined as ancestors of fruit varieties containing only few mutations in the ancient DNA sequences (Peffetti et al. 2007). Nowadays, Georgia represents the natural area of relict ancestor species domesticated as fruits and grape (Akhalkatsi et al. 2012). In spite of this priority, many landraces and ancient varieties of fruits and grape are disappeared in this country.

Besides the diminishing of the amount of agricultural products, the main threat to agrobiodiversity is the loss of the territory of Georgia. This territory in Neolithic/Eneolithic period was settled by Shulaveri-Shomu culture with archeological fossils of ancient crops starting ca. 6200 BC located in the south-eastern Georgia (Javakhishvili 1972). In the period of the 4th millennium BC, there was much large territory of Kura (Mtkvari)-Araxes-Culture with worldwide oldest gold mine named ***Sakdrisi***, Bolnisi district, which is more than 5400-year old and has great historical importance (Hauptmann and Klein 2009). This civilization was inhabited by people who spoke non-Indo-European languages and were spread from the South Caucasus till middle of the Asia Minor, where the dominant inhabitants were Hurrians and Hattians in the central Anatolia at that time (Suny 1994). By 2300 BC, the people of the Kura-Araxes area had already made contacts with the more advanced civilization of Akkadian Mesopotamia (Melikishvili 1970). At the end of the third millennium, the Indo-European population living already in the Hittites country entered in the eastern Anatolia and Georgia remain with western region of Colchis and eastern the Trialeti Culture till 1500 BC (Kavtaradze 1983). In the last centuries of the second millennium, people living in Armenia come from Hittite tablets inhabiting the Armenian plateau (Suny 1994). According to Assyrian inscriptions, the Hittite kingdom fell in about 1190 BC with participation in the destruction by proto-Georgian tribes notably the Kashkai and Tabal called as Muskhi or Meskhi (Melikishvili 1970). After this period, the Muskhi, who settled in the upper Euphrates, migrated in the east-central Anatolia (Edens 1995). The most important tribal formation of proto-Georgians in the post-Hittite period was formation of Diauehi (Diaokhi in Georgian language) in the twelfth century BC in the region to the north of present day Erzerum city in Turkey (Suny 1994). Later, Georgia was occupied by Armenian, Arabian, Mongolian, Persian, Turkish and Russian nations and finally, at 2008 year, the territory was diminished

by 20 % separated by Abkhazian and South Ossetian autonomy. The loss of the territory causes migration of local population and the area remains without traditions and linguistic names.

The occupation of the territories leads to the changes of traditional agriculture. For example, the territory of South Georgian region named Tao-Klarjeti was occupied by Turkey in 1580 AD, when agriculture was substituted by cattle breeding, which caused abandonment of cultivated fields and their transformation into pastures (Javakhishvili 1930). According to old administrative documents after occupation of Georgian part by Turks, in former Georgian village Sviri in Gurjistan Vilayet in Turkey, local population was paying taxes by crops, such as wheat, barley, rye, millet, chickpea, lentil, flax, alfalfa, etc. (Jalabadze 1972). During our expedition in Gurjistan Vilayet of Turkey in 2006, we did not find any of the old traditional field crops cultivated nowadays in the villages. The agriculture in this region is abandoned and substituted by cattle breeding. All vineyards are cut and remained grapes gone wild to make thicket at roadsides and at the edges of the forests. Some vegetables were grown in small house gardens, such as cabbage, sugar beat, carrot, cucumber, tomatoes, etc. However, seeds are bought in markets and there was no information on origin of the seed material, when they might be aboriginal varieties (Akhalkatsi 2009).

Additionally, there are several reasons for the genetic erosion of the ancient cultivars and the wide distribution of new varieties of introduced crops. Intensive Genetic erosion of ancient crops started in Georgia since 1950s, which was also a period of intense selection work in breeding stations in the whole of the Soviet Union. This process has started when '***kolkhoz***' reached extreme level of development in Soviet Republics, and almost all the local varieties of cereals (wheat, barley, rye, oat, Italian millet, and millet), legumes (peas, lentils, common vetch, and faba bean), and landraces of grapes have been replaced by breeding varieties. Recently, introduction of genetically modified (GM) crops is widespread in the territory of Georgia. The conservation of the full range of plant genetic diversity has historically often been associated with the conservation of socio-economically important species, because for these plant species the full range of genetic diversity is required for high market yields. Since 1990, the agricultural market of Georgia was reduced by export diminishing after independence period. This problem was depending on protection measures in the country, which are still not being implemented at an appropriate rate. First of all, new cultivars have higher yields and are therefore preferred both as a source of food for local people and as cash crops that determines local income. Recently, new breeder's varieties of wheat and other cereals with big harvest are introduced from different countries. The second reason why local peasants began to prefer cultivating GM plants may be explained by introduction of new diseases into Georgian agricultural fields in recent years, causing harm primarily to ancient crops and vegetables. However, the introduction of new parasites has revealed that endemic cultivated plants of Georgia contain valuable selective disease-resistant material for genetic engineering.

Otherwise, the real problem is that there are no enough data to assess either the current status of the local varieties or the information about domestication process

in Georgia. Germplasm of the landraces extinct in the local farms are stored only in the gene banks and in the living collections of Georgia and foreign countries. The fundamental work was done by the famous Georgian botanist Menabde (1938, 1948) on domestication and origin of wheat and barley in this region. The agricultural evidence was reported by several other Georgian authors (Ketskhoveli 1957; Khomizurashvili 1973; Akhalkatsi et al. 2012). It was studied domestication of grapevine (*Vitis vinifera* L.) from wild form (*V. vinifera* subsp. *sylvestris*) and pear varieties from wild Caucasian pear (*Pyrus caucasica*) using morphometric and systematic molecular methods confirming genetic relationships between wild populations and local cultivars of grape and pear (Ekhvaia and Akhalkatsi 2010; Asanidze et al. 2011, 2014; Imazio et al., 2013; Ekhvaia et al. 2014). However, complete evaluation of diversity of Georgian local cultivars and crop wild relatives (CWRs) has not yet been complete.

National policies and comprehensive measures are urgently needed to address the problem of conserving the genetic resources of ancient crops in Georgia. Thus, we suggest that it is necessary to establish a general overview of the types of crops that are current landraces and primitive varieties occurring in Georgia and to publish lists of indigenous landraces and CWRs of cereals, legumes, vegetables, and fruits representing direct ancestors, and endemic, rare or endangered species, in order to evaluate the sustainability of their traditional use in terms of nature conservation. Monitoring of crop diversity is now conducted by international nature conservation institutions and Georgian scientific and nongovernmental organizations to preserve the genetic resources of local cultivars. One of the problems is the deficit of information about the current state of ancient crops and recommendations for their conservation are inadequate. Therefore, it is necessary to assess research needs and implications for conservation and to formulate recommendations for the conservation and on-farm maintenance of Georgian landraces.

7.2 Diversity and Genetic Erosion of Landraces

In Georgia, the changes of agricultural land use mainly defects traditional landraces that are maintained within traditional or subsistence farming systems with small areas. Conservation of landraces is oriented on special genes derived from them for selection of modern cultivars of major crops (Zeven 1998). Landraces were widely expected to disappear with the introduction of modern cultivars, but pockets of landrace cultivation have survived, even in countries with the most industrialized and least biodiversity agriculture (Hammer et al. 1999). However, Maxted (2006) has argued that landrace diversity is the most highly threatened component of biodiversity today, and there is only little knowledge of how much diversity actually exists.

The most studied and detailed by archeology and history is the Near East. In spite of the fact that there are many cases of extinctions of landraces in Georgia, there are only a few reports for entire crop species, and there is no example of the

loss of a whole species. Monitoring in the area of Georgia needs report on arable lands ingredients or archeological excavations. The term genetic erosion is concerned to crop plants, and it will need contribution of scientific results to confirm the extinction and threats for landraces and local cultivars. It was basically grape, wheat, and barley agriculture although other crops like common millet, Italian millet, pea, lentil, chickpea, faba bean, etc. Therefore, it is necessary to investigate the landraces origin and use in the historically remnant country as Georgia.

7.2.1 Genetic Erosion of Grapevine Landraces, Vitis Vinifera L. (Vitaceae)

Worldwide, the earliest archeological finding of pips of grapevine cultivars (*V. vinifera*) is discovered in the vicinity of v. Shulaveri and Arukhlo excavations (Figs. 7.1 and 7.2a). This area is located near v. Dmanisi in south-east Georgia, where are found 1.7-Myr-old specimens of small-brained hominids, which is the earliest known hominid site outside of Africa (Gabunia and Vekua 1995). The detected grapevine pipes are dated to ~6000 BC (Ramishvili 1988), when Shulaveri-Shomu culture was located in this area (Javakhishvili 1972). Other archeological evidences of prehistoric winemaking are found near v. Shulaveri and Arukhlo excavations represented by clay vessels for wine storage call ***Qvevri*** in Georgian language (Fig. 7.2b). Other archeological findings of prehistoric winemaking are found near the proximity of the Caucasian region, such as the

Fig. 7.1 Map of Georgia. The administrative regions: *1*. Abkhazia; *2*. Samegrelo-Upper Svaneti; *3*. Guria; *4*. Adjara; *5*. Racha-Lechkhumi; *6*. Imereti; *7*. Samtskhe-Javakheti; *8*. Shida Kartli; *9*. Kvemo Kartli; *10*. Mtskheta-Mtianeti; *11*. Kakheti. The places of archeological excavations are indicated: Dikha-Gudzuba, Nokalakevi, Dzudzuana cave, Arukhlo, Dmanisi and Shulaveri

Fig. 7.2 **a** Archeological finding of pips of grapevine cultivars (*Vitis vinifera*) in the Arukhlo excavations at 6000 BC located in the National Museum of Georgia, Tbilisi; **b** Archeological finding of clay vessels *Qvevri* in the vicinity of v. Shulaveri "Khramis Didi Gora" dated to 6000 BC in the National Museum of Georgia, Tbilisi; **c** *Qvevri* put in the ground of the *Marani* of Nekresi monastery, VI century AD, Kakheti region; **d** stone carving on the medieval church Ananuri, Mtskheta-Mtianeti region, Georgia; **e** *Satsnekheli* for grape pressing made from tree -*Tilia begoniifolia,* trunks; **f** preparation of *Churchkhela* with grape juice of variety Rkatsiteli in Kakheti region, v. Shilda. *Photos* by Maia Akhalkatsi

northern Iran at the Hajji Firuz Tepe site in the northern Zagros mountains, dated ca. 5400–5000 BC (McGovern 2003), and in Levant and Jericho in the Near East, where archeological findings are dated from ca. 4000 to 3200 BC (Zohary and Spiegel-Roy 1975; Zohary and Hopf 1993, 2000). However, this type of wine storage is used in the Georgian lowland regions until today. These territories belonged

to the civilizations contacted to proto-Georgian nation. The name for wine in Indo-European languages was originally borrowed from the Georgian *Gwino* (Javakhishvili 1930). Therefore, Georgia is one of the oldest traditions in wine cultivation. Most researchers accept the opinion that a first domestication event occurred in Georgia (De Candolle 1882; Negrul 1946; Ketskhoveli et al. 1960; Vavilov 1992; Akhalkatsi et al. 2012; Imazio et al. 2013; Ekhvaia et al. 2014).

According to de Candolle (1882), the origin of domestication of crop plants should be determined using four type of evidence. These are archeological (or archaeobotany), botanical (the distribution of the wild, ancestral relatives), historical (a written record documenting the existence or importance of the crop) and linguistic evidence (the existence of words designating the crop or objects or concepts related to the crop in native languages). Although, additional scientific methods such as molecular systematic and radioautographic studies have increased the possibility to determine centers of crop domestication based on archeological and ethnobotanical arguments (Smith 1995). Therefore, crop origin determination needs knowledge on their history and linguistics or semiotics.

Wine is traditionally made and stored in houses called ***Marani***, where the ***Qvevri*** vessels owned by local families are located in ground (Fig. 7.2c). One of the *Qvevri* in each *Marani* is called ***Zedashe*** and contains wine that might be used only in religious rituals. The grapevine was a ritual plant and represented a tree of the Goddess of Sun in ancient religion. Nowadays, according to Georgian folk poetry, the sun is identified to mother called as "sun is my mother" (Javakhishvili 1930). Wine was used in ancient time for toasts at religious holidays. The toastmaster or *Tamada* is elected by the participants in order to present the planned toasts. When Georgian men and women drink wine, it is necessary to say a toast to the God. Ancient cups made of gold and silver as well as jewelery often display grapevines. With the Christianization (322–328 AD), St. Nino from Cappadocia introduced the first cross made from grapevine in the capital Mtskheta and, also stone carvings on Christian churches present grapes Fig. 7.2d). Ancient stone and wood constructions for the pressing of grapes called ***Satsnekheli*** in Georgian language made by tree trunk mainly by *Tilia* spp. (Fig. 7.2e). Grape is used for traditional dessert called ***Churchkhela*** made by cooking grape juice and wheat flour and added walnuts or hazelnuts as vertical lines on cotton thread (Fig. 7.2f). Dry *Churchkhela* is stored all year and used for dessert in all regions of Georgia.

The primary scientific argument of Vavilov (1992) on domestication of crops represents the idea that the centers of origin of cultivars should be characterized by high genetic and morphological variability of both wild and cultivated taxa. About 525 names of autochthonous grapevine landraces known from Georgia show greatest genetic and morphological variability characterized by a wide range of color gamma and shapes of berries and pips (Ketskhoveli et al. 1960; Akhalkatsi et al. 2012; Ekhvaia et al. 2014). These cultivars showed great ampelometric variability and broad adaptability to different climate and soil conditions (Ketskhoveli et al. 1960). It was a high importance to study aboriginal grape varieties in the place of its supposed domestication, and it was already determined genetic relations among native grapevine cultivars and local wild populations (Imazio et al.

2013; Ekhvaia et al. 2014). In the past, the wild grape species—*V. vinifera* subsp. *sylvestris*—providing an important initial impulse to the domestication of grapevine was abundant in the Minor and Greater Caucasus mountain regions (Ramishvili 1988). The distribution area was along the main river basins. The habitat types were riparian, oak-hornbeam, beech, and spruce forests up to 1000 m a.s.l. The populations nowadays are no longer as abundant after the invasion of *Phylloxera* in the middle of the nineteenth century and the current human impact by urbanization. Georgian wild grapevines showed high polymorphism (Ekhvaia and Akhalkatsi 2010). They are dioecious, showing high-variable frequency of female and male plants among populations. Some individuals have berries with white skin, while most have a blue–black coloration. White-fruited phenotype is considered to be determined by the variation present in the gene VvmybA1, a transcriptional regulator of anthocyanin biosynthesis (This et al. 2006). All five haplotypes detected using cpDNA microsatellite markers have been found in the Caucasian ecoregion suggesting that this area is possibly the center of origin of both wild and cultivated grapevines (Grassi et al. 2006). Several autochthonous Georgian varieties—'Saperavi,' 'Rkatsiteli,' 'Tavkveri,' 'Chvitiluri,' 'Kachichi,' 'Shonuri,' and 'Uchakhardani' are genetically related to wild grape populations located in gorges of River Mtkvari, R. Lekhura, and R. Alazani (Akhalkatsi et al. 2012). One of the oldest Georgian grape cultivar 'Krikina,' which is morphologically nearly identical to wild grapevine, shows the genetic similarity to the most ancient Georgian cultivars 'Meskhuri Shavi' cultivated on Meskhetian terraces.

Agricultural regions in Georgia have classified by production of wine (Javakhishvili 1930). Lowlands called *Bari* (0–1300 m a.s.l.) are oriented on wine production and high mountain lands called *Mta* (1300–2200 m a.s.l.) produce beer from barley. Winemaking was main business of agriculture in Georgia. Wine was exporting from Georgia since ancient times. The vineyards were cut down to reduce income for exporting the wine in neighbor countries during the occupation of the country by the Muslim nations. This process causes diminishing of autochthonous Georgian varieties. The other threats started in 1860, the *V. vinifera* was virtually wiped out in the places of its origin, when an aphid, *Phylloxera vastatrix* was accidentally introduced into France, and within a few years had ravaged all vineyards in Europe and in Georgia as well. In currently, almost all Georgian grape varieties have grafted on rootstocks of American grapevines—*V. riparia, V. rupestris,* and *V. berlandieri* and their hybrids, which are resistant to *Phylloxera*. This disaster made it necessary to undertake urgent steps for ex situ conservation of old, endangered and autochthonous grapevine varieties by establishing living collections in Georgia; this had begun in the 1930s. The collections of plant genetic resources were established in research institutes, which have been under reforms since 1990s and operating with diminishing funding to maintain the collections. In 2003, 949 varieties were protected in the living collections. Among them, 701 were cultivars obtained from selective breeding and only 248 of the 525 autochthonous Georgian varieties remain. Recently, these collections have been closed. Nevertheless, some effort has been made to establish new collections in Telavi (573 accessions), Skra (440), and Vachebi (312) in 2008 (Maghradze et al.

2010). The last collection was prepared by organization “Agro Kartu” in surrounding of v. Jighaura, Mtskheta district “Centre for Grapevine and Fruit Tree Planting Material Propagation” with ca 400 varieties. The University of Milan established the new collection in Italy (Maghradze et al. 2010). Some Georgian cultivars are in living collections abroad in Russia, Moldova, and Germany. A small living grapevine collection exists in the G. Eliava National Museum in Martvili district, Samegrelo province, founded in 1972 and containing 24 old Colchis grapevine varieties (Eliava 1992). Seven cultivars of Meskheti region have been collected in the research station of Biological Farming Association Elkana in village Tsnisi, Akhaltsikhe district. Many grape landraces are extinct and do not exist even in living collections.

Georgian native varieties are incorporated in Georgian plant breeding programs in Georgia as in other foreign countries (Imazio et al. 2013). Recently, 193 new varieties were bred in 15 countries, with the contribution of 13 Georgian native varieties (Vakhtangadze et al. 2010). Particularly interesting under this point of view seems to be the history of the Georgian variety ‘Saperavi’ extensively used in Ukraine breeding programs (Goryslavets et al. 2010). Many varieties in neighbor countries are exported from Georgia in Soviet period. Armenia and Azerbaijan have archeological remnants of grape from Kura-Arexes culture on their territories, but the traditions on viticulture these migrated nations did not had and Soviet time they started to produce wine and schnapps from Georgian varieties. Therefore, the name of grapevine cultivars remained as Georgian names, e.g., Armenian varieties ‘Kachet’ means region Kakheti, ‘Mskhali’ means pear in Georgian language, etc. The molecular comparison of varieties with different names in the South Caucasus leads to similarity of these varieties and Armenian ‘Kachet’ and Georgian ‘Kisi’ are located in one cluster of the dendrogram of genetic analyses (Vouillamoz et al. 2006). Historically, it is known that ‘Saperavi’ was exported from v. Tsinandali, Kakheti region to France in nineteenth century (Javakhishvili 1930). Thus, the knowledge on traditions and linguists data is necessary to carry out studies on the crop domestication.

The total area of vineyards in Georgia is 37,421 ha. The largest area of vineyards is located in Kakheti region (Fig. 7.3a) and intensively produced varieties are ‘Rkatsiteli’ for white wine and ‘Saperavi’ for red wine (Table 7.1). Tsolikauri and Tsitska are distributed in Imereti region of the west Georgia (Fig. 7.1). Total 35 autochthonous and 9 introduced varieties are distributed on arable lands. The home gardens contain other local varieties in small amount. Strongly diminished arable land area is in Meskheti, Samtskhe-Javakheti region (Fig. 7.3a). The vineyards in Meskheti was growing on the terraces of Mediterranean type (Fig. 7.3b) in the historic province of Tao-Klarjeti located now in southern Georgia and in the province of Artvin, Turkey. The vineyards of Meskheti were destroyed to the destruction of human settlements. Since fifteenth century, the Seljuk Turks occupied this territory and the vine terraces disappeared, and it was covered with trees or grasses (Fig. 7.3b). However, we have found peasants in some villages of Meskheti province searching for old cultivars in abandoned settlements and some landraces are replanted in house gardens. We have found ancient

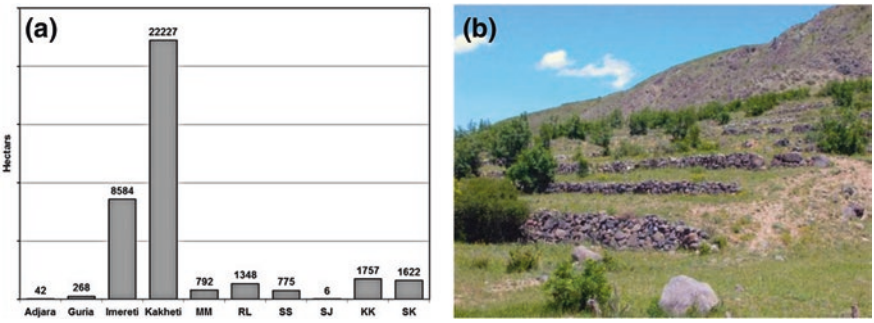

Fig. 7.3 **a** Hectares of vineyards in Georgian regions: *MM* Mtskheta-Mtianeti, *RL* Racha-Lechkhumi, *SS* Samegrelo-Upper Svaneti, *SJ* Samtskhe-Javakheti, *KK* Kvemo Kartli, *SK* Shida Kartli; **b** ancient agricultural terraces in Meskheti, Samtskhe-Javakheti region. *Photo* by Maia Akhalkatsi

Table 7.1 The hectare amounts of grapevine local and introduced varieties in areas of vineyards of Georgia

N	Georgian varieties	Area of vineyards, >20 ha	Georgian varieties	Area of vineyards, <20 ha	Introduced and hybrid varieties	Area of vineyards (ha)
1	Rkatsiteli	19,741	Kisi	20	Isabella (Adessa) Red	413
2	Tsolikauri	6161	Chkhaveri	20	Cabernet Sauvignon	223
3	Saperavi	4300	Shavkapito	10	Pinot Noir	171
4	Tsitska	2839	Kachichi	9	Isabella (Adessa) white	151
5	Chinuri	955	Tbilisuri	9	Aligote	97
6	Dzvelshavi	685	Skhalatubani	9	Pino Gris	91
7	Lomiauri	299	Avisirkhva	7	Chardonnay	2
8	Kakhuri mtsvane	249	Kartuli Tita	7	Chasselas	2
9	Goruli mtsvane	224	Ganjuri	6	Muscat white	1
10	Aleksandrouli	161	AsureTuli shavi	5	Muscat × Aleksandrouli	0.25
11	Rachuli tetra	152	Otskhanuri Sapere	5		
12	Ojaleshi	141	Budeshuri	2		
13	Mujuretuli	58	Mgaloblishvili	1		
14	usakhelouri	57	Khikhvi	1		
15	Aladasturi	46	Dondglabi	1		
16	Krakhuna	36	Kartuli Saadreo	0.01		
17	Tavkveri	29				
18	Orbeluri	25				

grapevine varieties growing before on terraces—'Samariobo Red,' 'Kharistvala Red,' 'Tskhenisdzudzu White,' 'Budeshuri White,' 'Chitiskvertskha White,' etc. Additionally, 'Meskhuri Shavi' (Red) and 'Meskhuri Mtsvane' (Green) are frost resistant and growing in high mountain areas in villages Zemo Vardzia (1322 m a.s.l.), Chachkari (1264 m a.s.l.), Aspindza district; and, Karzameti castle near boundary to Turkey, 1450 m a.s.l.

In conclusion, it should be mentioned that the Georgian cultivated and wild grapevines represent a unique and interesting genetic resources, which are characterized by a high similarity level between wild and cultivated grapevines. The admixture found among local Georgian cultivars and wild grapevine indicates the possibility that these cultivars are derived from ancestral domestication of local wild types. It should be noted that wild grapevine populations occurring nowadays on the territory of Georgia are threatened by different impacts in their natural habitats and need to be protected. Thus, the obtained data are supporting that Georgia is one of the oldest centers of domestication of grapevine and harbor of valuable genetic resources for grape breeding.

7.2.2 Genetic Diversity and Erosion of Cereals

The archeological findings from ancient period of cereal grains in Georgia were discovered from Arukhlo excavations, Nokalakevi settlement and Dikha-Gudzuba (Fig. 7.1). The date includes periods from sixth to second millennium BC (Melikishvili 1970). These archeological monuments presented ancient cities with many buildings contain a lot of gold jewelleries, linen and wool clothing, and many remnants of old food as well in burials (Javakhishvili 1972). The arable lands in Arukhlo excavations were irrigated. The cultivated cereals grains are presented in Arukhlo excavations by seven species of cultivated wheat—*Triticum aestivum, T. spelta, T. carthlicum, T. macha, T. monococcum, T. dicoccum, T. compactum* and one wild relative *Aegilops cylindrica* have been discovered (Menabde 1948). Other cereals: millet—*Panicum milleaceum*, barley—*Hordeum vulgare*, Italian millet—*Setaria italica*, oats—*Avena sativa*, wild lentil—*Lens ervoides,* and pea—*Pisum sativum* have been found in the same site and in Dikha-Gudzuba. Additionally, *T. macha* is archeologically findings in Dikha-Gudzuba and Shulaveri excavations dated by Neolithic period (Javakhishvili 1972) and was cultivated in Racha-Lechkhumi, Imereti, and Samegrelo up to 1950s (Dekaprelevich 1947). A wide range of carbonized seeds, including wheat (*Triticum* sp.), pea (*Pisum sativum*), rowan (*Sorbus* sp.), and walnut (*Juglans regia*), are found in soil samples in Nokalakevi, Western Georgia, dated to the Hellenistic period (Grant et al. 2009).

The cereals of Georgia were studied by Menabde (1948), who investigated origin and phylogenetic relationships of wheat and barley wild and cultivated species distributed in Georgia. The first sign of cereal domestication is the evidence that ears of cultivated cereal crops became less brittle in difference with their wild

relatives characterized by easy shattering of spikes into spikelets upon maturity, which is essential for seed dispersal and survival in the wild, whereas forms with non-brittle ears survive only under cultivation. It is generally assumed that most Triticeae crops have been domesticated from their wild relatives by selection of non-shattering individuals, which sporadically appear in wild populations as rare mutants (Zohary and Hopf 1993). Georgia gives rise to such important crops such as wheat, barley, lentil, chickpea, and pea.

Wheat—*Triticum* spp. According to Menabde (1948), historically distributed 16 cultivated wheat species, 144 varieties, and 150 forms were registered in Georgia in the 1940s (Table 7.2). Among them five species of wheat are Georgian endemics: (1) *Triticum timopheevii* (Zhuk.) Zhuk. subsp. *timopheevii* (***Chelta Zanduri*** in Georgian language), (2) *T. zhukovskyi* V.L. Menabde & Eritzjan (***Zanduri***), (3) *T. turgidum* L. subsp. *carthlicum* (Nevski) Á. Löve & D. Löve (***Dika***), (4) *T. turgidum* L. subsp. *palaeocolchicum* Á. Löve & D. Löve (***Kolkhuri Asli***), and (5) *T. aestivum* L. subsp. *macha* (Dekapr. & V.L. Menabde) Mackey (***Makha***). Seven species are with aboriginal varieties: (1) *T. monococcum* L. (***Gvatsa Zanduri***), (2) *T. turgidum* L. subsp. *dicoccon* (Schrank) Thell. (***Asli***), (3) *T. turgidum* L. subsp. *durum* (Desf.) Husn. (***Tavtukhi***), (4) *T. turgidum* L. subsp. *turgidum* (***Khorbali***), (5) *T. turgidum* L. subsp. *polonicum* (L.) Thell. (***Khorbali***), (6) *T. aestivum* L. subsp. *aestivum* (***Ipkli, Khulugo***), and (7) *T. aestivum* subsp. *compactum* (Host) Mackey (***Kondara, Chagvera, Nagala Puri***). Four species represent geographical races distributed in Georgia from historic periods: (1) *T. aestivum* subsp. *spelta* (L.) Thell., (2) *T. aestivum* subsp. *sphaerococcum* (Percival) Mackey, (3) *T. abyssinicum* Vav. and (4) *T. turgidum* L. subsp. *turanicum* (Jakubz.) Á. Löve & D. Löve.

Additionally, three species from the list are wild: (1) *T. boeticum* ($2n = 14$), (2) *T. dicoccoides* ($2n = 28$), and (3) *T. timopheevii* subsp. *armeniacum* ($2n = 28$); they were mixed with cultivars in the wheat fields and did not exist in natural habitats in Georgia (Menabde 1948). Sites of *T. boeoticum* are concentrated in the eastern Anatolia of Turkey. Studies on einkorn wheat domestication using amplified fragment length polymorphism (AFLP) show that *T. boeoticum* was domesticated in Turkey in the Karacadag Mountains close to city Diyarbakir (Heun et al. 1997). Old proto-Georgian kingdom Diauehi (Diaokhi) was adjacent region to this place in the twelfth century BC (Suny 1994). After migration of Georgian population to current regions, the wheat fields contained mixed wild spicies of *Triticum.* There is evidence that *T. boeoticum* was found in fields with *T. monococcum* in Georgia (Menabde 1948). Since the 1930s, their number has diminished and all of these species had disappeared after the 1960s, when non-aboriginal cultivars were introduced in *kolkhoz*—agricultural farming corporations in Soviet times, changing the species composition in wheat fields. At present, none of these species occurs in agricultural fields of Georgia.

The crop wild relative of wheat, *Aegilops* is related to wheat and a great number of cases have been reported documenting the transfer of genes from the wild relative to the crop, particularly for resistance characters (Hammer 1997). *Aegilops* is presented in Georgia by nine species, one subspecies, and one variety:

Table 7.2 List of wheat species distributed in Georgia by V. Menabde (1948, 1961)

No.	Taxon scientific name	*N* local varieties	Ploidy levels	2*n*	Genomic constitution	Status
1.	*T. boeoticum* Boiss.	1	2*n*	14	A^bA^b	W
2.	*T. monococcum* L.	6	2*n*	14	A^bA^b	PS
3.	*T. timopheevii* (Zhuk.) Zhuk. subsp. *timopheevii*	7	4*n*	28	A^bA^bGG	EG, PS
4.	*T. timopheevii* (Zhuk.) Zhuk. subsp. *armeniacum* (Jakubz.) Slageren	1	4*n*	28	A^bA^bGG	W
5.	*T. turgidum* L. subsp. *dicoccoides* (Körn. ex Asch. & Graebn.) Thell.	5	4*n*	28	A^uA^uBB	W
6.	*T. turgidum* L. subsp. *palaeocolchicum* Á. Löve & D. Löve	3	4*n*	28	A^uA^uBB	EG, SP
7.	*T. turgidum* L. subsp. *dicoccon* (Schrank) Thell.	1	4*n*	28	A^uA^uBB	SP
8.	*T. turgidum* L. subsp. *durum* (Desf.) Husn.	17	4*n*	28	A^uA^uBB	SP
9.	*T. turgidum* L. subsp. *turgidum*	21	4*n*	28	A^uA^uBB	SP
10.	*T. turgidum* L. subsp. *carthlicum* (Nevski) Á. Löve & D. Löve	4	4*n*	28	A^uA^uBB	EG, SP
11.	*T. turgidum* L. subsp. *polonicum* (L.) Thell.	4	4*n*	28	A^uA^uBB	SP
12.	*T. turgidum* L. subsp. *turanicum* (Jakubz.) Á. Löve & D. Löve	1	4*n*	28	A^uA^uBB	IS
13.	*T. dicoccon* subsp. *abyssinicum* Vavilov	1	4*n*	28	A^uA^uBB	IS
14.	*T. aestivum* L.	26	6*n*	42	A^uA^uBBDD	SP
15.	*T. aestivum* L. subsp. *macha* (Dekapr. & V.L. Menabde) Mackey	12	6*n*	42	A^uA^uBBDD	EG, PS
16.	*T. aestivum* subsp. *spelta* (L.) Thell.	12	6*n*	42	A^uA^uBBDD	IS
17.	*T. aestivum* subsp. *sphaerococcum* (Percival) Mackey	9	6*n*	42	A^uA^uBBDD	IS
18.	*T. aestivum* subsp. *compactum* (Host) Mackey	14	6*n*	42	A^uA^uBBDD	SP
19.	*T. zhukovskyi* V.L. Menabde & Eritzjan	1	6*n*	42	$A^bA^bA^bA^bGG$	EG, SP

The status of species is based on phylogenetic studies of V. Menabde (1948, 1961): *EG* endemic of Georgia; *W* wild; *PS* primary species; *SP* secondary species; *IS* introduced species. Ploidy levels and genomic constitution are indicated

Ae. tauschii Coss. subsp. *tauschii, Ae. tauschii* Coss. subsp. *strangulata* (Eig) Tzvelev, *Ae. tauschii* Coss. var. *meyerii* (Griseb.) Tzvelev, *Ae. biuncialis* Vis.; *Ae. columnaris* Zhuk.; *Ae. comosa* Sm., *Ae. cylindrica* Host; *Ae. geniculata* Roth, *Ae. neglecta* Req. ex Bertol.; *Ae. triuncialis* L.; and *Ae. umbellulata* Zhuk. Among them is *Ae. tauschii*, which is considered to be direct ancestor of bread wheat with highest level of gene diversity in populations (0.94) found in a group of accessions from Georgia, Armenia and Daghestan (Pestsova et al. 2000). The D genomes of all varieties of *T. aestivum* were found to be most closely related to accessions of the *Ae. tauschii* subsp. *strangulata* genepool (Fig. 7.4a), which is distributed in the south-eastern Georgia near the archeological areas of Arukhlo and Shulaveri.

The traditional wheat fields in all regions of Georgia usually contain several species and varieties (Eritzjan 1956; Zhizhizlashvili and Berishvili 1980). Bread wheat fields contain: *T. aestivum* var. *erythrospermum* 'Tetri dolis puri,' *T. aestivum* var. *ferrugineum* 'Tsiteli dolis puri,' *T. aestivum* var. *lutescens* 'Upkho tetri dolis puri,' *T. aestivum* var. *milturum* 'Upkho tsiteli dolis puri,' *T. compactum* 'Kondara khorbali.' Usually, this combination of wheat taxa is associated with wild weed ***Makhobeli***—*Cephalaria syriaca* (L.) Schrad. ex Roem. & Schult. (Dipsacaceae) occurring most often in such wheat fields (Fig. 7.4b). The seeds of this species are of the same size as wheat and after threshing, remain in the harvest. Seeds are ground into a powder used with wheat to make bread, cakes, etc. It adds a nice flavor but quickly goes rancid. Another combination of varieties was dominated by *T. durum* 'Shavpkha' composed by *T. durum* var. *apulicum, T. durum* var. *leucurum, T. durum* var. *murciense, T. aestivum* var. *erythrospermum, T. aestivum* var. *pseudo-barbarossa, T. aestivum* var. *lutescens, T. compactum* var. *erinaceum* (Menabde 1948). This population is adapted to dry climate in the lowland areas and in the high elevations up to 1800 m a.s.l. in Javakheti Plateau, where it is sown in early spring. The same character of adaptation to high elevation is typical for the wheat species *T. carthlicum* '*Dika,*' sown on high mountain areas in spring. The combination of varieties dominated by '*Dika*' is as follows: *T. carthlicum* var. *rubiginosum, T. carthlicum* var. *stramineum, T. aestivum* var. *erythrospermum, T. aestivum* var. *ferrugineum, T. compactum* var. *erinaceum* (Zhizhizlashvili and Berishvili 1980).

Wheat is main product for the bread in Georgia. Bread is called ***Puri*** (pronounced "poo-ree"), especially, the long-pointed bread called ***Shotis Puri*** or ***Dedas Puri***. Traditionally, bread is baked in a deep circular clay pot oven called a ***Tone*** (pronounced "ton-AY," Fig. 7.4c). Traditional bread is done from wheat flour with salt and water, otherwise, not used yeast. The technology of bread making is traditional for Georgia and started from Chalcolitic period. Two landraces of bread wheat—*T. aestivum* var. *erythrospermum* and *T. aestivum* var. *lutescens* are used for religious rituals in Svaneti (Girgvliani 2010). The flour of these cultivars is preserved separately from other reserves of bread wheat flour and used on religious holydays. Milled faba bean and kenaf seeds are added to the bread flour for baking ritual bread. There are barley cultivars: *H. vulgare* var. *pallidum* in Svaneti and *H. vulgare* var. *nutans* in Meskheti, used for traditional bread preparation added to the *T. carthlicum* 'Dika' flour.

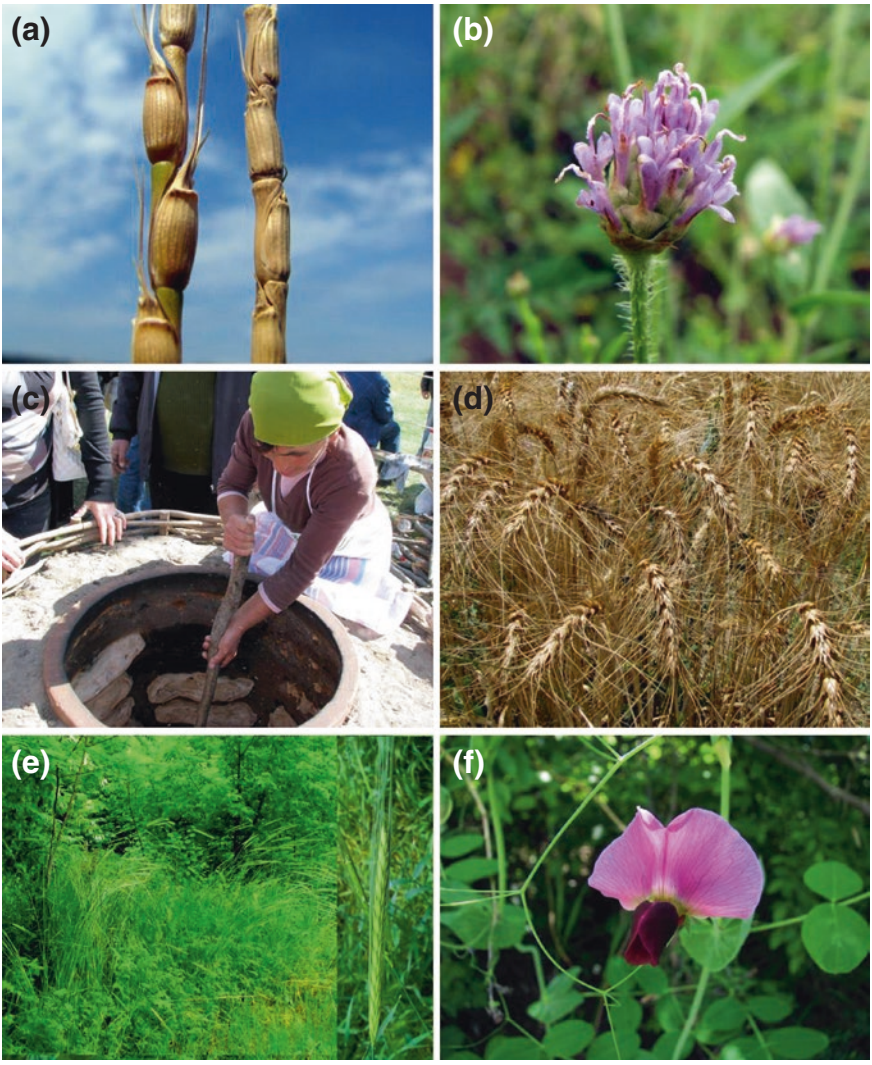

Fig. 7.4 **a** *Aegilops tauschii* subsp. *strangulata* (*left*) and *Ae. tauschii* (*right*) as genepool of **d** genomes of all varieties of *T. aestivum*; **b** wild weed ***Makhobeli***—*Cephalaria syriaca* (Dipsacaceae); **c** clay vessel ***'Tone'*** baking the bread; **d** restores landrace *T. aestivum* var. *ferrugineum* 'Akhaltsikhis tsiteli dolis puri'; **e** *Hordeum spontaneum* in River Vere bank; **f** *Pisum elatius* in Oak-Hornbeam forest edges near Nekresi monastery in Kakheti region. *Photos* by Maia Akhalkatsi

Wheat fields were planted throughout Georgia at elevations from 300 to 2160 m a.s.l. We have found this highest location of soft wheat field in the Eastern Greater Caucasus, village Chero in Tusheti (Akhalkatsi et al. 2010). At present, almost none of these traditional wheat varieties and species occur in the territory of Georgia. Only aboriginal varieties of bread wheat still exist in several high

mountain regions like Tusheti, Meskheti, Javakheti, and Svaneti (Pistrick et al. 2009). Living collections and gene banks preserve the local varieties. The living collection of the Biological Farming Association Elkana has many landraces in village Tsnisi, Akhaltsikhe district. In 2010, they sowed a 10 ha wheat field. The harvest from this field contained local cultivar *T. aestivum* var. *ferrugineum* 'Akhaltsikhis tsiteli dolis puri' (Fig. 7.4d) and weed ***Makhobeli*** (Fig. 7.4b). The flour was baked as bread in Tbilisi and as traditional bread in Meskheti.

Nowadays, there is only bread wheat, *T. aestivum* to be cultivated in Georgia on 94,865 ha. Some varieties are local cultivars breeding in Georgian Selection Stations during 1960–1985 years. These varieties produced from Georgia: 'Vardzia,' 'Dolis Puri 35-4,' 'Dzalisura 35-3,' 'Kakhi-8,' 'Tbilisuri-5,' 'Mukhranuli-7,' etc., are local breeding cultivars and are very similar to bread wheat landraces. Mainly, there are introduced American bread wheat varieties 'Copper' and 'Jagger,' Turkish 'Sultan-95,' Russian 'Basostaya-1' introduced from 1960s in *kolkhoz* remains in Eastern Georgia fields. The landraces seeds are protected in gene bank and living collection. Recently, Georgian monastery priests are oriented on cultivation of landraces of Georgian crops and a wheat variety are sowing from gene banks and in the future is expected restoration of local cultivars.

Barley—*Hordeum vulgare* L. (Poaceae) is the second most important cereal in Georgia after wheat and main crop in high mountain regions used for bread, forage and production of beer, as well as an attribute of religious rituals and in the folk medicine (Javakhishvili 1930). Two different names used for barley in Georgian language—*Krtili* and *Keri*. *Krtili* denotes six-row winter barley (*H. vulgare* subsp. *hexastichon* [L.] Čelak.), which is sowed in autumn; *Keri* refers to two-row summer barley (*H. vulgare* subsp. *distichon* [L.] Körn.), which is sowed in spring (Menabde 1938). The direct ancestor of barley—*H. spontaneum* K. Koch is distributed in River Kura (Mtkvari) valley with joint river gorges (Fig. 7.4e). Six-row barley is sowed in lowland areas. Two-row barley was cultivated mainly in high mountain regions. The cultivars of two-row barley *H. vulgare* var. *nutans* 'Akhaltesli' and *H. vulgare* var. *nigrum* Willd. 'Dzveltesli shavpkha' are distributed up to 2100 m a.s.l. in all high mountain areas. *H. vulgare* var. *nutans* is mixed in the field with wheat—*T. carthlicum* 'Dika,' and the flour is produced from mixed wheat and barley seeds. *H. vulgare* var. *nudum* Spenn. 'Kershveli' was cultivated in Meskheti and Svaneti. Four-row barley (*H. vulgare* subsp. *tetrastichon* [Stokes] Čelak.) is rare and the cultivar—*H. vulgare* var. *pallidum* Ser. 'Tetri Keri' occurs only in the high mountain region of Meskheti, Tusheti, and Svaneti up to 2130 m a.s.l. These cultivars persist today only in high mountain regions. However, their distribution has been seriously diminished. At present, introduced varieties of barley are widely cultivated in the lowlands and their names are unknown to the local population.

Rye—*Secale cereale* L. (Poaceae) is only a local cultivar of high mountain regions of Georgia (1800–2200 m a.s.l.). Fields of *S. cereale* ($2n = 14$) are now found only in Upper and Lower Svaneti and Meskheti. Rye was used for making alcohol and as forage. The wild species, *S. segetale* (Zhuk.) Roshev. ($2n = 42$), called 'Svila' is widespread in wheat and barley fields and is harvested together

with them. The bread of wheat with 'Svila' is considered to be very nutritious and has good taste. An endemic species of rye is *S. vavilovii* Grossh. ($2n = 14$). It is also called Caucasian rye. This species was found in wheat field in Georgia (Bockelman et al. 2002). We have monitored the place in village Beghleti, Khashuri district in 2008, where Georgian botanists had noted the presence of this species in the wheat fields, but cultivated plots no longer exist in that area. The village has lost of most of its residents and no agriculture is undertaken there. Introduced cultivars and commercial varieties of rye are not used in Georgia.

Oats—*Avena sativa* L. (Poaceae) is a traditionally cultivated plant distributed from 400 to 1400 m a.s.l. It is used only as forage for horses and poultry. Two varieties of oats have been described for Upper Svaneti—*A. sativa* var. *aurea* Körn. and *A. sativa* var. *krausei* Körn. (Ketskhoveli 1957). In lowlands, usually, the origin of the seeds is unknown to local farmers. It is purchased in the market and farmers receive no information about their origin.

Millet—*Panicum miliaceum* L. (Poaceae) is very old agricultural plant cultivated in all regions of Georgia. It was used as a supplementary feed (for animals and poultry) and for making alcoholic drinks. At present, it is cultivated only in high mountain regions (1000–1800 m a.s.l.). Several varieties are described in upper and lower Svaneti: *P. miliaceum* var. *aureum* V.M. Arnold & Shibaiev.—grain yellow or cream; *P. miliaceum* var. *subaereum* Körn.—grain gray; *P. miliaceum* var. *griseum* Körn.—grain brown; *P. miliaceum* var. *atrocastaneum* Batalin ex V.M. Arnold & Shibaiev.—grain black; *P. miliaceum* var. *badium* Körn.—grain white (Zhizhizlashvili and Berishvili 1980). The acreage of millet fields declined after introduction of maize in Georgia in seventh century. **Italian millet—*Setaria italica* (L.) P. Beauv. (Poaceae)** was cultivated in Colchis, Samegrelo since ancient times. The cultivar—*S. italica* subsp. *colchica* (Dekapr. & Kaspar.) Maisaya & Gorgidze was represented with 32 landraces (Maisaia et al. 2005). It was cultivated for a long time but was replaced by maize cultivated on 162,875 ha. It can currently be found in the Samegrelo region of western Georgia. Another subspecies—*S. italica* subsp. *moharia* (Alef.) H. Scholz., is called *Kvrima* in Georgian.

7.2.3 Biodiversity of Landraces and CWRs of Fruits and Vegetables

Extinct local landraces are detected as legumes—peas, lentils, chickpeas, faba beans, common vetch, bitter vetch, chickling vetch, alfalfa, sainfoin, and blue fenugreek containing CWRs in Georgia. The local cultivar of green pea, *P. sativum* subsp. *transcaucasicum* Govorov, has 14 varieties (Kobakhidze 1974). Another cultivar species, *P. arvense* is distributed only in home gardens with purple flowers, ridged dark colored seeds. One wild species *P. elatius* Steven ex M. Bieb. with dark purple flowers is often found in locations of old settlements, ruins of

monasteries, and churches and inside castle walls (Fig. 7.4f). Local varieties of Chickpea (*Cicer arietinum*) are rarely cultivated today. Three subspecies and 24 varieties were available in western Georgia—Racha-Lechkhumi, Svaneti and Imereti up to 1920s (Dekaprelevich and Menabde 1929). Chickpeas were traditionally available in Svaneti, but by the 1970s only one farmer was sowing it in Kala community village Khe (Zhizhizlashvili and Berishvili 1980). The Biological Farming Association Elkana is producing local cultivars of chickpea and selling them in market. Lentil (*Lens culinaris*) was represented in Georgia by two subspecies—*L. culinaris* subsp. *macrosperma* N.F. Mattos and *L. culinaris* subsp. *microsperma* N.F. Mattos; and 15 varieties (Kobakhidze 1974). Two wild species (*Lens nigricans* (M. Bieb.) Webb & Berth. *Lens ervoides* (Brign.) Grande) are available on the territory of Georgia. Lentil was cultivated in Meskheti till 1970s and in Svaneti till 2008. Now it is completally extinct and the Biological Farming Association Elkana is producing local cultivars of lentil for the market. Faba bean (*Vicia faba*) with three varieties and 31 subvarieties are described in Georgia with small (*V. faba* var. *minor* Beck.), medium (*V. faba* var. *equina* Pers.), and large (*V. faba* var. *major* Harz.) seeds (Kobakhidze 1974). At present, the large seed Faba bean is widely distributed only in upper and lower Svaneti. Chickling vetch (*Lathyrus sativus*) is used as human food in a soup to called s*hechamandi.* It is also green forage, used as silage and fed as seed flour to pigs and poultry. It is now available only at the research station of the Biological Farming Association Elkana. Bitter vetch—*Vicia ervilia*—is distributed in Meskheti and Javakheti. There are cultivated and wild species of this species. It is used as a forage and for soil enrichment with nitrogen. Common vetch (*Vicia sativa*) is used as forage and for hay, especially in upper and lower Svaneti and Javakheti. It is a valuable forage crop, rich in proteins. More often, it appears as a weed in the fields of high mountain areas among grain crops—millet, barley, and rye. Sainfoin (*Onobrychis* spp.), alfalfa (*Medicago sativa*), and clover (*Trifolium* spp.) are forage legumes. A local variety of *Onobrychis transcaucasica* Grossh. 'Akhalkalakuri,' is widely used. Blue fenugreek (*Trigonella caerulea*) is traditional spice plant used in almost all of the foods of Georgian cuisine. It is available in all regions of Georgia. CWR grain legumes such as *Phaseolus, Vicia, Vigna, Lens, Lathyrus, Cicer*, and some vegetables and industrial crops.

Vegetable and herb landraces are represented by sugar beets, spinach, carrots, radishes, turnips, onions, Welsh onion, leeks, garlic, parsley, coriander, tarragon, sweet basil, savory, gardencress pepperweed, dill, fennel, celery, garden lettuce, peppermint, etc. (Akhalkatsi et al. 2012). These landraces are not threatened as are cultivated in home gardens, and all villages contain these varieties.

Large agricultural product in Georgia is oriented on fruits and vegetables (Fig. 7.5a). Many cultivated plants have been introduced since ancient times to Georgia from other regions of the world (Javakhishvili 1930).

Some introduced crops have become very popular and widespread: cucumber (*Cucumis sativus)*, eggplant (*Solanum melongena*), marigold (*Tagetes patula*), and black pepper (*Piper nigrum*) were introduced from India; Watermelon (*Citrullus lanatus*) from South Africa; Maize (*Zea mays*), sunflower (*Helianthus annuus*),

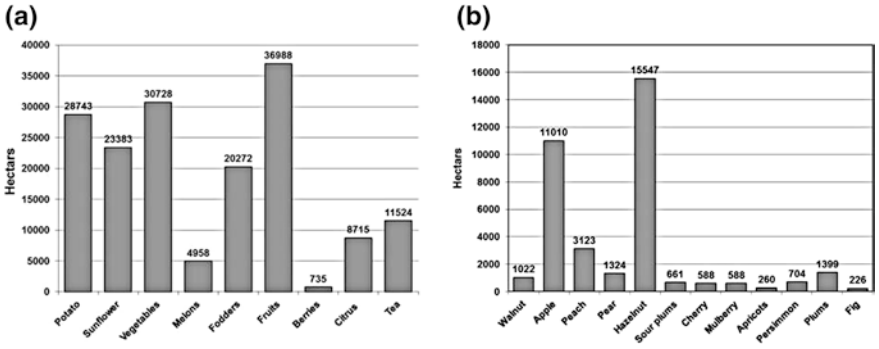

Fig. 7.5 **a** Hectares of vegetables and fruits in Georgia; **b** hectares of fruits in Georgia

tomato (*Solanum lycopersicum*), bean (*Phaseolus vulgaris*), pepper (*Capsicum annuum*), and potato (*Solanum tuberosum*) were introduced from the Americas at about the same time as in Europe (Javakhishvili 1930). Tea (*Camellia sinensis*) and citrus fruits (*Citrus limon, Citrus reticulata, and Citrus sinensis*) came from China in the 1830s. *Nicotiana rustica,* (tutuni in Georgian) has been cultivated for a long time and *N. tabacum*, was introduced during the Soviet period, and was cultivated in *kolkhoz* for commercial use. In spite of many introduced vegetables and fruits, there are local varieties of many cultivated plants, which are diminished and occur under threats.

Fruits are valuable landraces in Georgia. Most fruit trees in Georgia are wild in forests and have cultivars domesticated from these wild ancestors (Asanidze et al. 2011). Perennial fruits, nuts, and citruses are reduced by territories from 101,400 ha in 1990 to 60,000 ha at 2005. Mainly reduced the landraces and introduced varieties are added to apple and pear in high value (Fig. 7.5b).

Almost all landraces are associated to CWRs distributed on the territory of Georgia. Total of 20 plant families, 76 genera, and 479 species were identified as wild relatives of ancient crops in Georgia (Akhalkatsi et al. 2012). Most of these plant species are closely related genetically to landraces and might be their progenitor species, according to gene pool concept (Maxted et al. 2006). Some CWRs are identified as Primary Gene Pool (GP-1), within which GP-1A are the cultivated varieties and GP-1B are the wild or weedy forms of the crop; and Secondary Gene Pool (GP-2) which includes the coenospecies (less closely related species) from which gene transfer to the crop is possible but difficult using conventional breeding techniques. The GP-1 and GP-2 are determined for landraces of fruits, cereals, legumes, herbs and grape (Table 7.3). Twenty-five species are taxonomically similar as cultivars and CWRs with GP-1A but distributed in natural habitats. Twenty species are very close related to cultivars and are determined as GP1B. GP2 means possibility of gene transfer between cultivars and CWRs. Many fruits are domesticated in the Caucasus from wild ancestors representing Primary Gene Pool (GP-1B) to be the wild species of the trees. The fruit crops (GP1A) and ancestor species (GP-1B) are the following: Pome fruits—pear (*Pyrus*

Table 7.3 Gene pool and taxon group of CWRs to Georgian ancient crops

GP	TG	Crop	CWRs	Family
GP1A	TG1A	*Cannabis sativa*	*Cannabis sativa* L.	Cannabaceae
GP1A	TG1A	*Castanea sativa*	*Castanea sativa* Mill.	Fagaceae
GP1A	TG1A	*Carum carvi*	*Carum carvi* L.	Apiaceae
GP1A	TG1A	*Cornus mas*	*Cornus mas* L.	Rosaceae
GP1A	TG1A	*Diospyros lotus*	*Diospyros lotus* L.	Ebenaceae
GP1A	TG1A	*Ficus carica*	*Ficus carica* L.	Moraceae
GP1A	TG1A	*Fragaria vesca*	*Fragaria vesca* L.	Rosaceae
GP1A	TG1A	*Humulus lupulus*	*Humulus lupulus* L.	Cannabaceae
GP1A	TG1A	*Juglans regia*	*Juglans regia* L.	Juglandaceae
GP1A	TG1A	*Lepidium sativum*	*Lepidium sativum* L.	Brassicaceae
GP1A	TG1A	*Linum usitatissimum*	*Linum usitatissimum* L.	Linaceae
GP1A	TG1A	*Mespilus germanica*	*Mespilus germanica* L.	Rosaceae
GP1A	TG1A	*Morus alba*	*Morus alba* L.	Moraceae
GP1A	TG1A	*Morus nigra*	*Morus nigra* L.	Moraceae
GP1A	TG1A	*Petroselinum crispum*	*Petroselinum crispum* (Mill.) A. W. Hill	Apiaceae
GP1A	TG1A	*Pisum sativum*	*Pisum sativum arvense* (L.) Poir.	Fabaceae
GP1A	TG1A	*Prunus cerasifera*	*Prunus cerasifera* Ehrh. var. *divaricata* (Ledeb.)L.H.Bailey	Rosaceae
GP1A	TG1A	*Prunus domestica*	*Prunus domestica* L. subsp. *insititia* (L.) C. K. Schneid.	Rosaceae
GP1A	TG1A	*Punica granatum*	*Punica granatum* L.	Punicaceae
GP1A	TG1A	*Rubus idaeus*	*Rubus idaeus* L.	Rosaceae
GP1A	TG1A	*Secale cereale*	*Secacle cereale* L. subsp. *segetale* Zhuk.	Poaceae
GP1A	TG1A	*Staphylea colchica*	*Staphylea colchica* Steven	Staphyleaceae
GP1A	TG1A	*Staphylea pinnata*	*Staphylea pinnata* L.	Staphyleaceae
GP1A	TG1A	*Trigonella caerulea*	*Trigonella caerulea (L.) Ser.*	Asteraceae
GP1A	TG1A	*Vicia sativa*	*Vicia sativa* L.	Fabaceae
GP1B	TG1B	*Asparagus officinalis*	*Asparagus verticillatus* L.	Asparagaceae
GP1B	TG1B	*Asparagus officinalis*	*Asparagus caspius* Schult. & Schult. fil.	Asparagaceae
GP1B	TG1B	*Asparagus officinalis*	*Asparagus officinalis* L.	Asparagaceae
GP1B	TG1B	*Cerasus avium*	*Cerasus avium* (L.) Moench	Rosaceae
GP1B	TG1B	*Coriandrum sativum*	*Coriandrum sativum* L.	Apiaceae
GP1B	TG1B	*Corylus avellana*	*Corylus avellana* L.	Betulaceae
GP1B	TG1B	*Cydonia oblonga*	*Cydonia oblonga* Mill.	Rosaceae
GP1B	TG1B	*Daucus carota*	*Daucus carota* L.	Apiaceae
GP1B	TG1B	*Hordeum distichon*	*Hordeum spontaneum* K. Koch	Poaceae
GP1B	TG2	*Hordeum hexastichon*	*Hordeum bulbosum* L.	Poaceae
GP1B	TG1B	*Lens culinaris*	*Lens culinaris* Medik. subsp. *orientalis* (Boiss.) Ponert	Fabaceae

(continued)

Table 7.3 (continued)

GP	TG	Crop	CWRs	Family
GP1B	TG1B	*Linum usitatissimum*	*Linum bienne* Mill.	Linaceae
GP1B	TG1B	*Pisum sativum*	*Pisum elatius* M. Bieb.	Fabaceae
GP1B	TG1B	*Prunus domestica*	*Prunus spinosa* L.	Rosaceae
GP1B	TG1B	*Pyrus communis*	*Pyrus caucasica* Fed.	Rosaceae
GP1B	TG1B	*Pyrus communis*	*Pyrus balansae* Decne.	Rosaceae
GP1B	TG5	*Triticum aestivum*	*Aegilops tauschii* Coss. subsp. *tauschii*	Poaceae
GP1B	TG5	*Triticum aestivum*	*Aegilops tauschii* Coss. subsp. *strangulata* (Eig) Tzvelev	Poaceae
GP1B	TG5	*Triticum aestivum*	*Aegilops tauschii* Coss. var. *meyerii* (Griseb.) Tzvelev	Poaceae
GP1B	TG1B	*Vitis vinifera*	*Vitis vinifera* subsp. *sylvestris* (C.C.Gmel.) Hegi	Vitaceae
GP2	TG2	*Amygdalus communis*	*Amygdalus georgica* Desf.	Rosaceae
GP2	TG2	*Avena sativa*	*Avena barbata* Pott ex Link	Poaceae
GP2	TG2	*Avena sativa*	*Avena sterilis* L.	Poaceae
GP2	TG2	*Beta vulgaris*	*Beta maritima* L.	Chenopodiaceae
GP2	TG2	*Brassica oleracea*	*Brassica juncea* (L.) Czern.	Brassicaceae
GP2	TG2	*Brassica oleracea*	*Brassica napus* L.	Brassicaceae
GP2	TG2	*Brassica oleracea*	*Sinapis arvensis* L.	Brassicaceae
GP2	TG2	*Carum carvi*	*Carum caucasicum* (M. Bieb.) Boiss.	Apiaceae
GP2	TG2	*Carum carvi*	*Carum grossheimii* Schischk.	Apiaceae
GP2	TG2	*Carum carvi*	*Carum meifolium* (M. Bieb.) Boiss.	Apiaceae
GP2	TG2	*Carum carvi*	*Carum porphyrocoleon* (Freyn & Sint.) Woronow	Apiaceae
GP2	TG2	*Cicer arietinum*	*Cicer caucasica* Bornm.	Fabaceae
GP2	TG2	*Corylus avellana*	*Corylus colchica* Albov	Betulaceae
GP2	TG2	*Corylus avellana*	*Corylus iberica* Wittm. ex Kem.-Nath.	Betulaceae
GP2	TG2	*Corylus avellana*	*Corylus imeretica* Kem.-Nath.	Betulaceae
GP2	TG2	*Corylus avellana*	*Corylus kachetica* Kem.-Nath.	Betulaceae
GP2	TG2	*Corylus avellana*	*Corylus pontica* K. Koch	Betulaceae
GP2	TG2	*Fragaria vesca*	*Fragaria moschata* Duch.	Rosaceae
GP2	TG2	*Fragaria vesca*	*Fragaria viridis* Duch.	Rosaceae
GP2	TG2	*Lactuca sativa*	*Lactuca georgica* Grossh.	Asteraceae
GP2	TG2	*Lactuca sativa*	*Lactuca saligna* L.	Asteraceae
GP2	TG2	*Lactuca sativa*	*Lactuca serriola* L.	Asteraceae
GP2	TG2	*Lathyrus sativus*	*Lathyrus tuberosus* L.	Fabaceae
GP2	TG2	*Lens culinaris*	*Lens nigricans* (M. Bieb.) Webb & Berth.	Fabaceae
GP2	TG2	*Lens culinaris*	*Lens ervoides* (Brign.) Grande	Fabaceae
GP2	TG2	*Malus domestica*	*Malus orientalis* Uglitzk.	Rosaceae
GP2	TG2	*Panicum miliaceum*	*Panicum capillare* L.	Poaceae

(continued)

Table 7.3 (continued)

GP	TG	Crop	CWRs	Family
GP2	TG2	*Panicum miliaceum*	*Panicum sumatrense* Roth	Poaceae
GP2	TG2	*Panicum miliaceum*	*Panicum dichotomiflorum* Michx.	Poaceae
GP2	TG2	*Ribes rubrum*	*Ribes alpinum* L.	Grossulariaceae
GP2	TG2	*Ribes rubrum*	*Ribes caucasicum* M. Bieb.	Grossulariaceae
GP2	TG2	*Satureja hortensis*	*Satureja laxiflora* K. Koch	Lamiaceae
GP2	TG2	*Satureja hortensis*	*Satureja spicigera* (K. Koch) Boiss.	Lamiaceae
GP2	TG2	*Secale cereale*	*Secale strictum* subsp. *anatolicum* (Boiss.) K. Hammer	Poaceae
GP2	TG2	*Secale cereale*	*Secale strictum* subsp. *kuprijanovii* (Grossh.) K. Hammer	Poaceae
GP2	TG2	*Setaria italica*	*Setaria viridis* (L.) P. Beauv.	Poaceae
GP2	TG2	*Setaria italica*	*Setaria verticillata* (L.) P. Beauv.	Poaceae
GP2	TG2	*Setaria italica*	*Setaria glauca* (L.) P. Beauv.	Poaceae
GP2	TG2	*Setaria italica*	*Setaria intermedia* Roem. & Schult.	Poaceae
GP2	TG2	Spinacia oleracea	*Spinacea tetrandra* Stev.	Chenopodiaceae
GP2	TG5	*Triticum aestivum*	*Aegilops biuncialis* Vis.	Poaceae
GP2	TG5	*Triticum aestivum*	*Aegilops columnaris* Zhuk.	Poaceae
GP2	TG5	*Triticum aestivum*	*Aegilops comosa* Sm.	Poaceae
GP2	TG5	*Triticum aestivum*	*Aegilops cylindrica* Host	Poaceae
GP2	TG5	*Triticum aestivum*	*Aegilops geniculata* Roth	Poaceae
GP2	TG5	*Triticum aestivum*	*Aegilops neglecta* Req. ex Bertol.	Poaceae
GP2	TG5	*Triticum aestivum*	*Aegilops triuncialis* L.	Poaceae
GP2	TG5	*Triticum aestivum*	*Aegilops umbellulata* Zhuk.	Poaceae
GP2	TG2	*Vicia faba*	*Vicia johannis* Tamamsh.	Fabaceae
GP2	TG2	*Vicia faba*	*Vicia narbonensis* L.	Fabaceae

GP gene pool; *TG* taxon group (Maxted et al. 2006)

communis, P. caucasica), apple (*Malus domestica, M. orietalis*), quince (*Cydonia oblonga*); stone fruits—plum (*Prunus domestica, P. domestica* var. *insititia, P. spinosa*), myrobalan (*Prunus vachushti*), sour plum (*Prunus cerasifera* var. *divaricata*), cherries (*Cerasus avium, C. vulgaris*), cornel cherry (*Cornus mas*), medlar (*Mespilus germanica*), mulberry (*Morus alba, M. nigra*), pomegranate (*Punica granatum*); berries—red raspberry (*Rubus idaeus*), currant (*Ribes rubrum, R. nigra, R. alpinum, R. biebersteinii*), common fig (*Ficus carica*), bladdernut (*Staphylea pinnata*), and nuts—such as hazelnut (*Corylus avellana*), almond (*Amygdalus communis*), and walnut (*Juglans regia*), etc. Wild and cultivated fruits reveal high species and genetic diversity in Georgia and represent rich material for future breeding activities.

7.3 Genetic Erosion and Conservation Opportunity of Landraces

Agriculture land covers approximately 2.6 million hectares (ha) in Georgia including 839,709 ha of arable lands and 1,760,292 ha of pastures in alpine zone. In 1990–1995, since independence and conflicts during the collapse of the Soviet Union, the territories of agricultural arable lands diminished by 250 thousand ha. In 2004–2012, the next problem of the agriculture sector started by reduction of arable lands about 400 thousand ha depending as well on war and economic crisis, and, additionally, the government has pursued a policy of primary production as a result of neglect. Since 2013, the totally used arable lands reach ca. 480 thousands ha. The main lost arable lands are in mountain regions of the Great Caucasus, where villages are empty, and population in migrated to urban cities and left Georgia to work in foreign countries.

The decrease of agricultural area concerns permanent crops. In 1988, the area under orchards was 130.5 thousand ha and according to the statistical department, results in 2004 it equalled 37.0 thousand ha. The area under vineyards decreased from 117.7 thousand to 37.7 thousand ha. The area under citrus plantations diminished from 27.1 thousand to 8.7 thousand, and the area under tea plantations—from 64.8 thousand to 11.5 thousand. Otherwise, areas under some temporary crops have increased: for wheat from 88.5 thousand ha in 1988 to 94.9 thousand ha in 2004, for maize (for grain) from 108.8 thousand ha to 162.9 thousand ha, for sunflower from 12.4 thousand ha to 23.4 thousand ha. Recently the total area of agricultural land divided into Arable land (472,120 ha), land under perennial plants (100,215 ha) greenhouses (311 ha), and pasture/hay meadows in settlements (267,062 ha).

Very old archeological findings, cultural heritages and so far existing high morphological and genetic diversity of ancient crops and their wild relatives show that Georgia has very old agricultural traditions that have preserved to our times. The threat of agricultural reduction was detected to lose of territories of Georgia in historical time. In the early 1990th, until Georgia get independence, it was one of the main producers and exporters of agricultural products throughout the Soviet Union. Its exports were 70 % higher than its imports (Land 2011). Afterword, agricultural sector oriented in the past for export was destroyed. As a result, the active increase of import of agricultural food products caused almost complete collapse of agriculture in Georgia. In 2004, total agricultural production had fallen by more than half compared to the preindependence period (Land 2011). A severe impact on agricultural exports had as well the Russian Federation's embargo on Georgian products, imposed early in 2006, affecting the livelihoods of rural people. Since 2010, the export has begun to increase, although, import still represents very significant amount and value. In 2009, the imported agricultural food amounted to 1156 million US$ (79 %) and the export was only 246 million US$ (21 %; FAO 2009). Recently the export is increased mainly on wine exporting. Therefore, nowadays, opportunity of significance for Georgian entrepreneurs and

foreign investors in the food and agricultural sector might be presented by export opportunities either as part of an import substitution orientation.

There are several reasons for the genetic erosion of ancient cultivars and the wide distribution of new varieties of introduced crops. The reasons are new diseases into Georgian agricultural fields, causing harm primarily to ancient cereals and vegetables. However, the introduction of new parasites has revealed that the tetraploid and hexaploid endemic wheat species *T. timopheevii* and *T. zhukovskyi*, for example, are characterized by a high level of resistance to a new race (TTKS, commonly known as Ug99) and many other races of *Puccinia graminis* f. sp. *tritici* due to the wheat stem rust resistance gene Sr36 (Tsilo et al. 2008). *T. carthlicum* is characterized by immunity to diseases, a short growing period, and resistance to cold. Therefore, endemic cultivars of Georgian crop plants are important genomic species for breeding new cultivars with valuable selective disease-resistant material for genetic engineering.

Worldwide germplasm collections of crop plant species of Georgia maintained ex situ in gene banks and living collections. According to the National Biodiversity Action Plan of Georgia (Jorjadze 2005), international nature conservation institutions and Georgian scientific and nongovernmental organizations have taken care to preserve the genetic resources of local cultivars. The germplasm preserve is oriented on technologies, which generally means the generation of progressively larger amounts of genetic data. Genotyping individuals to identify the available allelic variation that makes up the phenotypes provide the groundwork on which genetic resources can be used in plant breeding (Barcaccia 2010). Phenotyping is very much linked to the usefulness of good molecular characterization, together forming the basis of progress in modern genomics research in crop plants (De Vicente et al. 2006).

Several gene banks and living collections occur in Georgia. There is one biggest genebank located at the Georgian Agrarian University Institute of Farming established in 2004 through support of International Centre for Agricultural Research in the Dry Areas (ICARDA). They owned a total 3057 accessions of local and introduced cultivars and CWRs in 2010. The other five gene banks are located in different research institutes unified with Agrarian University in 2011. Total number of germplasm accessions is 6286 in Georgian gene banks. However, the material kept in ex situ collections are not sufficient and need more contribution. Many seed banks worldwide contain about 7000 accessions of germplasm of Georgian cultivars and CWRs.

It should be emphasized that establishment and maintenance of ex situ collections and databases is just a first step in the conservation process of ancient crop varieties. The next step should be return of conserved seed material to the fields of local farmers. From 2004 to 2009, the Global Environmental Facility/United Nations Development Fund (GEF/UNDP) project “Recovery, Conservation and Sustainable Use of Georgia’s Agro-Biodiversity” was carried out with the aim of conservation and sustainable use of threatened local plant genetic resources in the oldest historical mountainous region of Georgia, Samtskhe-Javakheti. This project enabled establishment of sources of primary seed and planting material for

threatened crops and fruit varieties, and assisted farmers in accessing markets for organic products from such crops as lentil, pea, chickpea, faba bean, common millet, and Italian millet. Another project was the return of the Georgian wheat variety *T. aestivum* var. *ferrugineum* 'Akhaltsikhis Tsiteli Dolis Puri' in Meskheti province, where it was sown on 10 ha and produced bread that was introduced in shops featuring organic products in Tbilisi as of 2008. Afterward, this project was supported by the Georgian church, which expressed an interest in cultivating ancient crops on monastery grounds. However, these attempts have been realized only on a small scale and not in larger areas of the country.

7.4 Conclusions

The major activity of the corresponding governmental institutions should be directed on supporting local farmers in reintroducing ancient crops on the market and maintain maximum diversity of the target taxon's gene pool. In our opinion, the most important challenges in the near future are certainly the molecular characterization of germplasm collections for preserving them from genetic erosion and the identification of phenotypic variants potentially useful for breeding new varieties. The Georgian landraces originated in Neolithic period and existing until today represent unique genome to improve the multiplication of accessions and the maintenance of seed stocks for responding to an expected higher demand of materials. This will facilitate the use of, and add value to crop plant from germplasm resources. The importance of agricultural achievements not should be oriented only on high yield of crops but the traditional foods to which people have adapted a long time determines their healthy lifestyle. Thus, conservation and restoration of ancient landraces to modern agriculture can insure longevity of people.

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Chapter 8
Genetic Diversity and Heavy Metal Stress in Plants

Shilpi Srivastava and Atul Bhargava

Abstract Heavy metals are considered as potent pollutants due to their widespread occurrence and their acute and chronic toxic effect on plants, animals, and humans. Variation is of great theoretical importance because it is the raw material on which natural selection acts to influence the evolution of hyperaccumulation. Natural variation is also important basis for the development of hyperaccumulation technology as it indicates the potential for improvement of plant traits through selective breeding, and provides variable genetic markers that can be studied by crossbreeding and molecular techniques. Although some degree of hyperaccumulation occurs in all members of the species that can hyperaccumulate heavy metals, quantitative genetic variation in the ability to hyperaccumulate have been reported, both between and within populations. Genetic diversity and variability analysis have proved to be an effective method in grouping accessions for effective management and utilization in genetic improvement of plants for enhanced phytoextraction. The existing genetic diversity in crops can be used for phytoextraction by identifying easily cultivable, high biomass yielding plants, and practicing selection in future generations.

Keywords Heavy metals · Phytoextraction · Hyperaccumulation · Variability

8.1 Introduction

Stress is an environmental factor that limits crop productivity or causes a reduction in biomass (Grime 1979; Robert-Seilaniantz et al. 2010). Plants are exposed to a variety of stresses in natural environments that may occur singly or concurrently

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(Mittler and Blumwald 2010). Abiotic stress is defined as any environmental condition which reduces the growth, survival, and/or fecundity of plants below optimum levels (Boscaiu et al. 2008; Cramer et al. 2011). Abiotic stresses include parameters like temperature, humidity, light intensity, water supply, mineral availability, oxidative stress, and heavy metal toxicity, all of which determine the growth of a plant (Bhargava and Srivastava 2013). These stresses adversely affect growth and productivity, and trigger a series of morphological, physiological, biochemical, and molecular changes in plants (Ahmad et al. 2012a, b; Bhatnagar-Mathur et al. 2008). The stress factors are a menace for plants and prevent them from reaching their full genetic potential and limit crop productivity worldwide (Mahajan and Tuteja 2005). The effect of stresses is more pronounced in plants since the plants being sessile cannot escape from abiotic stress factors and are continuously exposed without any protection. The stress caused by abiotic factors alter plant metabolism leading to negative effects on growth, development, and productivity of plants (Rao et al. 2006). It is estimated that environmental stresses limit crop production by more than 50 % and as much as 70 % (Boyer 1982; Wang et al. 2003; Mittler 2006). If the stress becomes harsh or continues for longer periods it may lead to unbearable metabolic burden on cells, reduced growth and ultimately plant death. Thus, the losses worth hundreds of million dollars each year due to reduction in crop productivity and crop failure as a result of different stresses are threatening the sustainability of agricultural industry. However, plants have developed specific mechanisms that enable them to detect environmental changes and respond to complex stress conditions, minimizing damage while conserving valuable resources for growth and reproduction (Atkinson and Urwin 2012).

8.2 Heavy Metals

Different metals are required by plants in a wide range of concentrations. During the evolution of angiosperms, the metal requirements were strongly steered by the demands of physiological processes in different organelles, cells, tissues, and whole plants (Ernst 2006). Heavy metals, the ubiquitous environmental contaminants, are members of an ill-defined group of elements who have a specific gravity of more than 5 g/cm^3 in their standard state (Padmavathiamma and Loretta 2007; Bothe 2011; Bhargava and Srivastava 2014). According to this criterion, a total of 53 elements are regarded as heavy metals some of which are of importance to living forms while others are toxic. Heavy metals such as iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), cobalt (Co), or molybdenum (Mo) are essential for the growth of life forms while others have a single function such as vanadium (V) in some peroxidases and nitrogenases, or nickel (Ni) in the hydrogenases (Bothe 2011). Heavy metals like cadmium (Cd), lead (Pb), uranium (U), thallium (Tl), chromium (Cr), silver (Ag), and mercury (Hg) are toxic to organisms. Arsenic is a metalloid but is usually classified as a heavy metal. In the soil metals, it may exist in the following forms:

1. Bound to organic matter;
2. As ions occupying ion exchangeable sites and specifically adsorbed on inorganic soil constituents;
3. Free metal ions and soluble metal complexes in solution;
4. Precipitated or insoluble forms like oxides, sulfides, carbonates, and hydroxides; and
5. Entrapped in the structure of silicate minerals.

8.2.1 Sources of Heavy Metals

Contamination of soil, aqueous streams, and ground water with toxic metals poses a major environmental problem and is a hazard to human health (Bothe 2011; Bhargava et al. 2012a). This contamination is primarily due to human activities that have resulted in the increased release of heavy metals in the environment. Heavy metals in atmosphere, soils, water, and sediments pose a serious problem: they can enter and pass through the food chains, and in contrast to organic xenobiotics cannot be degraded by microorganisms. The problems with metal contamination are particularly pronounced in localities where industrial exploitation has led to accumulation of extreme concentrations of these substances, like the surroundings of smelters, tanneries, waste treatment plants, or mining sites (Baldrian and Gabriel 2002). Air emissions from combustion plants, oil, mining, smelting, electroplating, and military and waste practices are the common contributors of heavy metals in the environment (Sharma and Agrawal 2005; Bhargava et al. 2008, 2012a, b; Bhargava and Srivastava 2014).

8.2.2 Importance of Heavy Metals

Metals play a variety of roles in all living organisms. Metals are important for the living forms since they are the active centers of many enzymes. The chemical properties of the metal have been recruited for catalyzing key reactions and for maintaining protein structure. Metals are therefore required in minute amounts for normal cell metabolism, and their intake is subject to intricate homeostatic mechanisms (Bhargava et al. 2012b). Metals may act as structural elements, stabilizers of biological structures, components of control mechanisms (e.g., in nerves and muscles), and in particular are activators or components of redox systems. Some of the metals are essential elements, and their deficiency results in impairment of biological functions. An overview of the different uses of heavy metals in plants is provided in Table 8.1.

Table 8.1 The importance of heavy metals for plants

Metal	Beneficial effects of heavy metals	References
Cu	Important role in CO_2 assimilation and ATP synthesis Component of plastocyanin and cytochrome oxidase	Thomas et al. (1998) Demirevska-kepova et al. (2004)
Fe	Synthesis of chlorophyll Component of cytochromes	Miller et al. (1995), Spiller et al. (1982) Soetan et al. (2010)
Zn	Synthesis of cytochrome Synthesis of tryptophan and auxin Reduce the adverse effects of short periods of heat and salt stress	Tisdale et al. (1984) Alloway (2004), Brennan (2005) Disante et al. (2010), Tavallali et al. (2010)
Co	Inhibition of ethylene production Role in salt tolerance	Lau and Yang (1976) Ibrahim et al. (1989)
Mn	Activation of enzymes like decarboxylating malate dehydrogenase, isocitrate dehydrogenase, and nitrate reductase	Mukhopadhay and Sharma (1991)
Mo	Regulatory component in the maintenance of nitrogen fixation in legumes Integral part of molybdenum cofactor (Moco) which binds to molybdenum-requiring enzymes	Kaiser et al. (2005), Soetan et al. (2010) Bittner et al. (2001), Mendel and Haensch (2002), Kaiser et al. (2005)
Ni	Cofactor of enzymes involved in DNA biosynthesis and amino acid metabolism Component of the enzyme urease	Arinola et al. (2008) Aydinalp and Marinova (2009)
Hg	No beneficial effect reported	

8.3 Adverse Effects of Heavy Metals on Plants

Heavy metals are considered as soil pollutants due to their widespread occurrence and their acute and chronic toxic effect on plants grown on such soils (Yadav 2010; Manousaki and Kalogerakis 2011). Heavy metals are absorbed through the root systems and are known to induce changes in the plants at morphological, physiological, and molecular levels (Hall 2002; DalCorso et al. 2013). The toxicity in plants varies according to the species, type of metal, its concentration, chemical structure, and edaphic factors (Schützendübel and Polle 2002; Nagajyoti et al. 2010). Heavy metals induce destruction of chlorophyll, necrosis, turgor loss, reduced seed germination, and inhibition of root penetration and plant growth (Foy et al. 1978; Kim et al. 2003; DalCorso et al. 2008; Manousaki et al. 2008; Shakya et al. 2008; Aydinalp and Marinova 2009; Lamb et al. 2010; Singh et al. 2013). Heavy metals also influence homeostatic events like water uptake and transport, transpiration, and nutrient metabolism and leads to the deficiency of minerals like Ca, Mg, K, and P (Fodor 2002; Poschenrieder and Barceló 2004). Table 8.2 depicts the toxic effects of different heavy metals on plant growth and development. The uptake and accumulation of nutrients is influenced by alteration in the

Table 8.2 Toxic effects of heavy metals on plants

Metal	Toxic effect	References
Zn	Chlorosis Purplish-red color in leaves Inhibition of ribulose-1,5-bisphosphate-carboxylase/oxygenase (RuBisCO) Growth retardation in roots and shoots	Ebbs and Kochian (1997) Lee et al. (1996) Van Assche and Clijsters (1986) Choi et al. (1996), Ebbs and Kochian (1997), Fontes and Cox (1998)
Hg	Phytotoxicity and physiological disorders in plants Closure of leaf stomata and physical obstruction of water flow in plants	Zhou et al. (2007) Zhang and Tyerman (1999)
Cu	Growth retardation and leaf chlorosis Generation of oxidative stress, ROS, disturbance of metabolic pathways and damage to macromolecules	Enyedi et al. (1992), Lewis et al. (2001) Stadtman and Oliver (1991), Hegedus et al. (2001) Messer et al. (2005), Israr and Sahi (2006), Cargnelutti et al. (2006)
Co	Adverse effect on shoot growth and biomass	Li et al. (2009)
Mn	Chlorosis, puckering and crinkling of leaves, Leaf abscission, Loss of apical dominance Cytoplasmic injures and plasma membrane rupturing in the outer root cap and meristematic cells	El-Jaoual and Cox (1998), Sirkar and Amin (1974) Santandrea et al. (1998)
Pb	Inhibition of enzyme activities, water imbalance, alterations in membrane permeability and disturbs mineral nutrition	Sharma and Dubey (2005)
Cr	Inhibition of chlorophyll biosynthesis Inhibition of plant growth, chlorosis in young leaves, nutrient imbalance, wilting of tops, and root injury Induces oxidative stress by increasing the production of ROS	Vajpayee et al. (2000) Chatterjee and Chatterjee (2000), Dixit et al. (2002), Sharma et al. (2003), Scoccianti et al. (2006) Reddy et al. (2005)
Ni	Alteration in the lipid composition and H-ATPase activity of plasma membrane Chlorosis and necrosis Suppression of the hydrolysis of RNA and proteins by inhibiting the activity of ribonuclease (RNase) and protease	Ros et al. (1992) Pandey and Sharma (2002), Rahman et al. (2005) Maheshwari and Dubey (2007)
Cd	Inhibition of respiration Inhibition of photosynthesis Inhibition of calmodulin-dependent phosphodiesterase activity	Llamas et al. (2000) Kumar and Kumar (1999) Rivetta et al. (1997)
Mo	Inhibits tasseling, anthesis and the development of sporogenous tissues	Agarwala et al. (1978), Martin et al. (1995)

water absorption and solute permeability caused by the heavy metals (Hernández et al. 1997). The accumulation of heavy metals in plants and their subsequent release during decomposition facilitates their recycling in the ecosystem (Kim et al. 2003). This pathway regulates the level of toxic metals in the biosphere.

The response of plants upon exposure to heavy metal stress is primarily due to the generation of reactive oxygen species (ROS). Various metals either generate ROS directly through Haber–Weiss reactions or overproduction of ROS (Schützendübel and Polle 2002; Mithofer et al. 2004; Anjum et al. 2012). Thus, the occurrence of oxidative stress in plants could be the indirect consequence of heavy metal toxicity. The possible sequential events of ROS-induced damage development in sensitive plants in response to heavy metal stress are presented in Fig. 8.1. The indirect mechanisms include their interaction with the antioxidant system (Srivastava et al. 2004), disrupting the electron transport chain (Qadir et al. 2004), or disturbing the metabolism of essential elements (Dong et al. 2006). Heavy metals also cause membrane damage through various mechanisms like the oxidation of and cross-linking with protein thiols, inhibition of key membrane protein such as H^+-ATPase, or causing changes in the composition and fluidity of membrane lipids (Meharg 1993). Heavy metals may also impede plant growth indirectly by depriving plants of nutrients required for growth by inhibition of root growth and transpiration, or due to competition by the metal for uptake carriers. The reduction in root growth can limit nutrient uptake due to reduced root area

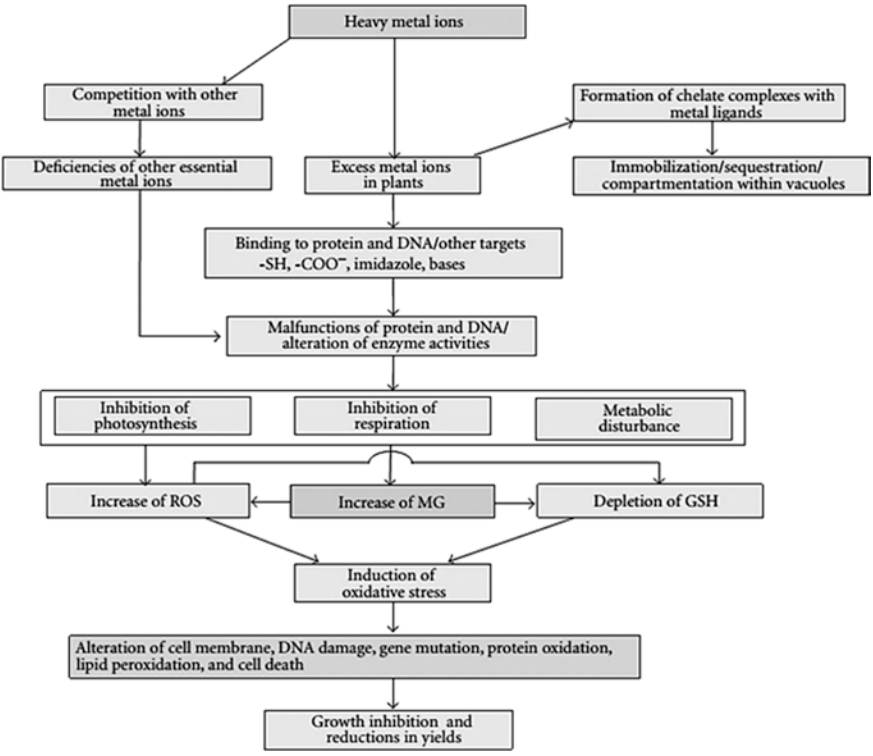

Fig. 8.1 Possible biochemical and molecular mechanisms of heavy metal-mediated ROS induction and damage to the development of higher plants (Hossain et al. 2012)

available for mineral absorption (Johnson et al. 2011). Another deleterious effects induced by heavy metals exposure in plants are lipid peroxidation, which can directly cause biomembrane deterioration and leakage of ions (Boominathan and Doran 2003).

8.4 Effect of Heavy Metals on Humans

Contamination of metals in the environment and human diet represents a persistent problem that is a burden on human health (EPA 2012). Humans are exposed to heavy metals in a variety of ways. Exposure generally occurs by ingestion or inhalation. People who live or work in an area near an industrial site which utilizes these metals are more prone to exposure. Similarly, those living near a site where these metals have been improperly disposed are at equal risk to exposure. Widely dispersed metals like mercury (Hg), lead (Pb), cadmium (Cd) and arsenic (As) have no beneficial effects in humans (Morais et al. 2012) but are generally considered most toxic to humans and animals. Moreover, no known homeostasis mechanism exists for them (Draghici et al. 2010; Vieira et al. 2011). Once absorbed in the human body, the heavy metals may induce several deleterious effects varying from irritation to acute to chronic ones. The nature of effects could be toxic (chronic, subchronic, or acute), neurotoxic, mutagenic, teratogenic, or carcinogenic (Richards 2007). In contrast, the essential elements do not produce toxic effects in plants and animals due to the presence of homeostatic mechanisms which regulate their level in the body (Oliveira da Silva et al. 2005). Table 8.3 provides an overview of the toxic effects of different heavy metals on human beings.

Cadmium (Cd) is one of the most important pollutants in terms of food chain contamination and has no role in human physiology. Cadmium is naturally present in air, soil, sediments, and unpolluted seawater. The element is emitted to air by mines, metal smelters, and industries using cadmium compounds for alloys, batteries, pigments, and in plastics (Harrison 2001). Human exposure to cadmium occurs through the ingestion of contaminated foodstuffs, by the incineration of municipal waste containing plastics and nickel–cadmium batteries and by cigarette smoking (Lewis et al. 1972; WHO 2004, 2006). Cadmium is known to accumulate in the kidney cortex and causes renal tubular dysfunction (Jarup et al. 1998a; Barbier et al. 2005; Nordberg 2009). Subchronic inhalation exposure to Cd leads to pulmonary effects like emphysema, bronchiolitis, and alveolitis, while high exposure leads to cadmium pneumonitis, an obstructive lung disease characterized by chest pain, bloody sputum, and death of lung tissues (Davison et al. 1988; Fernandez et al. 1996; Hendrick 2004). Cd exposure also leads to bone defects like osteomalacia, osteoporosis, spontaneous fractures, and skeletal demineralization (McKenna and Chaney 1991; Strehlow and Barltrop 1988; Jarup et al. 1998b; Staessen et al. 1999; Kazantzis 2004; Young 2005). Some studies have suggested an association of cadmium and renal cancer in humans (Il'yasova and Schwartz 2005) although later researchers have doubt over these findings.

Table 8.3 Toxic effects of heavy metals on human beings

Metal	Symptoms	References
Zn	Lethargy and focal neurological deficits Metal fume fever Epilepsy and transient global ischaemia	Murphy (1970) Kuschner et al. (1997) Weiss et al. (2000)
Hg	Sperm immotility High sister chromosome exchanges/cell and induced C-anaphases (abnormal mitosis)	Ernst and Lauritsen 1991 Rao et al. (2001)
Cu	*Tyrolean cirrhosis* Wilson's disease Alzheimer's disease	Muller et al. (1996) Brewer (2001) Brewer (2009, 2012)
Co	Abnormal lymphocyte function Hand tremor, incoordination, cognitive decline, depression, vertigo Arrhythmias and cardiomyopathy	Hart et al. (2006), Daou et al. (2011) Tower (2010a, b)
Mo	Acute psychosis with visual and auditory hallucinations	Momcilović (1999)
Pb	Memory and learning deficits Cognitive and behavioral impairments Chronic lead nephropathy Cancer	Needleman and Landrigan (1981) Devi et al. (2005) Brewster and Perazella (2004) van Wijngaarden (2012)
Cr	Dizziness, headache, and weakness Cancer of gastrointestinal tract and central nervous system	ATSDR (2000) Costa and Klein (2006), Zhitkovich (2011)
Ni	Nausea, vomiting, abdominal pain, diarrhea, giddiness Allergic contact dermatitis Nasal and lung cancer	Sunderman et al. (1988) EHC (1991), Cavani (2005) Costa et al. (2005), Lu et al. (2005)
Cd	Diabetes mellitus Neurodegeneration; vascular-type dementia (VD) High blood pressure and cardiovascular disease Reduced birth	Edwards and Prozialeck (2009) Mizuno and Kawahara (2013) Telisman et al. (2001)
As	Hypertension Anemia and leukopenia Diabetes mellitus	Lee et al. (2003), Yoshida et al. (2004) Tay and Seah (1975) Walton et al. (2004)

Lead has been used since centuries for building materials, pigments to glaze ceramics, water pipes, ammunition, glass and crystals, paints, protective coatings, acid storage batteries, gasoline additives, in cosmetics and as a preservative (Florea and Büsselberg 2006). However, it is also one of the oldest known and most widely studied occupational and environmental toxins (Gidlow 2004). Lead contamination is one of the greatest concerns for human health. Human exposure to lead occurs primarily through drinking water, airborne lead-containing particulates (especially in cigarette smoke and fumes of petroleum products), and lead-based paints. The danger of Pb is more pronounced due to its low mobility even under high precipitation. The half-life of lead in blood is about 1 month and in

the skeleton 20–30 years (WHO 1995). The toxicology of organolead has been extensively reviewed by Grandjean and Nielsen (1979). Tetraethyllead (TEL) and tetramethyllead (TML) are the main constituents in organolead. Both the tetraethylated or methylated forms are degenerated in the body to the trivalent organic forms, which are highly toxic. The toxicity of organolead differs from inorganic lead compounds depending on alkylation, while the toxic effects of TEL and TML are essentially similar, although the toxicities of these compounds seem to vary by species in animal experiments (Grandjean and Nielsen 1979; Florea and Büsselberg 2006). In adults, inorganic lead does not penetrate the blood–brain barrier, whereas this barrier is less developed in children. The children are especially susceptible to lead exposure and subsequent brain damage due to higher permeability of the blood–brain barrier due to which adverse effects of Pb occur at lower threshold levels than in adults. Lead toxicity causes dysfunction of the kidneys, reproductive, and cardiovascular systems; inhibition of hemoglobin synthesis; and damage to the central nervous systems (Kantor 2006; Ogwuegbu and Muhanga 2005). Some recent reports have suggested a correlation between lead exposure and carcinogenicity (Siddiqui et al. 2002; Xu et al. 2006; Rousseau et al. 2007; Alatise and Schrauzer 2010).

Soil is contaminated with zinc (Zn) emanating from sewage sludge or urban composts, fertilizers, emissions from municipal waste incinerators, residues from metalliferous mining, the metal smelting industry, and other human activities (Yadav 2010). Of the 2–3 g Zn in human body, about 90 % of Zn is found in muscles and bones, while prostate, liver, the gastrointestinal tract, kidney, skin, lung, brain, heart, and pancreas also contain estimable concentrations of the metal (Wastney et al. 1986; Llobet et al. 1988; Bentley and Grubb 1991; He et al. 1991). Zn causes the same signs of illness as does lead and is often mistaken as lead poisoning. Common signs of Zn toxicosis include diarrhea, vomiting, anemia, epigastric pain, and abdominal cramps (Brown et al. 1964; Porea et al. 2000; Haase et al. 2008).

Arsenic (As), a metalloid, occurs in two oxidation states: a trivalent form, arsenite (As_2O_3; As III), and a pentavalent form, arsenate (As_2O_5; As V) (Ratnaike 2003). Arsenic is often present in plants and animals without any adverse health effect, its toxicity usually depending on the oxidation state and chemical species. The primary route of exposure of inorganic arsenic is through underground drinking water with elevated arsenic concentrations which gradually leads to chronic arsenicosis (Chakraborti et al. 2004; Bhattacharya et al. 2007; Mudhoo et al. 2011). Drinking water contaminated with arsenic has been found in both developed and developing countries and is a global health problem affecting millions of people, especially in South Asia (Ahsan et al. 2000; Mazumder et al. 1998; Sun 2004). The major source of organic arsenic is mainly fish and seafood, but the organic exposure appears to be much less toxic than the inorganic forms (Uneyama et al. 2007). Arsenic is known to form complexes with coenzymes leading to inhibition of production of adenosine triphosphate, the main energy yielding molecule in the body. Arsenic toxicity causes an immune disorder wherein the body's immune system attacks part of its own peripheral nervous system resulting

in muscle weakness. Arsenic is carcinogenic in its oxidation states and high exposure often causes death.

Mercury (Hg) is a unique metal due to its existence in different forms e.g., HgS, Hg^{2+}, Hg°, and methyl-Hg. Hg released to the soil mainly remains in solid phase through adsorption onto sulfides, clay particles, and organic matters. Methylmercury, the common organomercurial species, is of particular concern because of its toxicological characteristics, a long biological half-life and biomagnification through the trophic chain. Mercury is used as a pharmaceutical, in the gold industry, as a component of barometers, thermometers, dental products, electrical equipment, control devices, and in fungicides. The high usage of mercury has resulted in the widespread occurrence of mercury contamination in the entire food chain. The 'Minamata disease,' first reported from Japan in 1956, is the most known incident of organic mercury poisoning which was caused by the release of methylmercury in the industrial wastewater (Weiss 1996). Oral exposure to organomercurial compounds reportedly leads to gastrointestinal and associated disorders like diarrhea, irritation, blisters in the gastrointestinal tract, vomiting, abdominal pain, constipation, and gastritis (Jalili and Abbasi 1961; Al-Saleem 1976; Pfab et al. 1996; Castoldi et al. 2003; Oliveira Da Silva et al. 2005). Exposure to mercury is known to induce genotoxicity (Rao et al. 2001; Bonacker et al. 2004) and adversely affect the nervous system (Olivieri et al. 2002; Counter and Buchanan 2004; Johnson 2004), renal system (Ellingsen et al. 2000), reproductive system (Dickman and Leung 1998), immune system (Vimercati et al. 2001; Prochazkova et al. 2004), and the cardiovascular system (Sorensen and Murata 1999).

8.5 Response of Plants to Heavy Metals

Plants are sensitive to heavy metals in a variety of ways that are enumerated below:

1. Uptake and accumulation of metals by binding to extracellular exudates and constituents of the cell wall.
2. Extrusion of metals from cytoplasm to the extranuclear compartments.
3. Complexation of the metal ions inside the cells by complex molecules.
4. Concentration of osmolytes and osmoprotectants and induction of enzyme systems.
5. Alteration of plant metabolism (Cho et al. 2003).

Baker (1981) has classified the plants growing on metalliferous soils into three categories:

(i) Excluders—These plants prevent uptake of toxic metals into root cells (de Vos et al. 1991). As a result the metal concentrations in the shoot are maintained up to a critical value, at a low level across a wide range of soil concentration.

(ii) Accumulators—Accumulators do not prevent metals from entering the roots and allow bioaccumulation of high concentration of metals mainly in the aboveground plant parts. For example, members of the order Caryophyllales show a general ability to accumulate metals in their shoot (Broadley et al. 2001).
(iii) Indicators—In these plants the internal concentration reflects the external levels (McGrath et al. 2002).

Hyperaccumulators are a subgroup of accumulator species often endemic to naturally mineralized soils, which accumulate high concentrations of metals in their foliage, while storing lower concentrations in their roots (Reeves and Brooks 1983; Brooks 1987; Baker and Brooks 1989; Raskin et al. 1997; Macnair 2003). Bioconcentration factor (BCF) is the ratio of metal concentration in the shoot tissue to the soil (McGrath and Zhao 2003). Hyperaccumulators have a BCF greater than 1, sometimes reaching as high as 50–100, while most other plants have metal BCF values of less than 1, which means that it takes longer than a human lifespan to reduce soil contamination by 50 % (Peuke and Rennenberg 2005). Hyperaccumulation of heavy metal ions is a striking phenomenon exhibited by approximately <0.2 % of angiosperms and reported to occur in over 450 species of vascular plants from 45 angiosperm families with most plants belonging to the families Asteraceae, Brassicaceae, Caryophyllaceae, Cyperaceae, Cunoniaceae, Fabaceae, Flacourtiaceae, Lamiaceae, Poaceae, Violaceae, and Euphorbiaceae (Baker and Whiting 2002; Padmavathiamma and Li 2007; Rascio and Navari-Izzo 2011; Bhargava et al. 2012a). Metal hyperaccumulators come from a wide range of taxonomic groups and geographic areas, and as such have a wide diversity of morphological, physiological, and ecological characteristics (Pollard et al. 2002).

8.6 Genetic Diversity

Genetic differentiation between individuals is the basis for the evolutionary change of species, populations, and lineages. Biological diversity is defined as the variation present in all species of plants and animals, their genetic material and the ecosystems in which they occur (Rao and Hodgkin 2002). Diversity can occur at three levels: genetic diversity (variation in genes and genotypes), species diversity (species richness), and ecosystem diversity (communities of species and their environment). The Rio Convention of 1993 on biodiversity has also recognized 3 levels of biological variability viz. (i) diversity of ecosystems and landscapes, (ii) species richness, and (iii) genetic variation within species. Genetic diversity is the amount of genetic variability among individuals of a variety, or population of a particular species (Brown 1983). It forms the basis for survival and adaptation, and makes it possible to advance the adaptive processes on which evolutionary success depends (Rao and Hodgkin 2002). Study of genetic diversity is the process by which variation among individuals or groups of individuals or populations

is analyzed by a specific method or a combination of methods (Mohammadi and Prasanna 2003). Assessment of genetic diversity is invaluable in genetic improvement of plants as it helps in the identification of diverse parental combinations to create segregating progenies with maximum genetic variability and facilitates introgression of desirable genes from diverse germplasm into the available genetic base (Barrett and Kidwell 1998; Thompson et al. 1998; Bhargava et al. 2007, 2008; Fuentes and Bhargava 2011). Many tools are now available for studying variability and the relationships among accessions that include seed protein electrophoresis, isozymes, and various types of molecular markers.

8.7 Genetic Diversity and Heavy Metals

Genetic diversity is a prerequisite for adaptive evolution. Variation is of great theoretical importance, because it is the raw material on which natural selection acts to influence the evolution of hyperaccumulation (Pollard et al. 2002). The relationship between hyperaccumulation and tolerance can be easily understood by studying the patterns of variation in these types. Natural variation is also important basis for the development of hyperaccumulation technology as it indicates the potential for improvement of plant traits through selective breeding, and provides variable genetic markers that can be studied by crossbreeding and molecular techniques (Pollard et al. 2002). Although some degree of hyperaccumulation occurs in all members of the species that can hyperaccumulate heavy metals, quantitative genetic variation in the ability to hyperaccumulate have been reported, both between (Pollard and Baker 1996; Bert et al. 2000; Escarré et al. 2000; Pollard et al. 2002; Assunção et al. 2003; Roosens et al. 2003) and within populations (Pollard and Baker 1996; Meerts and van Isacker 1997; Escarré et al. 2000; Pollard et al. 2002). Such variation does not appear to correlate positively with either the concentration of heavy metals in the soil or the degree of metal tolerance in the plants.

The existence of genetic difference in heavy metal uptake and accumulation, as well as tolerance has been found in diverse crop plants. Rice is one of the most utilized cereals for edible purposes in different parts of the world. In rice, several reports are available that show enormous variation for heavy metal tolerance (Aniol and Gustafson 1990; Yang et al. 2000; Zhang et al. 2000; Arao and Ae 2003; Liu et al. 2003). In fact, Cheng et al. (2006) found significant genotypic variation for Cd, Cr, As, Ni, and Pb in the grains while investigating nine rice genotypes grown in six locations for two successive years. A comparative study on cadmium uptake by several rice cultivars was carried out by Morishita et al. (1987) in Andisols with a low-total cadmium concentration in soil. It was observed that japonica brown rice varieties had the lowest average uptake rate compared to the other three varieties namely, javanica, indica, and Hybrid. Average cadmium levels in brown rice ranged from 2.1 to 27.0 mcg kg^{-1} among 28 japonica varieties and from 4.1 to 55.5 mcg kg^{-1} among 23 indica varieties. Arao and Ishikawa (2006) reported that 49 varieties of rice were cultivated in Cd-polluted soils; the japonica

varieties were categorized into the low grain Cd group. Several indica or indica–japonica varieties accumulated considerable amounts of Cd in grains as well as in straw (Arao and Ishikawa 2006). Liu et al. (2003) conducted a study on 20 rice cultivars of different genotypes and origins on cadmium supplemented soils. The result showed that the effects of Cd on rice growth and development varied greatly among cultivars. Some varieties were highly tolerant to soil stress imposed by cadmium, while others were very sensitive. Differences existed among the cultivars for Cd uptake and distribution of rice plants (Liu et al. 2003). Liu et al. (2007) conducted pot soil experiments with two rice cultivars at different levels of Cd to understand certain mechanisms causing the variations between rice cultivars with regard to Cd uptake and accumulation. The results showed that the rice cultivar with higher concentrations of LMWOA (low-molecular-weight organic acids) in soil accumulated more Cd in the plants. The results indicated that LMWOA secretion by rice root, especially in Cd-contaminated soils, was likely to be one of the mechanisms determining the plant Cd uptake properties of rice cultivars (Liu et al. 2007).

In *Brassica juncea*, high variability between species and between cultivars within a species for the accumulation of heavy metals has been documented by Salt et al. (1995). Kastori et al. (2010) observed high genetic variability between populations of wild sunflower species and hybrids in the uptake and tissue concentration of heavy metals. Coefficient of variation of concentration of nonessential microelements in wild populations varied from 7.7 to 73.8. The average coefficient of variation was the highest for Cr, Ni, and Zn in hybrids and for Cd, Ni, and Cr in wild species.

Genetic diversity for heavy metal accumulation has been reported in underutilized crops like *Chenopodium* and *Amaranthus* (Shukla et al. 2006; Bhargava et al. 2008, 2010). In chenopods, significant genotypic differences have been reported in the heavy metal uptake by plants both at inter and intraspecific level (Bhargava et al. 2008). The study was undertaken to characterize and classify the qualitative variation among the chenopod germplasm based on mineral composition of the foliage for 10 minerals (Table 8.4). The analysis of variance exhibited significant differences for all the 10 minerals under study (data not shown) indicating the presence of large amount of variation for different minerals among the accessions. Principal component analysis (PCA) showed that the first 4 PCs (Principal Component) accounted for 74.70 % of the total variance among the accessions (Table 8.5). The first PC (PC1) accounted for 41.96 % of the total qualitative variation and had nickel, zinc, and chromium with high positive and copper with high-negative coefficients. Cluster analysis grouped the accessions into 4 major clusters. The first cluster, which showed maximum diversity, had 17 accessions, all of *Chenopodium quinoa* having high content of most of the heavy metals viz. zinc, chromium, nickel, and cadmium. Cluster II was the largest consisting of 18 accessions which had low content of nickel, cadmium, and chromium. Cluster III contained three accessions that had lowest amount of calcium, iron, magnesium, and zinc, while accessions in cluster IV were characterized by high levels of calcium, sodium, magnesium, nickel, chromium, and cadmium.

Table 8.4 Mineral composition (mg/100 g dry weight) of 40 accessions of *Chenopodium* spp (Bhargava et al. 2008)

	K	Ca	Na	Fe	Mg	Zn	Cu	Ni	Cr	Cd
C. quinoa Willd. CHEN 33/84	7923	1159	5479	84.57	831	16.46	12.51	9.23	3.94	3.12
C. quinoa Willd. CHEN 92/91	6475	1074	2864	82.63	776	30.67	11.16	14.28	3.94	1.36
C. quinoa Willd. CHEN 67/78	8363	1133	6510	87.50	856	11.45	12.45	15.48	7.43	1.76
C. quinoa Willd. CHEN 84/79	7645	1126	3531	81.57	829	22.72	11.61	8.75	4.62	2.78
C. quinoa Willd. PI 510532	5708	1064	15,181	85.13	842	14.35	11.45	10.61	5.57	13.42
C. quinoa Willd. PI 510536	7807	1172	9164	86.50	842	31.82	12.32	13.26	13.52	3.44
C. quinoa Willd. PI 510537	5177	1038	2226	83.73	848	12.22	11.71	10.68	5.92	2.35
C. quinoa Willd. PI 510547	8527	1250	4294	79.20	848	11.77	11.86	11.68	8.59	3.36
C. quinoa Willd. PI 587173	7712	1154	9660	83.07	836	18.58	12.21	14.10	3.79	6.92
C. quinoa Willd. PI 614881	6214	1055	1065	89.40	833	35.24	13.43	18.38	5.61	1.90
C. quinoa Willd. PI 614883	6965	1149	6627	87.20	832	21.15	11.86	7.84	8.21	4.04
C. quinoa Willd. Ames 13719	6434	1197	7754	85.20	847	36.17	10.39	18.41	18.80	6.42
C. quinoa Willd. Ames 13762	7275	1019	11,750	83.80	585	5.80	11.88	9.63	4.35	0.57

(continued)

Table 8.4 (continued)

	K	Ca	Na	Fe	Mg	Zn	Cu	Ni	Cr	Cd
C. quinoa Willd. Ames 22156	2081	1005	2734	85.30	824	24.95	13.75	22.19	13.34	1.71
C. quinoa Willd. Ames 22158	7375	1010	5683	86.33	830	13.82	12.52	6.23	1.38	4.41
C. quinoa Willd. PI 584524	6876	1154	3048	84.63	795	10.90	13.56	16.00	3.26	3.61
C. quinoa Willd. PI 433232	6810	1120	7720	84.17	814	21.69	10.53	8.56	8.44	2.35
C. quinoa Willd. PI 478410	6629	1137	6944	83.63	836	10.84	13.37	6.99	5.85	4.69
Mean (*C. quinoa* group)	6777.56	1112	6235.22	84.64	816.89	19.48	12.14	12.35	7.03	3.79
C. berlandieri subsp. *nuttalliae* PI 568155	7794	1029	10,087	92.33	823	14.91	14.07	0.00	2.64	0.95
C. berlandieri subsp. *nuttalliae* PI 568156	7234	1148	8657	91.33	828	15.52	14.13	0.00	1.72	1.03
Mean (*C. berlandieri* subsp. *nuttalliae* group)	7514	1088.50	9372	91.83	825.50	15.21	14.10	0.00	2.18	0.99
C. bushianum Ames 22376	5186	1532	14,274	84.03	854	9.72	12.46	12.72	1.88	1.90
C. giganteum CHEN 86/85	5629	1625	15,640	82.07	830	15.49	13.86	16.38	3.35	4.48
C. giganteum *Ames 86650*	5210	1430	12,937	80.23	797	5.30	14.03	0.20	2.93	0.67
C. giganteum PI 596372	4335	1083	8100	81.40	838	11.96	14.66	2.71	1.87	0.81
C. giganteum 'local'	5256	1224	8744	81.00	832	10.56	13.92	2.28	1.80	1.34

(continued)

Table 8.4 (continued)

	K	Ca	Na	Fe	Mg	Zn	Cu	Ni	Cr	Cd
Mean (*C. giganteum* group)	5107.50	1340.50	11355.25	81.18	824.25	10.83	14.12	5.39	2.49	1.83
C. murale 'local'	4632	1149	11,650	75.60	834	12.84	14.17	0.00	2.17	0.98
C. album 'local red'	4385	1051	10,554	80.03	396	3.39	14.63	0.00	1.03	1.47
C. album 'Chandanbathua'	6791	1085	8644	85.50	824	11.77	14.25	1.70	1.62	1.22
C. album 'Chandigarh'	7837	1079	10,350	83.30	818	12.35	14.48	0.00	1.70	1.72
C. album 'Mexico'	6944	1118	12,200	78.17	823	12.16	13.85	1.20	2.17	0.94
C. album PRC 9801	7103	1164	8850	82.13	396	3.15	13.73	0.00	1.44	1.27
C. album PRC 9802	5520	1219	8530	83.73	793	10.03	14.34	0.00	1.76	0.96
C. album PRC 9803	6325	1187	8527	82.87	756	11.45	14.16	0.00	2.08	1.48
C. album PRC 9804	7583	1138	8477	81.93	751	12.33	13.55	0.00	2.73	0.67
C. album IC 107296	4590	1014	9094	87.80	831	9.98	14.43	0.00	2.29	1.90
C. album IC 107297	6579	1204	8470	85.83	801	12.41	14.05	0.00	2.12	0.87
C. album PI 605700	4692	1176	8160	84.53	831	12.02	14.56	0.00	2.48	0.98
C. album 'local 6x'	2640	1145	10,814	86.93	826	10.45	14.51	1.12	2.31	1.03
C. album 'Iowa'	7092	1151	9184	80.80	801	16.72	13.83	0.00	2.47	0.48
Mean (*C. album* group)	6006.23	1133.15	9373.38	83.35	742.08	10.63	14.18	0.31	2.02	1.15
C. ugandae CHEN 77/78	7814	1199	9827	81.53	823	10.13	14.31	3.94	1.59	0.59

Table 8.5 Eigenvalues, proportion of variability and agronomic traits that contributed to the first four PCs of *Chenopodium* spp (Bhargava et al. 2008)

Components	PC_1	PC_2	PC_3	PC_4
Root	0.926	0.263	0.256	0.204
% variance explained	41.96	11.91	11.58	9.25
Cumulative variance	41.96	53.87	65.45	74.70
Coefficients of variates				
Potassium	0.103	0.023	0.366	0.202
Calcium	−0.039	−0.300	−0.068	0.066
Sodium	−0.231	−0.323	0.015	0.056
Iron	0.110	0.146	−0.060	0.164
Magnesium	0.170	−0.035	−0.179	0.320
Zinc	0.380	0.078	−0.098	0.073
Copper	−0.472	0.095	−0.230	0.053
Nickel	0.538	−0.093	−0.111	−0.117
Chromium	0.352	−0.018	−0.054	−0.056
Cadmium	0.192	−0.142	0.044	0.013

Extensive variation for cadmium tolerance and accumulation has also been observed among populations of the partridge pea (*Chamaecrista fasciculata*), a leguminous pioneer species native to the eastern United States (Henson et al. 2013). At the germination stage, *C. fasciculata* did not exhibit between-population variation for tolerance. However, between-population variation for tolerance was noted in plant growth, as reflected by their tolerance indices. *C. fasciculata* accumulated cadmium throughout all plant parts specifically noted for their role in interspecific interactions: stems, leaves, pollen, seeds, and root nodules. It was concluded that the potential of *C. fasciculata* for use in remediation or restoration varied significantly across populations, demonstrating the importance of considering seed source when screening populations of *C. fasciculata* for utilization in phytoremediation (Henson et al. 2013).

Variability for heavy metal tolerance has also been reported extensively in tree species like willows (*Salix* sp.). Willows have shown significant variations in tolerance across species, varieties, and clones. Significant variations in metal tolerance were found among willow species and clones exposed to cadmium, copper, or arsenic (Punshon and Dickinson 1999; Kuzovkina et al. 2004; Purdy and Smart 2008; Magdziak et al. 2011). Numerous studies have indicated high capacity for cadmium and zinc uptake in *Salix integra* (Yang and Chen 2008; Harada et al. 2010; Liu et al. 2011). Wang et al. (2014) examined the variations in lead (Pb) tolerance and accumulation of three cultivated varieties of *S. integra*, a shrub willow native to northeastern China, using hydroponic culture in a greenhouse. The tolerance and accumulation of Pb varied among the three willow varieties depending on the Pb concentration. All three varieties had a high-tolerance index (TI) and EC50 value but a low-translocation factor (TF), indicating that Pb sequestration is mainly restricted in the roots of *S. integra*. Among the three varieties, Dahogntou was more sensitive to the increased Pb concentration than the other two varieties,

Table 8.6 Variability for heavy metal tolerance/accumulation in diverse plant species

Plant species	Heavy metal	References
Helianthus annus	Cd	Li et al. (1995), (1997)
Populus nigra	Cd	Gaudet et al. (2011)
Averrhoa carambola	Cd	Dai et al. (2011)
Thlaspi caerulescens	Cd, Zn Ni, Zn	Zha et al. (2004) Richau and Schat (2009)
Thlaspi pindicum	Ni, Zn	Taylor and Macnair (2006)
Ipomoea aquatica	Cd	Wang et al. (2009)
Dianthus carthusianorum	Zn, Pb	Wójcik et al. (2013)
Chenopodium quinoa	Cd	Bhargava et al. (2008)
Chenopodium giganteum	Cd, Zn, Ni	Bhargava et al. (2008)
C. album	Mg, Zn, Cd	Bhargava et al. (2008)
Oryza sativa	Cd	Liu et al. (2005), Wang et al. (2011)
Triticum aestivum	Cd	Stolt et al. (2006)
Triticum turgidum L. var. durum	Cd	Li et al. (1997)
Linum usitatissimum	Cd	Li et al. (1997), Hocking and McLaughlin (2000)
Brassica juncea	Ni Cr	Ansari et al. (2015) Diwan et al. (2008)
Brassica rapa	Zn, Fe, Mn	Wu et al. (2007)
Brassica oleracea	Zn, Fe	Kopsell et al. (2004)
Apium graveolens	Cd, Pb	Zhang et al. (2013)
Amaranthus tricolor	Zn, Fe, Ni, Mn	Shukla et al. (2006)
Arabidopsis thaliana	Cu Co, Ni, Cu, Cd, Mo	Kobayashi et al. (2008) Baxter et al. (2008, 2012)
Pteris vittata	Zn, Cd	Wu et al. (2009)

with the lowest EC50 and TI for root and above-ground tissues. The three varieties revealed various toxicity symptoms of leaf wilting, chlorosis, and inhibition of shoot and root growth under the higher Pb concentrations.

Table 8.6 depicts the variability for heavy metal accumulation and tolerance reported in diverse plant species.

8.8 Implications of Heavy Metals on Genetic Diversity

The genetic composition of natural populations is constantly modified by natural events (Ungherese et al. 2010). Anthropogenic impact of pollutants can cause severe alterations in the genetic structure of populations. Therefore, the effect of pollutants on genetic variability is fundamental in preserving the evolutionary potential of natural populations. Among the various groups of contaminants, heavy metals seem to strongly affect genetic variability, both directly (via germ

cell mutations) and indirectly (via somatic mutations or ecological and physiological effects) (Bickham et al. 2000; Belfiore and Anderson 2001; De Wolf et al. 2004). Heavy metal exposure can alter the genetic composition of a population by favoring more tolerant genotypes and causing demographic bottlenecks leading to a decrease of genetic variability known as 'genetic erosion' (Van Straalen and Timmermans 2002; Ribeiro et al. 2012; Ribeiro and Lopes 2013). In genetic erosion, small populations become increasingly subject to genetic drift and inbreeding, resulting in loss of genetic variation and a decrease in fitness. Genetic drift will cause allele frequencies to fluctuate, which over time leads to random loss and fixation of alleles and an increase in homozygosity (Bijlsma and Loeschcke 2011). A special case of genetic drift is population bottleneck which occurs when the size of a population is significantly reduced leaving a small collection of genotypes as founders for recovery and expansion (van Straalen and Timmermans 2002). Some recent studies have pointed toward an increase in the genetic diversity in metal-polluted environments and a possible role in evolution. In polluted environments, intra- and interpopulation changes at the molecular level proceed rapidly and lead to the formation of new ecotypes in a relatively short time (Słomka et al. 2011). A recent study used ISSR PCR fingerprinting data to analyze the genetic diversity and genetic structure of seven populations of *Viola tricolor*: four growing on soil contaminated with heavy metals (Zn, Pb, and Cd; waste heaps) and three from control soil (Słomka et al. 2011). The populations from the polluted sites showed higher genetic polymorphism (%(poly) = 84 %) and gene diversity (H(T) = 0.1709) than the control populations (%(poly) = 75 % and H(T) = 0.1448). The number of private markers detected within metallicolous (MET) populations was more than double that found within nonmetallicolous (NON) populations (15 vs. 7). The STRUCTURE and UPGMA analyses showed clear genetic differences between the NON and MET populations. Based on broad analyses of the genetic parameters, it was concluded that the effect of these polluted environments on the genetic diversity of the MET populations, separating them from the NON populations, is evidence of microevolutionary processes at species level, leading to species divergence and the emergence of local ecotypes better adapted to their different environments.

Sites contaminated by heavy metals (metalliferous sites) are places where microevolutionary processes accelerate due to colonization of the contaminated sites by plants that have a small genome size and have evolved an r-life strategy with the crucial ability to reproduce quickly, owing to fast flowering, seed ripening, and much greater flower and seed yields (Wierzbicka and Rostański 2002; Grześ 2007; Vidic et al. 2009). The toxicity of metal pollution can affect the genetic diversity of exposed populations through various means like plant survivorship, recruitment, reproductive success, mutation rates, and migration (Anderson et al. 1994; Bickham and Smolen 1994; Fox 1995; Deng et al. 2007). The populations of plants growing at heavy metal contaminated sites are often genetically distinct from the populations of the same species in uncontaminated locations (Assunção et al. 2003; Dubois et al. 2003). However, conflicting results

have been obtained when genetic variation has been studied among metal-tolerant and nonmetal-tolerant populations. The genetic diversity of the uncontaminated population was found to be similar to that of the contaminated population in *Silene paradoxa* (Mengoni et al. 2000), *Agrostis stolonifera* (Wu et al. 1975) and *Arrhenatherum elatius* (Ducousso et al. 1990). On the contrary, the reduction of genetic diversity was found in some species like *Deschampsia cespitosa* (Bush and Barrett 1993) and *Armeria maritima* (Vekemans and Lefèbvre 1997).

Deng et al. (2007) undertook a detailed study to assess the impact of heavy metal contamination on genetic variation of *Sedum alfredii*, a fleshy perennial herb. *S. alfredii* has been reported to be a Pb accumulator (He et al. 2002) and hyperaccumulator for Zn and Cd (Yang et al. 2002, 2004). The genetic diversity and population structure of seven populations of *S. alfredii* growing in lead/zinc (Pb/Zn) mine spoils or in uncontaminated soils were investigated using random amplified polymorphic DNA (RAPD) technology. A significant reduction of genetic diversity was detected in the mining populations. Analysis of molecular variance (AMOVA) and the unweighted pair group method with arithmetic mean (UPGMA) tree derived from genetic distances further corroborated that the genetic differentiation between mine populations and uncontaminated populations was significant (Deng et al. 2007) (Fig. 8.2). Reduction in genetic diversity of a mine population was theoretically expected because of the strong bottleneck as a result of strong selection pressure on plants due to heavy concentration of Zn, Cd, and Pb (Bradshaw 1984; Lefèbvre and Vernet 1990). The reduction of genetic diversity might be caused by a bottleneck effect which preserved the tolerant individuals and decreased the number of sensitive ones (Bickham et al. 2000).

Babst-Kostecka et al. (2014) investigated the genetic variability of *Biscutella laevigata* L. (Brassicaceae), a perennial, strictly outcrossing species, among all 16 known low and high elevation provenances from locations in southern Poland using nine microsatellite markers to assess historical and evolutionary processes shaping its genetic structure. Populations clustered into two groups which corresponded to their edaphic origin and diverged 1200 generations ago. The authors detected a significant decrease in genetic diversity and evidence for a recent bottleneck in metallicolous populations. Environmental conditions, especially the metal concentrations in the soil, appeared to more strongly influence the genetic structure rather than geographic distance (Babst-Kostecka et al. 2014). A significant reduction in the genetic diversity (founder and bottleneck effects) in metallicolous compared to nonmetallicolous populations was associated with the colonization of polluted sites and/or evolution of metallicolous populations. As a consequence, populations from natural and anthropogenic locations have adapted to different environmental conditions and have genetically diverged.

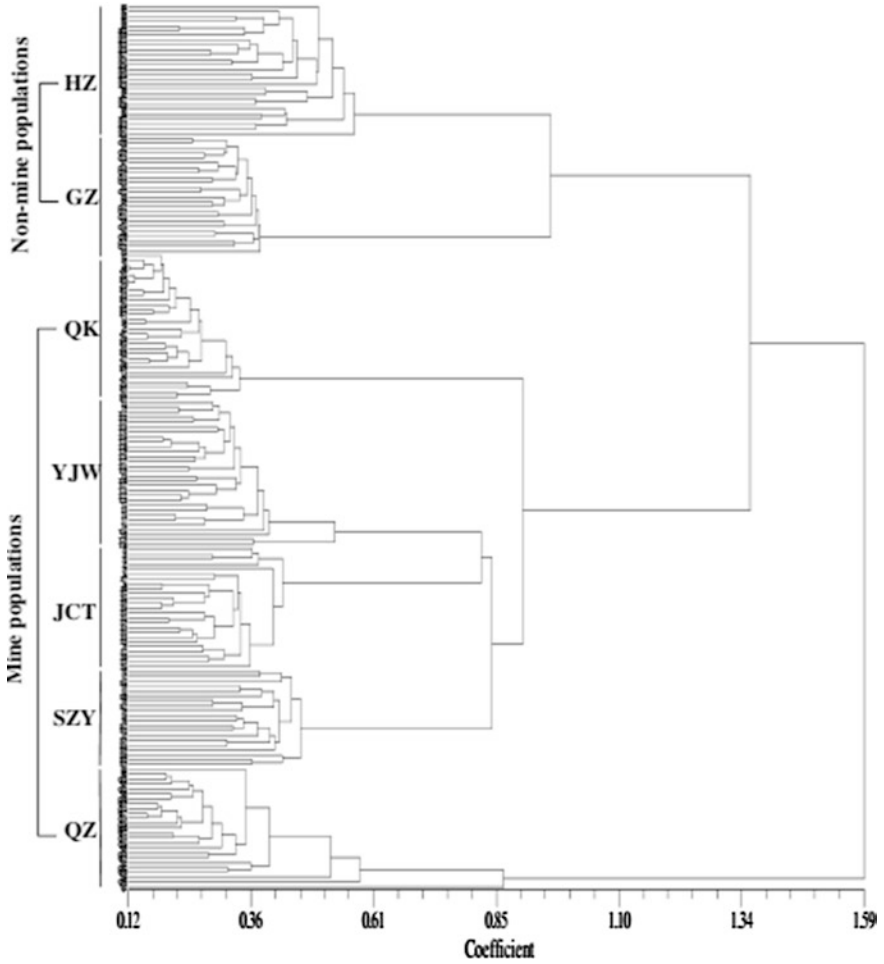

Fig. 8.2 The UPGMA tree plot of genetic distance among *S. alfredii* populations grown on metal contaminated soils based on RAPD analysis (Deng et al. 2007)

8.9 Implications of Genetic Diversity/Variability for Phytoremediation

Most of the known hyperaccumulators are small, slow growing, and often are rare species of limited population size and restricted distributions. If desirable traits can be identified in natural hyperaccumulator plants, they could be selected either by conventional breeding techniques, or using new methods of hybridization such as protoplast fusion, or by the manipulation of gene expression in transgenic plants. The ideal phytoremediation plant should combine rapid growth and high biomass along with high metal accumulation in the shoot tissues (Chaney et al. 2000; Lasat

2002; McGrath et al. 2002). Thus, understanding the genetic mechanism of metal accumulation in hyperaccumulator species is important because it facilitates the use of various approaches to genetic improvement of plants for metal uptake (Bhargava et al. 2012a). Efficient management and utilization of germplasm requires detailed knowledge of the genetic diversity of agronomic traits for proper characterization of populations to facilitate efficient synthesis of breeding populations that are designed to accomplish specific objectives (Bhargava et al. 2007, 2008). From the viewpoint of a breeder, the presence of sufficient genetic variability in the base population is a prerequisite for any crop-breeding program. The characters of economic importance are generally quantitative in nature and exhibit a considerable degree of interaction with the environment. Thus, it becomes necessary to compute variability present in the breeding material and its partitioning into genotypic, phenotypic, and environmental ones. The available and potential qualitative variability is interesting for potential users of the germplasm in relation to prospect of isolating different genotypes for phytoextraction of heavy metals. Genetic diversity and variability analysis have proved to be an effective method in grouping accessions for effective management and utilization in genetic improvement of plants for enhanced phytoextraction. The existing genetic diversity in crops can be used for phytoextraction by identifying easily cultivable, high biomass yielding plants, and practicing selection in future generations (Bhargava et al. 2012a).

8.10 Conclusions

Since the mineral homeostasis in plants is under complex genetic control, there is likely to be substantial genetic variation for this control, which opens new avenues for the improvement of mineral accumulation and tolerance by classical breeding or genetic engineering approaches. The available and potential qualitative variability may be of immense interest for potential users of the germplasm in relation to prospect of isolating different genotypes for effective phytoextraction.

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Chapter 9
Diversity and Erosion in Genetic Resources of Spices

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Abstract Genetic resources are global assets of inestimable value to human kind, which holds the key to increasing food security. The loss of variation in crops due to the modernization of agriculture has been described as genetic erosion. The current status of the genetic diversity and erosion in spice crops is discussed in this chapter. Human intervention into the natural habitats of wild and related species in centers of diversity, diseases, and pests plays an important role in the loss of older species and varieties. This is further complicated by climate change and reproductive behavior of crop species. The Genetic erosion of cultivated diversity is reflected in a modernization bottleneck in the diversity levels that occurred during the history of the crop. Two stages in this bottleneck are recognized: the initial replacement of landraces by modern cultivars and further trends in diversity as a consequence of modern breeding practices. The factors contributing to erosion is due to the enormous diversity in cultivated plants, population growth, deforestation, erosion, changing land use, and climate factors are major threats to the existing biodiversity of the region. Urbanization is increasing and agriculture is changing from subsistence based on highly market-driven farming. Although

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these changes have increased incomes of the populations of wild habitants to certain extent, not all of them are for the good. In particular, biodiversity is declining as a result of some of these changes. It is mandate to conserve the vanishing plant genetic resources and to understand better the linkages between agricultural and economic system that affect diversity and sustainable production. Genetic erosion may occur at three levels of integration: crop, variety, and allele. Thus, genetic erosion is reflected in the reduction of allelic richness in conjunction with events at variety level. This requires immediate efforts to understand and implement the effective multiplication and conservation strategies using both conventional and modern technologies to save the loss of the valuable genetic resources and preserve them for posterity. An important aspect is also to include genetic resource conservation as an important part in our social life.

Keywords Genetic resources · Genetic erosion · Crop diversity · Black pepper · Spices · Cardamom · Ginger · Turmeric · Vanilla · Cinnamon · Nutmeg · Clove · Coriander · Cumin · Fennel · Fenugreek · GIS · In situ conservation · In vitro conservation · Cryo preservation · DNAbank · Pollen bank

9.1 Introduction

Plant genetic resources—constituting genotypes or populations of cultivars (landraces, advance/improved cultivars), genetic stocks, wild and weedy species which are maintained in the form of plants, seeds, tissues, etc.—hold key to food security and sustainable agricultural development (Iwananga 1994). Genetic diversity is an essential resource for crop breeding and reservoirs of identified and unidentified genes are essential for the study of the breeders of all generations. The primary and secondary centers of origin are the source for germplasm due to the natural hybridization and flow of genes throughout their existence. Detailed study on germplasm gives us the source material for resistance to biotic and abiotic stresses which can be further used in the improvement aspect.

India is the land of spices and is the primary or secondary center of origin to major spices especially black pepper, cardamom, ginger, turmeric, cinnamon, tamarind, and garcinia, where genetic diversity is rich and their wild forms still exist. In others, the diversity is limited. The important spices relevant in Indian context are black pepper, cardamom, ginger, turmeric, coriander, cumin, fennel, fenugreek, cinnamon, turmeric, cloves, allspice, garcinia, vanilla, and a few herbal spices (Ravindran et al. 2005b; Peter and Nirmal Babu 2006; Ravindran et al. 2006).

9.2 Origin, Distribution, and Diversity

9.2.1 Black Pepper

Black pepper one of the oldest spices known to the world (*Piper nigrum* L.) is a native of the humid tropical forests of the Western Ghats, from where it has spread throughout the tropics. *P. nigrum* L. belongs to the pepper family Piperaceae of the series Microembryeae of Monochlamydeae. The genus *Piper* is generally distributed in the tropical and subtropical regions of the world. The main centers of distribution are Central and South America and South Asia (Trelease and Yuncker 1950). The main center of distribution for Neotropical species is Central America. In the Central American forests, the genera are distributed in four different habitats, viz. edge of the semi-deciduous forests, inside the semi-deciduous forest, edge of the swampy forest, and inside the swampy forests. The greatest diversity of *Piper* species occurs in Tropical America with over 700 species followed by southern Asia with over 400 species (Fig. 9.1). Diversity of *Piper* is also occurring in South Pacific (40 spp.) and in the African tropics (15 spp.) (Jaramillo and Manos 2001).

About 114 species are reported from the Indian subcontinent (Table 9.1), of which about 18 species are found in sub-mountainous tracts of Western Ghats and adjacent peninsular and coastal region (Ravindran and Nirmal Babu 1994; Ravindran 2000; Ravindran et al. 2000, 2005; Tyagi et al. 2004; Ravindran and Kallupurackal 2012). In India, the north-eastern region and the south-western

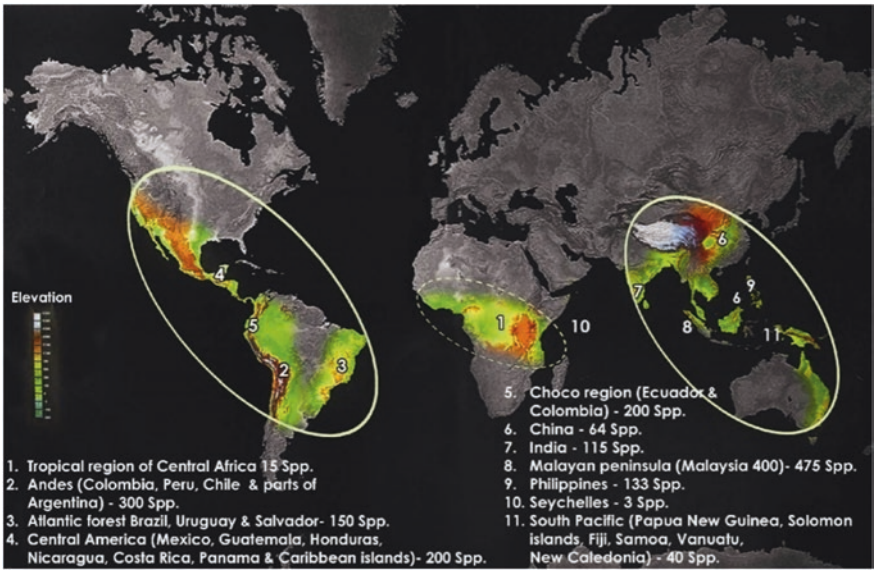

Fig. 9.1 The global geographical distribution of *Piper* and probable centers of origin of genus *Piper* (Saji 2006)

Table 9.1 *Piper* species reported from India

Sl. No.	Species	Sl. No.	Species	Sl. No.	Species
1.	*P. accrescens* Van Heurck & Müll.Arg.	39.	*P. gibsonii* C.DC	77.	*P. ovato-acuminatum* C.DC.
2.	*P. acutistigmum* C.DC.	40.	*P. glabramentum* C.DC.	78.	*P. ovatostemon* C.DC.
3.	*P. anisotis* Hook.f.	41.	*P. glabrirhache* C.DC.	79.	*P. pallidum* Van Heurck & Müll.Arg.
4.	*P. arborescens* Roxb.	42.	*P. grandipeduculum* C.DC.	80.	*P. parvilimbum* C.DC.
5.	*P. arborigaudens* C.DC.	43.	*P. griffithii* C.DC.	81.	*P. pedicellatum* C.DC.
6.	*P. argyrophyllum* Miq.	44.	*P. guigual* Buch.-Ham. ex D.Don	82.	*P. peepuloides* Roxb.
7.	*P. arunachalensis* Gajurel, Rethy & Y.Kumar	45.	*P. hamiltonii* C.DC.	83.	*P. petiolatum* C.DC.
8.	*P. attenuatum* Buch. Ham. ex Wall.	46.	*P. hapnium* Buch.-Ham.	84.	*P. phalangense* C.DC.
9.	*P. aurantiacum* Wall.	47.	*P. haridasanii* Gajurel, Rethy & Y.Kumar	85.	*P. pseudonigrum* K.C.Velayudhan & V.A. Amalraj
10.	*P. aurorubrum* C.DC	48.	*P. hookeri* Miq.	86.	*P. puberulirameum* C.DC.
11.	*P. bababudani* Rahiman.	49.	*P. hymenophyllum* (Miq.) Wight	87.	*P. pykarahense* C.DC.
12.	*P. barberi* Gamble	50.	*P. isopleurum* C.DC.	88.	*P. retrofractum* Vahl.
13.	*P. bengalense* C.DC.	51.	*P. japvonum* C.DC.	89.	*P. rhytidocarpum* Hook.f.
14.	*P. betle* L.	52.	*P. jenkinsi*i C.DC.	90.	*P. ribesioides* Wall.
15.	*P. betleoides* C.DC.	53.	*P. kapruanum* C.DC.	91.	*P. sarmentosum* Roxb.
16.	*P. boehmeriaefolium* Wall.	54.	*P. khasianum* C.DC.	92.	*P. schmidtii* Hook.f.
17.	*P. brachystachyum* Wall.	55.	*P. laeve* Vahl	93.	*P. saxatile* Wall.
18.	*P. calvilimbum* C.DC.	56.	*P. lainatakanum* C.DC.	94.	*P. sikkimense* C.DC.
19.	*P. caninum* Blume	57.	*P. lanatum* Roxb.	95.	*P. silentvalleyensis* Ravindran, M.K. Nair & Asokan Nair
20.	*P. carnistigmum* C.DC.	58.	*P. laxivenum* C.DC.	96.	*P. siriboa* L.
21.	*P. caudilimbum* C.DC.	59.	*P. longum* L.	97.	*P. subpeltatum* Willd. –
22.	*P. chaba* Hunter	60.	*P. maingayi* Hook.f.	98.	*P. sugandhi* Babu et Naik
23.	*P. clarkei* C.DC.	61.	*P. makruense* C.DC.	99.	*P. suipigua* Buch.-Ham. ex D.Don

(continued)

Table 9.1 (continued)

Sl. No.	Species	Sl. No.	Species	Sl. No.	Species
24.	*P. clypeatum* Wall.	62.	*P. malamiris* L.	100.	*P. subrigidilimbum* C.DC.
25.	*P. cornilimbum* C.DC.	63.	*P. mannii* C.DC.	101.	*P. sylvaticum* Roxb.
26.	*P. crassistipes* C.DC.	64.	*P. meeboldii* C.DC.	102.	*P. sylvestre* Lam.
27.	*P. crenulatibracteum* C.DC.	65.	*P. mullesua* Buch.-Ham.	103.	*P. syringifolium* Vahl
28.	*P. cubeba* L. f.	66.	*P. muneypo-rense* C.DC.	104.	*P.t albotii* C.DC.
29.	*P. curtistipes* C.DC.	67.	*P. mungpooa-num* C.DC.	105.	*P. tenuibracteum* C.DC.
30.	*P. dekkoanum* C.DC.	68.	*P. nagaense* C.DC.	106.	*P. tenuiflorum* Vahl
31.	*P. diffusum* Blume ex Miq.	69.	*P. nepalense* Miq.	107.	*P. thermale* Vahl
32.	*P. exasperatum* Vahl	70.	*P. nigramentum* C.DC.	108.	*P. thomsoni* Hook.f.
33.	*P. falconeri* C.DC.	71.	*P. nigrum* L.	109.	*P. trichostachyon* C.DC.
34.	*P. filipedunculum* C.DC.	72.	*P. obtusistig-mum* C.DC.	110.	*P. trioicum* Roxb.
35.	*P. galeatum* C.DC.	73.	*P. oldhamii* C.DC.	111.	*P. tristachyon* C.DC.
36.	*P. gallatlyi* C.DC.	74.	*P. ootacamun-dense* C.DC.	112.	*P. voigtii* C.DC.
37.	*P. gamblei* C.DC.	75.	*P. opacilimbum* C.DC.	113.	*P. wightii* Miq.
38.	*P. gammiei* C.DC.	76.	*P. ovatistigmum* C.DC.	114.	*P. zuccarinii* C.DC.

(Western Ghats) region are recognized as two independent centers of diversity. *Piper* species occurring in India are unisexual, but the Central and South American species are generally bisexual types. However, the cultivated black pepper is bisexual. Probably, the bisexual types might have originated from the wild unisexual ones as a result of domestication and conscious and continuous selection for high-yielding types and their maintenance by vegetative propagation by people through the ages.

Over 100 cultivars of black pepper are known to India. The Dutch in the seventeenth and eighteenth centuries brought pepper cultivation on to Java, Sumatra, Borneo, Sarawak, the Malay Peninsula, Siam, Philippines, and later into the West Indies on a plantation scale. Black pepper is believed to be introduced to America during the middle of eighteenth century (Gentry 1955).

In addition to black pepper, the other economically important species of *Piper* are Indian long pepper (*P. longum* L), betel vine (*P. betle* L.), Java long pepper (*P. chaba* Hunter), tailed pepper (*P. cubeba* L.), Kawa pepper (*P. methysticum*

Forster), West African pepper (*P. clusi* C. DC.), Benin pepper (*P. guineense* Schum. & Thonn.) and Japanese pepper (*P. kadzura* (Choisy) Ohwi.).

The community composition, species richness, relative abundances of different species, and species diversity in a community were studied by Saji (2006) using Shannon diversity index (H) which indicated that the Western Ghats region of South India could be divided into seven, based on relative abundance of different species and species diversity in a community. Southern tip of India has highest diversity, while the coastal regions have low diversity. The hilly regions of Kerala and Tamil Nadu are the two regions with maximum diversity—especially the mountainous regions between the states (Fig. 9.2). The effort by the Government of India to protect these natural habitats as bio-parks is helping in conservation of biodiversity in these regions. This is helping many *Piper* species getting protected and they multiply into viable populations due to prevailing vegetative propagation. Because of these efforts, we find some endangered species like *Piper wightii* and *P. schmidtii* which are multiplying in Nilgiri reserves in Tamil Nadu and recently we discovered an ecological niche of *Piper barberi* a critically endangered species of *Piper* in the forest region of Idukki dist of Kerala, in India.

The cultivar diversity is very high in India where over 100 cultivars of pepper have been reported (Ravindran et al. 2000) from Western Ghats (Table 9.2). It is probable that the cultivated forms in different regions have originated from wild peppers of the same region.

India has assembled a world collection of black pepper germplasm with over 3500 accessions of cultivars, related species, land races, and maintains them in ex situ clonal field repositories. In addition, over 17 improved varieties are released for cultivation with good characters like high yield, bold berries, resistance to

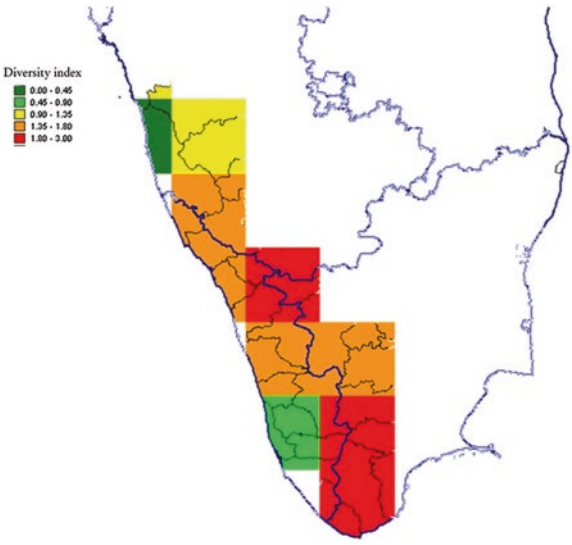

Fig. 9.2 Diversity indices of distribution of *Piper* species in Western Ghats of India

Table 9.2 Important cultivars of black pepper in India and other countries

Country	Name of the cultivar
India	Aimpiriyan, Arakkulam munda, Arimulaku, Aralumuriyan, Aranavalan, Arasinagunda, Arasinamuratta, Arikotta, Arivally, Balankotta, Cheppukulamundi, Cheriyakaniakkadan, Champakkara, Chankupazhuppan, Charadfupiriyan, Cheruvally (Cherukodi), Chettanvally, Cholamundi, Chumala, Doddigae,, Dadasinikulu, Doddalae, Ghantuvalli, Giddaghere, Irumaniyan, Jeerakamundi, Kalluvally, Karimkotta, Karimunda, Karimundi, Karivilanchi, Kottanadan, Kurimalai, Kuriyalmundi, Kuthiravally, Kallubalankotta, Kallumunda, Knajirakodan, Kanjiramundi, Kapplangamundi, Karimkodi, Karimuratta, Karivally, Konomkara, Kotta, Kottan, Kudirugunda, Kumbhachola (Kumbhakodi), Kumbhanadan, Kuppakkodi, Kuttiyanikodi, Kuzhuvelikodi, Malamundi, Maligesara, Mundi, Malanadan, Manjamundi, Marampadathi, Marankodi, Maeramodiyan, Motaghere, Munda, Murithothan, Narayakkodi, Nedumchola, Neelamundi, Nadesankodi, Nastigunda, Neyyattinkaramundi, Orumaniyan, Perambramunda, Perumkodi, Poonjaran munda, Padappan, Perumkarimunda, Pirimundi (Pirimunda), Ppunchakodi, Sagar Local, Shimoga, Sulla, Thevanmundi, Thommankodi, Thulamundi, Thekkan, Thippalimundi, Thottamundi, Uddaghere, Uthirankotta, Vadakkan, Valiakaniakkadan, Vattamundi, Vellanamban, Velliyaranmunda, Vally, Varikkakodi, Vellamunda, Veluthakaniakkadan, Vokkalginja, Vokkalu, Wynadan, Yohannakodi
SriLanka	Palulata, Panniyur 1, Kuching
Malaysia	Kuching, Semongok Perak, Semongok Emas
Indonesia	Belantung, Natar-1, Natar-2, Bulok Belantung, Jambi, Kerinci, Lampung Daun Lebar (LDL), Bangka (Muntok) and Lampung Daun Kecil (LDK), Petaling 1. Petaling 2, Choenuk, Bengkayang
Brazil	Chingapura, Bragantina, Iaçará and Guajarina,Panniyur 1

Phytophthora and nematodes, high dry recovery, high piperine, oleoresin and essential oil contents (Table 9.11). However, due to the preference of high-yielding varieties and debilitating diseases like *Phytophthora* foot rot, many of the old primitive cultivars are being replaced and are slowly disappearing from cultivation.

9.2.2 Small Cardamom

Small Cardamom is the dried fruit of *Elettaria cardamomum* Maton, belonging to Zingiberaceae. Cardamom occurs in its native state only in the tropical evergreen forests of the Western Ghats. The wild populations of cardamom gradually declined because of the large-scale destruction of forest habitats. *E. cardamomum* in India is monotypic genus with only one species. Its closest species is the Sri Lankan wild cardamom *E. cardamomum* namely, var. major. Seven other species of *Elettaria* were reported from southeast Asia.

The species *E. cardamomum* comprises a freely interbreeding population, and the genus *Elettaria* seems to be a "Cenospecies," in India, with a single "ecospecies" corresponding to the taxonomic species, *E. cardamomum* Maton. The

ecotype can be divided into three mini local populations as local types they are '*Travancoria,' 'Oblongata,' and* '*Kanarensis.*' Cardamom consists of three morphologically distinct types, namely, Malabar, Mysore and Vazhukka. Cardamom being a cross-pollinated crop, a lot of phenotypic variants exists in nature. Some of exceptional variants in cardamom have panicles of various types, terminal panicle, branched raceme, female sterility and cleistogamy. Good variability exists in cardamom with regard to various morphological characters such as fruit (capsule) size, shape, leaf, and plant pubescence and quality characters, such as essential oil and its components, such as 1, 8-cineole and alpha-terperyl acetate. (Madhusoodanan et al. 1994, 2002) reported that a wide range of variation was observed between and within cultivars of small cardamom for economically important characters. In general, the 'Vazhukka' and 'Mysore' types are robust compared to Malabar types (Ravindran and Madhusoodanan 2002; Korikanthimath et al. 2006; Parthasarathy and Prasath 2012).

Over 900 accessions of cardamom germplasm are maintained as clonal repositories at various centers in India. The characters which are found to least occur are compound panicle, basal branching of panicles, and red pseudostem pigmentation. The diversity is very narrow with respect to biotic and abiotic stresses. In addition, over 10 improved varieties are released for cultivation with good characters like high yield, bold capsules, high-quality attributes, resistance to viruses, rhizome rot and drought (Table 9.11). However, due to the preference for newer high-yielding varieties and debilitating viral disease and fungal diseases like katte and rhizome rot, many of the low yielding cultivars are being replaced.

9.2.3 Ginger

Ginger (*Zingiber officinale* Roscoe) belongs to family Zingiberaceae. The northeastern region is a major producer of ginger. Indo-Malayan region is the native home of this family. Ginger is not found in the truly wild state. It is believed to have originated in southeast Asia, but was under cultivation from ancient times in India (Purseglove et al. 1981; Mohanty and Panda 1994). There is no definite information on the primary center of origin or domestication. It was brought to the Mediterranean region from India by traders during the first century AD. During the thirteenth century AD, the Arabs took ginger to eastern Africa from India. Later, it was spread to West Africa by the Portuguese for commercial cultivation. Because of the ease with which ginger rhizomes can be transported long distances, it has spread throughout the tropical and subtropical regions in both hemispheres. The main areas of ginger cultivation are India, China, Nigeria, Indonesia, Jamaica, Taiwan, Sierra Leone, Fiji, Mauritius, Brazil, Costa Rica, Ghana, Japan, Malaysia, Bangladesh, Philippines, Sri Lanka, Solomon Islands, Thailand, Trinidad, Tobago, Uganda, Hawaii, Guatemala and many Pacific Ocean islands.

The genus *Zingiber*, consisting of about 150 species, is widely distributed in tropical and subtropical Asia. Some important species of *Zingiber* (Sabu and

Table 9.3 Species of *Zingiber* and their distribution

Species	Origin
Z. officinale Roscoe, Z. *zerumbet* (L.) Sm., *Z. purpureum* Roscoe, *Z. roseum* (Roxb.) Roscoe, *Z. wightianum* Thw., *Z. macrostachyum* Dalz., *Z. cernuum* Dalz., *Z. capitatum* Roxb., *Z. cylindricum* Moon, *Z. montanum* (Koenig) Link ex, *Z. intermedium* Baker, *Z. nimmonii, Z. odoriferum* Blume, *Z. ligulatum* Roxb., *Z. spectabilis* Griff., *Z. clarkii* King ez Benth, *Z. marginatum* Roxb., *Z. intermedium* Baker, *Z. chrysanthum* Roscoe, *Z. rubens* Roxb., *Z. squarrosum* Roxb, *Z. elatum* R. Br	India
Zingiber americanus Blume*., Zingiber argenteum* J. Mood & I. Theilade, *Z. acuminatum* Valeton, *Z. albiflorum* R.M. Sm., *Z. aurantiacum* I. Theilade, *Z. chlorobracteatum* Mood & Theilade, *Z. citrinum* Ridley, *Z. curtiisii* Holttum, *Z. flammeum* I. Theilade & J.Mood, *Z. fraseri* I. Theilade, *Z. georgei* J. Mood & I. Theilade, *Z. gracile* Jack, *Z. flagelliforme* J. Mood & I. Theilade, *Z. incomptum* Burtt & R.M. Sm., *Z. kuntstleri* Ridley, *Z. lambii* J. Mood & I. Theilade, *Z. latifolium* J. Mood & I. Theilade, *Z. leptostachyum* Valeton, *Z. malaysianum* C.K. Lim, *Z. martini* R.M. Sm., *Z. multibracteatum* Holttum, *Z. pendulum* J. Mood & I. Theilade, *Z. phillippsii* J. Mood & I. Theilade, *Z. pseudopungens* R.M. Sm., *Z. sulphureum* Burkill ex I.Theilade, *Z. velutinum* J. Mood & I. Theilade, *Z. vinosum* J. Mood & I. Theilade, *Z. viridiflorum* J. Mood & I. Theilade	Malaysia
Z.aromaticum Valeton	Tropical Asia
Z. bradleyanum Craib,, *Z.citriodorum* J. Mood & I. Theilade, *Z. corallinum* Hance, *Z. flavovirens* I. Theilade, *Z. larsenii* I. Theilade, *Z. longibracteatum* I. Theilade, *Z. newmanii* I. Theilade, *Z. peninsulare* Theilade, *Z. smilesianum* Craib, *Z. villosum* I. Theilade	Thailand
Z. mioga Roscoe	Japan
Z. barbatum Wall	Myanmar, Thailand
Z. chrysostachys Ridley, *Z. spectabile* Griffith, *Z. wrayii* Ridley, *Z. petiolatum* (Holttum) I. Theilade	Thailand, Malaysia
Z. collinsii I. Theilade & J. Mood	Vietnam
Z. eborium J. Mood & I. Theilade	Malaysia, Indonesia
Z. gramineum Noronha ex Blume	Thailand, Cambodia, Sumatra
Z. griffithii Baker	Malaysia, Thailand, Singapore
Z. junceum Gagnep	Cambodia, Thailand, Laos
Z. kerrii Craib	Thailand, Indochina
Z. koshunense C.T. Moo	Taiwan
Z. longipedunculatum Ridley	Australia
Z. neglectum Valeton, *Z. niveum* J. Mood & I. Theilade	United States
Z. ottensii Valeton	Southeast Asia
Z. pachysiphon B.L. Burtt & R.M. Sm.	Malaysia, Australia
Z. parishii Hook. f.	India, Myanmar, Thailand

(continued)

Table 9.3 (continued)

Species	Origin
Z. pellitum Gagnepo	Thailand, Laos, Malaysia
Z. puberulum Ridley	Malaysia, Thailand, Singapore
Z. rubens Roxb	India, Thailand, Vietnam, China
Z. squarrosum Roxb	India, Myanmar

Skinner 2005, Ravindran and Nirmal Babu 2005a) are given in Table 9.3. In India, variability for cultivated ginger exists mainly in the north-eastern region and Kerala. A botanically distinct variety *Z. officinale* var. rubrum having pink outer skin of rhizome is under cultivation in Malaysia. The genus *Zingiber* includes many species grown as ornamentals, but some are cultivated for valuable medicines. They bear showy, long-lasting inflorescences and often brightly colored bracts and floral parts; they are widely used as cut flowers in floral arrangements. Some of them are good foliage plants due to their arching form and shining leaves.

Cultivated ginger *Zingiber officinale* does not occur in wild but maintained only under cultivation. Ginger has no seed set and is only propagated vegetatively. There is moderate varietal/cultivar diversity in India. In India, over 1200 accessions are maintained in clonal repositories. The cultivars are often named after the locality. Good variation with respect to plant height, days to maturity, dry recovery, rhizome shape, size, yield, fiber content, color and quality attributes was observed (Ravindran et al. 2005a; Nirmal Babu et al. 2011a; Valsala 2012). This is due to accumulated natural mutations maintained in the population efficient vegetative propagation. Chemical variations in essential oil, oleoresin and gingerol, shogaol contents, have been reported. However, genetic diversity for biotic and abiotic resistances is almost absent making this crop susceptible for diseases and pests. In addition, over 12 improved varieties are released for cultivation with good characters like high gingerol and shogaol content and dry recovery. High/low fiber, plumpy rhizomes, vegetable types, and high dry recovery (Table 9.11). Rhizome rot caused by *Pythium* spp. and bacterial wilt caused by *Ralstonia* spp. are the most destructive diseases affecting ginger plantations. As no resistance source is reported so far in ginger or related species, many of the local cultivars are facing threat of elimination.

9.2.4 Turmeric

Turmeric *Curcuma longa* (L.) belongs to Zingiberaceae, and is one of the most ancient spices used in India. India is the largest producer and exporter of turmeric. Turmeric is believed to have originated in the Indo-Malayan region.

The genus *Curcuma* consists of about 70–110 (true identity is unclear) species distributed chiefly in southeastern Asia (Skornickova et al. 2007). In addition to *C. longa*, the other economically important species of the genus are *C. aromatica*, which is used in medicine and in toiletry articles; *C. kwangsiensis*, *C. ochrorhiza*, *C. pierreana*, *C. zedoaria* and *C. caesia*, which are used in folk medicines of the southern and southeastern Asian nations; *C. alismatifolia*, *C. elata* and *C. roscoeana*, with floricultural importance; *Curcuma amada*, which is used as medicine and in a variety of culinary preparations, pickles, and salads; *C. zedoaria*, *C. pseudomontana*, *C. montana*, *C. angustifolia*, *C. rubescens*, *C. haritha* and *C. caulina* which are all used in manufacturing arrowroot powder. The other species of minor importance are *C. purpurescens*, *C. mangga*, *C. heyneana*, *C. zanthorrhiza*, *C. phaeocaulis* and *C. petiolata* (Nirmal Babu et al. 1993; Rama Rao and Rao 1994; Velayudhan et al. 1999; Ravindran et al. 2007a, b; Skornickova et al. 2007).

The greatest diversity of the genus occurs in India, Myanmar and Thailand and extends to Korea, China, Australia and the South Pacific. This genus is also distributed in Cambodia, Indonesia, Malaysia, Laos, Madagascar and the Philippines. Many species of *Curcuma* are economically valuable and different species are cultivated in China, India, Indonesia and Thailand and throughout the tropics, including tropical regions of Africa, America and Australia. Genus *Curcuma* has about 42 species distributed in India, out of which *C. longa* is cultivated for turmeric, *C. aromatica* is grown for use in toiletry articles, and *C. amada* (mango ginger) is cultivated in limited areas for use as a vegetable. The country of origin of cultivated turmeric (*C. longa*) is presumed to be the southeast Asia. India is the single largest producer and exporter of turmeric in the world (Manohar Rao et al. 2006; Nirmal Babu et al. 2011b).

Different species of turmeric are used in folk medicine, as a spice, as a vegetable in a variety of culinary preparations, pickles, and salads, in the production of arrowroot powder, and in toiletry articles. Many *Curcuma* species are highly valued as ornamentals. Turmeric oil is also now used in aromatherapy and the perfume industry. Many *Curcuma* species were recognized by local and tribal people all over Asia as valuable sources of medicine. Distributions of *Curcuma* species in southeast Asia and India are given in Tables 9.4 and 9.5.

Good cultivar diversity occurs in India, with over 1500 accessions are conserved in various centers. In India, turmeric set seeds and seedling populations of over 300 progenies supplement the existing germplasm. There is a high variation with regard to morphology, yield, quality attributes, and dry recovery. In addition, over 34 improved varieties are released for cultivation with good characters like short duration, resistance to rhizome rot, plant height, high curcumin, and oleoresin content and dry recovery (Table 9.11).

Table 9.4 Distribution of *Curcuma* species in Indo—Malayan region

Curcuma species	Distribution
C. aeruginosa Roxb.	India, Thailand, Indochina, Malaysia, Indonesia, Sri Lanka, Myanmar
C. albiflora Thwaites, *C. oligantha* Trimen	Sri Lanka
C. alismatifolia Gagnep	Thailand, Laos, Cambodia
C. amarissima Roscoe	India, China
C. aurantiaca Zijp	India, Java, Thailand, Malaysia
C. australasica Hook. f.	Australia
C. bicolor Mood & Larsen, *C. burtii* K. Larsen & Smith, *C. ecomata* Craib., *C. glans* K. Larsen & Mood, *C. gracillima* Gagnep., *C. thorelii* Gagnep	Thailand
C. comosa Roxb., *C. cordata* Wall., *C. petiolata* Roxb., *C. sessilis* Gage, *C. strobilifera* Wall. ex. Baker	Myanmar
C. elata Roxb., *C. rubrobracteata* Skornick., M. Sabu & Prasanthk	India, Myanmar
C. exigua N. Liu & S.J. Chen, *C. kwangsiensis* S.G. Lee & C.F. Liang, *C. sichuanensis* X. X. Chen, *C. yunnanensis* N. Liu & S.J. Chen	China
C. ferruginea Roxb.	India, Bangladesh
C. flaviflora S. Q. Tong	China, Thailand
C. harmandii Gagnep	Thailand, Cambodia
C. larsenii C. Maknoi & T. Jenjittikul	Thailand, Laos, Vietnam
C. longa L.	Asia
C. mangga Valeton & Zijp	India, Malaysia, Indonesia
C. parviflora Wall	Thailand, Myanmar, Malaysia
C. picta Roxb. ex. Skornick	India, Thailand, Sri Lanka, Peninsular Malaysia
C. rhabdota Siriirugsa & M.F. Newman	Thailand, Laos
C. rhomba Mood & K. larsen	Vietnam, Thailand
C. roscoeana wall	India, Bangladesh, Myanmar, Thailand
C. rubescens Roxb.	India, Thailand, Bangladesh
C. sparganifolia Gagnep	Indochina, Cambodia, Thailand
C. viridiflora Roxb.	Indonesia, Malaysia, Thailand, China, Sumatra
C. zanthorrhiza Roxb.	India, Java, Peninsular Malaysia, Vietnam, China, Thailand, Philippines
C. zedoaria (Christm.) Roscoe	India, Myanmar, Thailand, Malaysia

9.2.5 Vanilla

Vanilla planifolia [syn. *Vanilla fragrans*) is a member of Orchidaceae, the only commercially important spice in this family. Vanilla is a crop of great commercial importance as the source of natural vanillin; a major component of flavor industry.

Table 9.5 Distribution of *Curcuma* species in India[a]

Sl. No.	Species	Region of occurence	Sl. No.	Species	Region of occurence
1.	*C. aeruginosa* Roxb.	NE	17.	*C. latifolia* Rosc.	NE
2.	*C. albiflora* Thw.	Ka	18.	*C. longa* L.	
3.	*C. amada* Roxb.	IND	19.	*C. lutea*	Ke/Ka
4.	*C. amarassima* Rosc.	NE	20.	*C. malabarica*	Ka/Ke
5.	*C. aromatica* Salisb.	Ka/Ke	21.	*C. montana* Roxb.	AP
6.	*C. aurantiana*	Ke	22.	*C. nilamburensis* Velayudhan et al.	Ke
7.	*C. brog*	NE	23.	*C. nilgherrensis* Wight	SI
8.	*C. caesia* Roxb.	NE	24.	*C. oligantha* Trimen	Ke
9.	*C. cannanorensis* Ansari et al.	Ke	25.	*C. pseudomontana* Graham	SI & M
10.	*C. comosa* Roxb.	NE	26.	*C. raktakanta* Mangaly and Sabu	Ke
11.	*C. coriaceae* Mangaly & Sabu	Ke	27.	*C. soloensis*	NE
12.	*C. decipiens* Dalzell	Ke	28.	*C. sylvatica*	NE/Ke
13.	*C. ecalcarata* Sivarajan and Indu.	Ke	29.	*C. thalakkaveriensis* Velayudhan et al.	Ka
14.	*C. haritha* Sabu	Ke	30.	*C. vamana* Sabu & Mangaly	Ke
15.	*C. karnatakensis* Velayudhan et al.	Ka	31.	*C. vellanikkariensis* Velayudhan et al.	Ke
16.	*C. kudagensis* Velayudhan et al.	Ka	32.	*C. zedoaria* (Christm.) Roscoe	IND

Ke Kerala, *Ka* Karnataka, *NE* North-eastern region *M* Maharashtra, *SI* South India, *AP* Andhra Pradesh, *IND* India
[a]Velayudhan et al. (1999)

It originated in Mexico but is grown in many Pacific Ocean islands, Indonesia and many African countries. The genus Vanilla comprises about 110 species, distributed in tropical parts of the world (Purseglove et al. 1981; Cuvelier and Grisoni 2010; De Guzman and Zara 2012). Few important vanilla species are *V. andamanica, V. aphylla* syn. *V. vatsalana, V. pilifera, V. tahitensis, V. pompon, V. wightiana, V. parishii,* and *V. walkeriae* (Table 9.6).

The germplasm available in vanilla in India is very narrow. The primary gene pool of *V. planifolia* is narrow and is evidently threatened due to destruction of its natural habitats making the secondary gene pool important as a source of desirable traits especially for resistance to diseases. The species diversity in the country is represented by five species, viz., *V. aphylla, V. walkeriae, V. wightiana, V. pilifera,* and *V. andamanica* and most of them are considered endangered. Intense works of selection, breeding, and conservation of genetic resources are required to overcome the narrow genetic base of this vegetatively propagated crop. Effective

Table 9.6 Some important species of Vanilla

Vanilla	*Vanilla abundiflora* J.J. Sm., *V. acuminata* Rolfe, *V. africana* Lindl., *V. albida* Blume, *V. mexicana* Mill., *V. aphylla* Blume, *V. andamanica* Rolfe, *V. angustipetala* Schltr., *V. annamica* Gagnep., *V. appendiculata* Rolfe, *V. vellozoi* Rolfe, *V. anomala (Ames & L.O.Williams)* Garay, *V. barbellata* Rchb.f., *V. bahiana* Hoehne, *V. bertoniensis* Bertoni, *V. bicolor* Lindl., *V. borneensis* Rolfe, *V. bradei* Schltr. ex Mansf., *V. calopogon* Rchb.f., *V. calyculata* Schltr., *V. gardneri* Rolfe, *V. chalotii* Finet, *V. chamissonis* Klotzsch, *V. claviculata* Sw., *V. cobanensis* Archila, *V. columbiana* Rolfe, *V. coursii* H.Perrier, *V. crenulata* Rolfe, *V. cribbiana* Soto Arenas, *V. cristagalli* Hoehne, *V. odorata* C. Presl, *V. dietschiana* Edwall, *V. dilloniana* Correll, *V. dressleri* Soto Arenas, *V. dubia* Hoehne., *V. dungsii* Pabst, *V. edwallii* Hoehne, *V. poitaei* Rchb.f., *V. fimbriata* Rolfe, *V. francoisii* H. Perrier, *V. gardneri* Rolfe, *V. giulianettii* F.M. Bailey, *V. griffithii* Rchb.f., *V. guatemalensis* Archila, *V. guianensis* Splitg., *V. hamata* Klotzsch, *V. hartii* Rolfe, *V. havilandii* Rolfe, *V. helleri* A.D.Hawkes, *V. imperialis* Kraenzl., *V. inodora* Schiede, *V. insignis* Ames, *V. kaniensis* Schltr., *V. kempteriana* Schltr., *V. kinabaluensis* Carr, *V. madagascariensis* Rolfe, *V. martinezii* Soto Arenas, *V. montana* Ridl., *V. moonii* Thwaites, *V. nigerica* Rendle, *V. organensis* Rolfe, *V. ovata* Rolfe, *V. palembanica* Teijsm. & Binn.,*V. parvifolia* Barb. Rodr., *V. perrieri* Schltr., *V. phaeantha* Rchb.f., *V. planifolia* Jacks. ex Andrews, *V. platyphylla* Schltr., *V. pompona* Schiede, *V. ramificans* J.J. Sm., *V. ramosa* Rolfe, *V. ribeiroi* Hoehne, *V. roscheri* Rchb.f., *V. savannarum* Britton, *V. seretii* De Wild., *V. somae* Hayata, *V. sprucei* Rolfe, *V. trigonocarpa* Hoehne, *V. utteridgei* J.J. Wood, *V. walkeriae* Wight, *V. wariensis* Schltr., *V. wightii* Lindl. ex Wight, *V. zanzibarica* Rolfe, *V. yersiniana* Guillaumin & Sigaldi

Source Vanilla Species [*Catalogue of Life: 22nd December 2014, Integrated Taxonomic Information System (IT IS)*]

procedures for micropropagation and in vitro conservation by slow growth in selected species of vanilla are available.

Although vanilla is cultivated throughout the tropics, its natural populations in Southern Mexico—the most critical sources of novel genetic diversity—are on the verge of disappearing due to deforestation and over collection (Lubinsky 2003). Since the narrow primary gene pool is evidently threatened, the secondary gene pool comprising the close relatives of *V. planifolia*, which is also equally threatened, becomes important as a source of desirable traits—especially for self-pollination, higher fruit set, and disease resistance (Minoo 2002; Minoo et al. 2006; Bory et al. 2010). Many species of vanilla are considered endangered (Table 9.6) and there is urgent need to conserve them. The recent International Congress on vanilla emphasized the need to conserve these species before they go extinct (International Congress on Vanilla 2003). Thus a major challenge is to conserve the vanilla gene pool from the onslaught of habitat destruction, over collection, climate changes and destructive diseases in monocultures.

Recent advances in conservation have paved the way to safeguard plant biodiversity with a biotechnological approach, which can be regarded as complementary to the traditional clonal orchards and seed banks. Traditionally, Vanilla germplasm is conserved in clonal repositories belonging to botanical gardens and

in scientific institutions. However, the high costs of this traditional conservation system limit the number of accessions that can be preserved. In order to stem the flow of loss of biodiversity, an attempt to conserve *Vanilla* species, in vitro has been made (Minoo et al. 2006).

9.2.6 Tree Spices

There are many tree spices which are important. The most important ones, in Indian context, are cinnamon (*Cinnamomum verum syn: Cinnamomum zylanicyum*), nutmeg (*Myristica fragrans*), clove (*Syzygium aromaticum Syn: Eugenia Cariophyllus*), and garcinia (*Garcinia* sp.) (Ravindran et al., 2004a, b, 2005a), some of which are native and others introduced (Krishnamoorthy and Rema 1994).

9.2.6.1 Nutmeg

Nutmeg tree is the only plant that produces two separate spices, namely nutmeg (kernel of the seed) and mace (aril covering the seed). Nutmeg belongs to Myristicaceae and the species is believed to have originated in the Moluccas Islands of Indonesia. The important species occurring in India are *M. amygdalina, M. andamanica, M. attenuata, M. dactyloides, M. beddomeii, M. gibbosa, M. glabrae, M. glaucescensr., M. irya Gaertn., M. kingii., M. longifolia.,* and *M. magnifica.* Being a dioecious plant, good variability exists in nutmeg, especially for characters such as fruit size and shape, mace, and seed volume. (Krishnamoorthy et al. 1996). The chemical composition also shows quantitative variations for major quality components. Myristicin, elemicin, and 1,8-cineole are the important constituents in nutmeg. There is an increase in genetic diversity in cultivated nutmeg due to variation through segregating progenies. But the nutmeg population (nutmeg swamps) are slowly disappearing. Over 475 accessions are maintained at various centers in India. About six varieties of high-yielding, high-quality cultivars were recommended for release in India (Table 9.11).

9.2.6.2 Cinnamon

True cinnamon is obtained from *C. verum* belonging to Lauraceae; indigenous to Sri Lanka and Southern Western Ghats of India. Cassia cinnamon is obtained from various sources, the most important being *C. cassia* (Chinese cassia, Vietnam cassia or Saigon cassia). The other cassia cinnamons are Indonesian (Javan) cassia (*C. burmanii*), Saigon (Vietnam) cassia (*C. loureirii*), and Indian cassia (*C. tamala*). The genus is a native of south-western tropical India and SriLanka, consisting more than 250 species distributed in southeast Asia, China and Australia. Seychelles and Malagay Republic are the major cinnamon-producing

countries besides Sri Lanka. Some important species of *Cinnamomum* are given in Table 9.7. Over 26 species occur in India. Endemic species are *C. macrocarpum* Hk. F., *C. malabathrum* Bl., *C. nicolsonianum* Manilal and Shylaja, *C. riparium* Gamble, *C. keralaense* Kosterm, *C. travancoricum* Gamble, *C. wightii* Meiss., *C. heyneanum* Nees, *C. gracile* (Miq.), and *C. chemungianum* Mohan and Henry. Non-endemic species are *C. citriodorum*Thw., *C. filipedicellatum* Kosterm., *C. goaense* Kosterm, *C. perottetii* Meiss., *C. sulphuratum* Nees, and *C. walaiwarense* Kosterm (Haldankar et al. 1994; Krishnamoorthy et al. 1996; Tyagi et al. 2004; Ravindran et al. 2004a, b).

Cinnamon trees are naturally cross-pollinated and as a result much variation exists in natural populations for morphological, chemical as well as bark characters. The quality of cinnamon depends on the essential oil content and composition of leaf and bark oil. The leaf oil contains eugenol as the chief component, while the bark oil has cinnamaldehyde. Over 430 accessions are maintained at various centers in India. About six varieties of high-yielding, high-quality cultivars were recommended for release in India (Table 9.11).

9.2.6.3 Clove

Clove, belonging to the family Myrtaceae, is a native of Moluccas Islands and was introduced to India. Because of the limited introductions that have taken place and due to self-pollinating nature of the species, the genetic base of germplasm available in India is very narrow for use in any meaningful crop improvement program. The spice is dried, mature, unopened flower buds (Nurdjannah and Bermawie 2012). The clove buds contain around 15–17 % volatile oil, the main component of which is eugenol (about 70–90 %). There are many species of *Syzygium* occurring in India. Over 250 accessions are maintained at various centers in India.

9.2.6.4 Garcinia

The genus *Garcinia* of the family Clusiaceae is a large genus of evergreen polygamous trees, shrubs, lianas, and herbs. It consists of over 200 species distributed in the tropics of the world chiefly in Asia, Africa, and Polynesia. *Garcinia* is native to old world tropics and maximum concentration of *Garcinia* species occurs in Asian countries. It is hypothesized that the genus *Garcinia* has originated before the continental drift followed by separate diversification in canters in the Afro-Madagascar and Indo-Malayan areas. About 35 species occur in India, many of which are endemic and economically important including *G. mangostana, G. indica G. gummi-gutta, G. cowa, G. pedunculata, G. xanthochymus* Hk.f, with immense medicinal properties. *Garcinia* is the source for a natural diet ingredient hydroxy citric acid (HCA) which is an anti-obesity compound. However, lack of awareness coupled with habitat destruction, is leading to genetic erosion of this forest resource and many species are threatened.

Table 9.7 Species diversity in tree spices

Genus	Species
Cinnamomum	*Cinnamomum alainii (C.K. Allen) A.H. Liogier, C. alexei Kosterm., C. alternifolium Kosterm., C. altissimum Kosterm., C. amoenum (Nees) Kosterm., C. amplexicaule* (Cham. & Schltdl.) Kosterm., *C. anacardium* Kosterm., *C. angustitepalum* Kosterm., *C. antillanum* (Meisn.) Kosterm., *C. appelianum* Schewe in Hand.-Mazz., *C. arbusculum* Kosterm., *C. archboldianum* C.K. Allen, *C. areolatum* (Lundell) Kosterm., *C. arfakense* Kosterm., *C. arsenei* (C.K. Allen) Kosterm., *C. asomicum* S.C. Nath & Baruah, *C. aubletii* Lukmanoff, *C. aureofulvum* Gamble, *C. auricolor* Kosterm., *C. austrosinense* Hung T. Chang, *C. austroyunnanense* H.W. Li, *C. baileyanum (*F. Muell. ex F. M. Bailey) Francis, *C. balansae* Lecomte, *C. bamoense* Lukmanoff, *C. barbeyanum* (Mez) Kosterm., *C. bejolghota* (Buch.-Ham.) Sweet, *C. bhamoensis* M. Gangop., *C. bhaskarii* M. Gangop., *C. bintulense* Kosterm., *C. birmanicum* A.J.G.H. Kostermans, *C. bishnupadae* M.Gangop., *C. blandfordii* M.Gangop., *C. bodinieri* H. Lév., *C. bonii* Lecomte, *C. borneense* Miq., *C. brachythyrsum* J. Li, *C. bractefoliaceum* F.G. Lorea-Hernandez, *C. breedlovii* (Lundell) Kosterm., *C. brenesii* (Standley) Kosterm., *C. brevifolium* Miq., *C. burmannii* (Nees & T. Nees) Bl., *C. calciphilum* Kosterm., *C. calyculatum* Miq., *C. cambodianum* Lecomte, *C. camphora* (L.) J. Presl, *C. cappara-coronde* Bl., *C. caratingae* I. de Vattimo-Gil, *C. carolinense* Koidz., *C. caryophyllus* (Lour.) S. Moore, *C. cassia* (L.) Presl, *C. caudifolium* Kosterm., *C. cebuense* Kosterm., *C. celebicum* Miq., *C. champokianum* Baruah & S.C. Nath, *C. chartophyllum* H.W. Li, *C. chavarrianum* (Hammel) Kosterm., *C. chemungianum* M. Mohanan & A.N. Henry, *C. chiapense* (Lundell) Kosterm., *C. citriodorum* Thw., *C. clemensii* C.K. Allen, *C. concinnum* F.G. Lorea-Hernandez, *C. contractum* H.W. Li, *C. cordatum* Kosterm., *C. coriaceum* Cammerloher, *C. corneri* Kosterm., *C. costaricanum (*Mez & Pittier) Kosterm., *C. crenulicupulum* Kostermans, *C. crispulum* Kosterm., *C. culilaban* (L.) Kosterm., *C. cupulatum* A.J.G.H. Kostermans, *C. curtisii* Lukmanoff, *C. curvifolium (*Lour.) Nees, *C. cuspidatum* Miq., *C. cyrtopodum* Miq., *C. damhaensis* Kosterm., *C. daphnoides* Sieb. & Zucc., *C. dasyanthum* Miq., *C. degeneri* C.K. Allen, *C. dictyoneuron* Kosterm., *C. doederleinii* Engl., *C. dubium* Nees, *C. durifolium* Kosterm., *C. ebaloi* Kosterm., *C. effusum (Meisn.)* Kosterm., *C. ehrenbergii (Mez)* Kosterm., *C. ellipticifolium* A.J.G.H. Kostermans, *C. elongatum* (Nees) Kosterm., *C. endlicheriicarpum*Kosterm., *C. englerianum* Schewe, *C. erythropus* (Nees & Mart.) Kosterm., *C. eugenoliferum* Kosterm., *C. falcatum* (Mez) R.A. Howard, *C. filipedicellatum* Kosterm., *C. filipes* (Rusby) Kosterm., *C. fitianum* (Meisn.) A. C. Sm., *C. floccosum* van der Werff, *C. formicarium* van der Werff & Lorea-Hern., *C. fouilloyi* Kosterm., *C. foveolatum* (Merr.) H.W.Li & J.Li, *C. frodinii* Kosterm., *C. fruticosum* (Lundell) Kosterm., *C. glanduliferum* (Wall.) Nees, *C. glaucescens* (Buch.-Ham. ex Nees) Hand.-Mazz., *C. glaziovii* (Mez) Kosterm., *C. glauciphyllum* A.J.G.H. Kostermans, *C. goaense*Kosterm., *C. glossophyllum* F.G. Lorea-Hernandez, *C. gracillimum* Kosterm., *C. grandiflorum* Kosterm., *C. grandifolium* Cammerloher, *C. grandis* Kosterm., *C. grisebachii* F.G. Lorea-Hernandez, *C. hartmannii* (Johnston) Kosterm., *C. hatschbachii* Vattimo, `*C. hausknechtii* (Mez) Kosterm., *C.helferii* Lukmanoff, *C. heterantherum* (Ruiz & Pav.) Kosterm., *C. heyneanum* Nees, *C. hkinlumense* A.J.G.H. Kostermans, *C. ilicioides* A. Chev., *C. impressicostatum* Kosterm., *C. impressinervium* Meisn., *C. inconspicuum* Kosterm., *C. iners* Reinw. ex Bl., *C. inunctum* (Nees) Meisn., *C. japonicum* Sieb. ex Nakai, *C. javanicum* Bl., *C. jensenianum* Hand.-Mazz., *C. johnstonii* (C.K. Allen) Kosterm., *C. kami* Kosterm., *C. keralaense* Kosterm., *C. kerangas* Kosterm., *C. kerrii* Kosterm., *C. kinabaluense* Heine, *C. kingdon-wardii* A.J.G.H. Kostermans, *C. kotoense* Kaneh. & Sasaki, *C. kruseanum* O. Téllez-Valdés & J.L. Villaseñor, *C. kunstleri* Ridl., *C. kwangtungense* Merr., *C. lampongum* Miq., *C. lanaoense* Kosterm., *C. lanigerum* van der Werff, *C. lanuginosum* Kosterm., *C. laubatii* F. Muell., *C. lawang* Kosterm., *C. ledermannii* Schewe, *C. leptophyllum* F. G. Lorea-Hernandez, *C. leptopus* A.C. Sm., *C. litseifolium* Thw., *C. lohitensis* M.Gangop., *C. longepedicellatum* Kosterm., *C. loureiroi* Nees, *C. macrophyllum* Miq., *C. mairei* H. Lév., *C. malabatrum* (Burm. f.) Presl, *C. mathewsii* (Mez) Kosterm., *C. melastomataceum* Kosterm., *C. microphyllum* Ridl., *C. molle* (Mez) Kosterm., *C. oblongum* Kosterm., *C. osmophloeum* Kaneh., *C. parthenoxylon (*Jack) Meisn., *C. polyadelphum* (Lour.) Kosterm., *C. quadrangulum* Kosterm., *C. racemosum* Kosterm., *C. rigidum* Gillespie, *C. sessilifolium* Kanehira, *C. stenophyllum* (Meisn.) I. de Vattimo, *C. subsessile* (Meisn.) Kosterm., *C. tamala* (Buch.-Ham.) Th. G.G. Nees, *C. tazia* (Buch.-Ham.) Kosterm. ex M. Gangop., *C. tenuifolium* J. Sugimoto, *C. velveti* F.G. Lorea-Hernandez, *C. verum* J.S. Presl, *C. xylophyllum* Kosterm., *C . wightii* Meisn., *C. yabunikkei* H.Ohba, *C. zapatae* F.G. Lorea-Hernandez

(continued)

Table 9.7 (continued)

Genus	Species
Myristica	*Myristica acsmithii* W.J.J.O. de Wilde, *M. acuminata* (Lam.) Warb.,*M. fatua subsp. affinis* (Warb.) de Wilde, *M. agusanensis, M. alba* W.J.J.O. de Wilde, *M. hollrungii* Warb., *M. fragrans* Houtt., *M. bancana* (Miq.) J. Sinclair, *M. andamanica* Hook. fil., *M. archboldiana* A. C. Sm., *M. argentea* Warb., *M. basilanica* W.J.J.O. de Wilde, *M. beddomei, M. beccarii* Warb., *M. bialata* Warb., *M. bifurcate, M. brachypoda* W.J.J.O. de Wilde, *M. brassii* A. C. Sm., *M. brevistipes* W.J.J.O. de Wilde, *M. cagayanensis* Merr., *M. carrii* J. Sinclair, *M. cerifera* A. C. Sm., *M. ceylanica* A. DC., *M. chartacea* Gillespie, *M. chrysophylla, M. cinnamomea* King, *M. clemensii* A.C.Sm., *M. concinna* J. Sinclair, *M. corticata* W.J.J.O. de Wilde, *M. crassa* King, *M. cucullata* Markgra, *M. cumingii* Warb., *M. elliptica* Wall. ex Hook. fil. & Thoms., *M. ensifolia* J. Sinclair, *M. extensa* W.J. de Wilde, *M. fallax* Warb., *M. fasciculata* W.J.J.O. de Wilde, *M. firmipes* J. Sinclair, *M. garciniifolia* Warb., *M. gibbosa* Hook. fil. & Thoms., *M. gracilipes* J. Sinclair, *M. grandifolia* A. DC., *M. guatteriifolia* A. DC., *M. hollrungii* Warb., *M. impressa* Warb., *M. iners* Bl., *M. ingrata subsp. Ingrate, M. inopinata* J. Sinclair, *M. johnsii* W.J.J.O. de Wilde, *M. kalkmanii* W.J.J.O. de Wilde, *M. koordersii* Warb., *M. laevifolia* W.J.J.O. de Wilde, *M. macrantha* A. C. Sm., *M. maingayi* Hook. fil., *M. malabarica* Lam., *M. maxima* Warb., *M. mindanaensis* Warb., *M. nana* W.J.J.O. de Wilde, *M. neglecta* Warb., *M. ornata* W.J.J.O. de Wilde, *M. pachyphylla* A. C. Sm., *M. pedicellata* J. Sinclair, *M. petiolata* A. C. Sm., *M. philippensis* Lam., *M. pumila* W.J.J.O. de Wilde, *M. rubrinervis* W.J. de Wilde, *M. rumphii* (Bl.) Kosterm., *M. sarcantha* W.J. de Wilde, *M. schleinitzii* Engl., *M. smythiesii* J. Sinclair, *M. sphaerosperma* A. C. Sm., *M. subalulata* Miq., *M. sumbawana* Warb., *M. trianthera* W.J.J.O. de Wilde, *M. tubiflora* Bl., *M. umbrosa* J. Sinclair
Syzygium	*Syzygium abbreviatum*Merr., *S. aborense* (Dunn) Rathakr. & N.C.Nair, *S. abortivum* (Gagnep.) Merr. & L.M.Perry, *S. abulugense* Merr., *S. aciculinum* Merr. & L.M.Perry, *S.* acre (Pancher ex Guillaumin) J.W.Dawson, *S. acrophilum* (C.B.Rob.) Merr., *S. acuminatissimum* (Blume) DC., *S. acuminatum* (Roxb.) Miq., *S. acutangulum* Nied., *S. acutatum* (Miq.) Amshoff, *S. adelphicum* Diels, *S. adenophyllum* Merr. & L.M.Perry, *S. aegiceroides* (Korth. ex Miq.) Korth., *S. aemulum* (Blume) Amshoff, *S. aeoranthum* (Diels) Merr. & L.M.Perry, *S. affine* Merr., *S. agastyamalayanum* M.B.Viswan. & Manik., *S. aggregatum* J.W.Dawson, *S. aksornae* Chantaran. & J.Parn.,*S. alatoramulum* B.Hyland, *S. alatum* (Lauterb.) Diels, *S. albayense* Merr., *S. album* Q.F.Zheng, *S. alternifolium* (Wight) Walp., *S. alutaceum* (Diels) Merr. & L.M.Perry, *S. alyxiifolium* (Ridl.) I.M.Turner, *S. amieuense* (Guillaumin) J.W.Dawson, *S. amplifolium* L.M.Perry, *S. ampullarium* (Stapf) Merr. & L.M.Perry, *S. andamanicum* (King) N.P.Balakr., *S. angkae* (Craib) Chantaran. & J.Parn., *S. angulare* (Elmer) Merr., *S. anisatum* (Vickery) Craven & Biffin, *S. anisosepalum* (Duthie) I.M.Turner, *S. anthicoides* P.S.Ashton, *S. antonianum* (Elmer) Merr., *S. apiarii* P.S.Ashton, *S. arboreum* (Baker f.) J.W.Dawson, *S. argyrocalyx* (Warb.) Merr. & L.M.Perry, *S. aromaticum* (L.) Merr. & L.M.Perry, *S. assamicum* (Biswas & Purkay.) Raizada, *S. attenuatum* (Miq.) Merr. & L.M.Perry, *S. aurantiacum* (H.Perrier) Labat & Schatz, *S. auriculatum* Brongn. & Gris, *S. avene* Miq., *S. barnesii* (Merr.) Merr., *S. bartonii* (F.M.Bailey) Merr. & L.M.Perry, *S. beddomei* (Duthie) Chithra, *S. benjaminum* Diels, *S. bicolor* Merr. & L.M.Perry, *S. blumei* (Steud.) Merr. & L.M.Perry, *S. borbonicum* J.Guého & A.J.Scott, *S. brachiatum* (Roxb.) Miq., *S. brachybotryum* Miq., *S. brackenridgei* (A.Gray) Müll.Stuttg., *S. bracteosum* Merr. & L.M.Perry, *S. brevifolium (A.Gray) Müll.Stuttg., S. bruynii* (Diels) Merr. & L.M.Perry, *S. bujangii* P.S.Ashton, *S. cacuminis* (Craib) Chantaran. & J.Parn.,*S. calcicola* (Merr.) Merr.,*S. calyptrocalyx* P.S.Ashton, *S. cameronum* I.M.Turner, *S. capillaceum (*Brongn. & Gris) J.W.Dawson, *S. capitatum* (Merr.) Merr. & L.M.Perry, *S. capituliferum* Merr. & L.M.Perry, *S. caryophyllatum* (L.) Alston, *S. caryophylloides* (Lauterb.) Merr. & L.M.Perry, *S. caudatum* (Merr.) Airy Shaw, *S. cephalophorum* (Ridl.) Merr. & L.M.Perry, *S. christmannii* Merr. & L.M.Perry, *S. cinctum* Merr. & L.M.Perry, *S. circumscissum* (Gagnep.) Craven & Biffin, *S. clementis* (C.B.Rob.) Merr.,*S. coccineum* J.W.Dawson, *S. cordatum* Hochst. ex Krauss, *S. cordifoliatum* (Ridl.) I.M.Turner, *S. cormiflorum* (F.Muell.) B.Hyland, *S. cornifolium* (Blume) Merr. & L.M.Perry, *S. corymbosum* (Blume) DC., *S. cumini* (L.) Skeels, *S. curtiflorum* (Elmer) Merr., *S. cylindricum* (Wight) Alston, *S. dansiei* B.Hyland, *S. dealbatum* (Burkill) A.C.Sm., *S. diffusiflorum* Merr., *S. durifolium* Merr. & L.M.Perry, *S. duthieanum* (King) Masam., *S. ebaloii* Merr., *S. elegans (*Brongn. & Gris) J.W.Dawson, *S. emirnense*

(continued)

Table 9.7 (continued)

Genus	Species
	(Baker) Labat & Schatz, *S. endophloium* B.Hyland, *S. eucalyptoides* (F.Muell.) B.Hyland, *S. eugenioides* (F.Muell.) Biffin & Craven, *S. eximiiflorum* (Diels) Merr. & L.M.Perry, *S. faciflorum* P.S.Ashton, *S. filiforme* Chantaran. & J.Parn., *S. ischeri* (Merr.) Merr.,*S. formosum* (Wall.) Masam., *S. frutescens* Brongn. & Gris, *S. fruticosum* DC., *S. gardneri* Thwaites, *S. gigantifolium* (Merr.) Merr., *S. glabratum* (DC.) Veldkamp, *S. glaucum* (King) Chantaran. & J.Parn.,*S. grande* (Wight) Walp., *S. grijsii* (Hance) Merr. & L.M.Perry, *S. guineense* (Willd.) DC., *S. halophilum* (Merr.) Masam., *S. handelii* Merr. & L.M.Perry, *S. hebephyllum* Melville, *S. heloanthum* Diels, *S. hoseanum* (King) Merr. & L.M.Perry, *S. hughcumingii* Merr., *S. ilocanum* (Merr.) Merr., *S. inasense* (King) I.M.Turner, *S. inophyllum* DC., *S. inopinatum* Amshoff, *S. isabelense* (Quisumb.) Merr.,*S. jaffrei* J.W.Dawson, *S. jasminifolium* (Ridl.) Chantaran. & J.Parn., *S. kanarense* (Talbot) Raizada, *S. kanneliyensis* Kosterm., *S. koordersianum* (King) I.M.Turner, *S. lacustre* (C.B.Rob.) Merr., *S. lakshnakarae* Chantaran. & J.Parn., *S. lamii* Merr. & L.M.Perry, *S. latifolium* (Poir.) DC., *S. leptoneurum* Diels, *S. leucanthum* L.M.Perry, *S. longifolium* (Brongn. & Gris) J.W.Dawson, *S.* longipedicellatum (Merr.) Merr., *S. luteum* (C.B.Rob.) Merr., *S. macgregorii* (C.B.Rob.) Merr., *S. macranthum* Brongn. & Gris, *S. macrocalyx* Merr. & L.M.Perry, *S. makul* Gaertn., *S. megacarpum* (Craib) Rathakr. & N.C.Nair, *S. melanophilum* H.T.Chang & R.H.Miao, *S. micrandrum* (Ridl.) Merr. & L.M.Perry, *S. mimicum* (Merr.) Merr.,*S. muelleri* (Miq.) Miq.,*S. mulgraveanum* (B.Hyland) Craven & Biffin, *S. myrtoides* (A.Gray) R.Schmid, *S. nanum* J.W.Dawson, *S. nitidum* Benth., *S. oblanceolatum* (C.B.Rob.) Merr., *S. occlusum* Miq., *S. odoratum* (Lour.) DC., *S. ovale* Korth., *S. palembanicum* Miq., *S. pallens* Merr. & L.M.Perry, *S. paniculatum* Gaertn., *S. parkeri* (Baker) Labat & Schatz, *S. patens* Korth., *S. pendulinum* J.W.Dawson, *S. perryae* I.M.Turner, *S. platypodum* Diels, *S. polyanthum* (Wight) Walp., *S. pondoense* Engl., *S. pseudojambolana* Miq., *S. pseudomolle* (M.R.Hend.) I.M.Turner, *S. pullei* Diels, *S. purpuriflorum* (Elmer) Merr., *S. quadratum* (King) I.M.Turner, *S. ramiflorum* Airy Shaw, *S. rehderianum* Merr. & L.M.Perry, *S. rigidifolium* Merr., *S. robustum* Miq., *S. rubens* (Roxb.) Walp., *S. rugosum* Korth., *S. salicifolium* (Wight) J.Graham, *S. samoense* (Burkill) Whistler, *S. setosum* (King) I.M.Turner, *S. squamatum* Merr. & L.M.Perry, *S. steenisii* Merr. & L.M.Perry, *S. subcapitulatum* Miq., *S. subnodosum* Miq., *S. sulitii* Merr., *S. taeniatum* Diels, *S. tahanense* (Ridl.) I.M.Turner, *S. tectum* (King) I.M.Turner, *S. tenuifolium* (Ridl.) Airy Shaw, *S. thomsenii* (Diels) Merr. & L.M.Perry, *S. tolypanthum* Diels, *S. trachyphloium* (C.T.White) B.Hyland, *S. tricolor* (Diels) Merr. & L.M.Perry, *S. triste* (Kurz) N.P.Balakr., *S. umbilicatum* (Koord. & Valeton) Amshoff, *S. vaccinifolium* Merr., *S. vaupelii* Whistler, *S. venosum* DC., *S. viburnoides* Diels, *S. virotii* J.W.Dawson, *S. waterhousei* Merr. & L.M.Perry, *S. xanthophyllum* (C.B.Rob.) Merr., *S. xylopiaceum* (Diels) Merr. & L.M.Perry, *S. zeylanicum* (L.) DC., *S. zollingerianum* (Miq.) Amshoff
Garcinia	*Garcinia acuminata* Planch. & Triana, *G. acutifolia* N. Robson, *G. afzelii* Engl., *G. amabilis* Kanehira & Hatusima, *G. andamanica* King, *G. angustifolia* A. C. Sm., *G. apetala* Pierre, *G. aphanophlebia* Baker, *G. aristata* (Griseb.) A. Borhidi, *G. balansae* Pierre, *G. balica* Miq., *G. basacensis* Pierre, *G. benthamii* Pierre, *G. blumei* Pierre, *G. brasiliensis* C. Martius, *G. caloneura* Boerl., *G. calophylla* Pierre, *G. caudiculata* Ridl., *G. cordata* Merrill, *G. cuspidata* King, *G. dioica* Bl., *G. echinocarpa* Thw., *G. elliptica* Choisy, *G. emarginata* Lauterb., *G. esculenta* Y.H. Li, *G. fabrilis* Miq., *G. fruticosa* Lauterb., *G. grandifolia* (Choisy) Pierre, *G. gummi-gutta* (L.) N. Robson, *G. hanburyi* Hook. f., *G. holttumii* Ridl., *G. indica* (Thouars) Choisy, *G. kingaensis* Engl., *G. korthalsii* Pierre, *G. leptophylla* Bittrich, *G. lucida* Vesque, *G. macrantha* A. C. Sm., *G. macrophylla* Mart., *G. mangostana* L., *G. microcarpa* Pierre, *G. morella* (Gaertn.) Desr., *G. neglecta* Vieill., *G. oligantha* Merr., *G. pachyantha* A. C. Sm., *G. pacifica* Merrill, *G. parvifolia* (Miq.) Miq., *G. prainiana* King, *G. smithii* A.J.G.H. Kostermans, *G. speciosa* Wall., *G.versteegii* Lauterb., *G. volkensii* Engl., *G. xylosperma* Pierre, *G. zeylanica* Roxb.

Source Species [*Catalogue of Life: 22nd December 2014, Integrated Taxonomic Information System (IT IS)*]; The Plant List http://www.theplantlist.org/browse/A/Myrtaceae/Syzygium/

Parthasarathy et al. (2013) reported that using GIS technique mapping of potential distribution of wild species of *Garcinia* of Western Ghats with the help of GIS techniques was done. Collection sites were plotted on map with the help of ArcGIS software. Based on the GIS prediction surveys, the authors found that *Garcinia cambogia* is distributed throughout the Western Ghats, whereas *G. indica* is predominantly seen in the northern parts of Western Ghats. This indicated that their distribution and population size is reduced to dangerous levels. Unless located and preserved, these species may quickly become endangered. There is considerable variation in yield and other characters studied.

9.2.7 Seed Spices

The major seed spices grown in India are coriander, cumin, fennel and fenugreek which are grown on a commercial scale. Cultivation of the remaining seed spices is limited to certain areas only. Three of the major seed spices, coriander (*Coriandrum sativum* L.), cumin (*Cuminum cyminum* L.), and fennel (*Foeniculum vulgare* Mill), belong to family Apiaceae, whereas fenugreek (*Trigonella foenum-graecum* L.) belongs to Fabaceae. Most of the seed spices cultivated in India are Mediterranean in origin. In none of the seed spices, wiled relatives, which could contribute by way of hybridization to cultivated forms, are known to exist in India. Most of the germplasms, therefore, exist in the form of traditional varieties. Most of such varieties have been subjected to natural selection for local adaptation and therefore, these are expected to pose valuable genes for resistance against biotic and abiotic stresses. Good collections are also maintained in China (Coriander-99, Fennel-35)2, Australia (coriander and fenugreek), Germany (Coriander and fennel), Netherlands (Coriander and Fennel), USA (Coriander, fenugreek, fennel, and cumin), as well as the countries of Mediterranean region namely Morocco, Egypt, Iran, as well as horn of Africa (Ethiopia). Most of the European and North American as well as Australian collections are the introductions from either India or Mediterranean countries. Cumin is a major crop in Syria, and hence the country is expected to have good genetic diversity in cumin. (Sharma 1994; Malhotra and Vijay 2003; Singhania et al. 2005a, b, c; Sastry 2009, Agarwal and Sharma 1990).

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9.2.7.1 Coriander

Coriander is native to southern Europe, Asia Minor, and Caucasus where it also grows wild. Now India is a major producer of coriander. The diversity of coriander

is rather limited in India. *C. sativum* var *indicum* belongs to India. The small fruited types are recognized as C. *sativum* L. var. *microcarpum* and the large fruited one are described as *C. sativum* L. var. *vulgare* (Diederichsen and Hammer 2003). The sub-species of *C. sativum* Subsp. *Sativum* are var. *sativum* and var. *africanum* Stolet. The Subsp. of *C. sativum* Subsp. *asiaticum* are var. asiaticum, var. *anatolicum* and var. *afghanicum.* The other sub-species are *C. sativum* Subsp. *vavilovii* var. *vavilovii* and *C. sativum* Subsp. *pygmaeum* Stolet.

Dried ripe coriander seeds contain both steam volatile oil and fixed oil. The aromatic odor and taste of coriander fruit is due to its volatile oil, which is a clear, colorless to light yellow liquid. The flavor of the oil is warm, spicy aromatic, sweet and fruity. The oil contents of seeds vary widely with geographical origin. Higher volatile oil content is found in Norwegian coriander (1.4–1.7 %) followed by Bulgarian coriander (0.1–0.5 %). Indian seeds are poor in volatile oil content (0.1–0.4 %) (Agrawal and Sharma 1990). Major components of essential oil are linalool (67.7 %), followed by α-pinene (10.5 %), γ- terpinene (9.0 %), geranyl acetate (4.0 %), camphor (3.0 %) and geraniol (1.9 %). Minor components in the oil are β-pinene, camphene, myrcene, limonene, *p*-cymol, dipentene, α- terpinene, *n*-decylaldehyde, borneol, and acetic acid esters.

There is good generic diversity in coriander with respect to morphological characters, quality attributes, and resistance to biotic and abiotic stresses. Over 2130 accessions are maintained at various centers in India (Karla et al. 2006; Sharma and Sharma 2012).

Thirty-five high-yielding coriander cultivars are released for cultivation in India (Table 9.8). These varieties exhibit diversity for fruit shape, size, and plant type. Many of them are resistance to biotic and abiotic stresses like wilt, powdery mildew, stem gall, grain mold, tolerance to drought, field tolerance to white fly, mites and aphids early maturity, dual-purpose types, resistant to lodging and shattering, etc.

9.2.7.2 Cumin

Cumin is native to Egypt and Syria, Turkistan and Eastern Mediterranean region. Cuminaldehyde is the major component in cumin oil. Oil content is low in indigenous germplasm but high in exotic collections (Sharma 1994). Cumin is an aromatic spice with stimulating properties. It has a characteristic strong flavor and is slightly bitter in taste. Seeds contain 2–5 % volatile oil of which 40–65 % is cuminaldehyde (cuminic aldehyde). Over 590 accessions are maintained at various centers in India (Patel et al. 2006; Amin 2012).

Fourteen high-yielding cumin cultivars are released for cultivation in India (Table 9.8). The cv. RZ-19 is moderately resistant to wilt, having attractive fruits. Gujarat cumin-4 is wilt resistant and is the most important variety in India. Diversities for high yield, fruit shape and size, high quality, tolerance to *Fusarium* wilt, *Alternaria* blight and powdery mildew and rich in essential oil content exist among these improved varieties.

Table 9.8 Species diversity in seed spices

Genus	Species
Coriandrum	*Coriandrum didymum* Stokes*, C. diversifolium* Gilib.*, C. globosum* Salisb.*, C. latifolium* Crantz, *C. majus* Garsault, *C. melphitense* Ten. & Guss.*, C. radians* Prantl, *C. sativum* L.*, C. seselifolium* DC. ex DC.*, C. setifolium* Koso-Pol.*, C. testiculatum* Lour.*, C. tordylium* (Fenzl) Bornm.
Cuminum	*Cuminum aegyptiacum* Mérat ex DC., *C. aethiopicum* Royle, *C. borszczowi Koso-Pol., C. brevisetum Koso-Pol., C. crinitum Koso-Pol., C. cuminodes Kuntze, C. cyminum* L.*, C. hispanicum Mérat ex DC., C. maroccanum P.H.Davis & Hedge, C. minimum Steud., C. odorum Salisb., C. officinale Garsault, C. ramosissimum Koso-Pol., C. regium Royle, C. sativum J.Sm., C. setifolium Koso-Pol., C. sudanense H. Wolff*
Foeniculum	*Foeniculum capense* DC., *F. divaricatum* Griseb.*, F. dulce* Mill.*, F. giganteum* Lojac.*, F. graecum* Calest.*, F. kraussianum* Meisn.*, F. luteum* Fisch. ex Sweet, *F. multiradiatum* K.Koch, *F. peucedanoides* Benth. & Hook.f., *F. piperitum* J.Presl, *F. rigidum Brot.* ex Steud., *F. salsum* Calest.*, F. scoparium* Quézel, *F. subinodorum* Maire Weiller & Wilczek, *F. tortuosum* Benth. & Hook.f., *F. virescens* Benth. & Hook.f., *F. vulgare* Mill.*, F. webbii* Benth. & Hook.f.
Trigonella	*Trigonella adscendens* (Nevski) Afan. & Gontsch.,*T. afghanica* Vassilcz., *T. anguina* Delile, *T. aphanoneura* Rech.f., *T. arabica* Delile, *T. aristata* (Vassilcz.) Sojak, *T. astroides* Fisch. & C.A.Mey., *T. badachschanica* Afan, *T. balachowskyi* Leredde, *T. balansae* Boiss. & Reut.,*T. berythea Boiss. & Blanche, T. bicolor* (Boiss. & Balansa) Lassen, *T. cachemiriana Cambess, T. caelesyriaca* Boiss.*, T. caerulea (L.) Ser., T. capitata Boiss., T. calliceras* Fisch.*, T. carica* Hub.-Mor.*, T. cariensis* Boiss.*, T. cassia* Boiss.*, T. cedretorum* Vassilcz.*, T. cephalotes* Boiss. & Balansa, *T. coerulescens* (M.Bieb.) Halacsy, *T. cretica* (L.) Boiss., *T. cylindracea* Desv*, T. dasycarpa* (Ser.)Vassilcz., *T. elliptica* Boiss.*, T. emodi* Benth.*, T. esculenta* Willd.*, T. edelbergii* (Sirj. & Rech.f.) Rech.f., *T. falcata* Balf.f., *T. filipes* Boiss.*, T. fimbriata* Benth.*, T. foenum-graecum* L.*, T. freitagii* Vassilcz.*, T. geminiflora* Bunge, *T. gharuensis* Rech.f., *T. glabra* Thunb.*, T. gladiata* M.Bieb., *T. gontscharovii* Vassilcz.*, T. gracilis* Benth.*, T. graeca* (Boiss. & Spruner)Boiss.*, T. grandiflora* Bunge*, T. griffithii* Boiss.*, T. halophila* Boiss.*, T. hamosa, T. heratensis* Rech.f., *T. hierosolymitana* Boiss.*, T. ionantha* Rech.f., *T. iskanderi* Vassilcz.*, T. isthmocarpa* Boiss. & Balansa, *T. kafirniganica* Vassilcz.*, T. koeiei* Sirj. & Rech.f., *T. korovinii* Vassilcz.*, T. kotschyi* Benth.*, T. laciniata* L., *T. latialata* (Bornm.)Vassilcz., *T. laxiflora* Aitch. & Baker, *T. laxissima* Vassilcz.*, T. lilacina* Boiss.*, T. linczevskii* Vassilcz.*, T. lipskyi* Sirj.*, T. lunata* Boiss.*, T. lycica* Hub.-Mor., *T. macroglochin* Durieu, *T. macrorrhyncha* Boiss.*, T. marco-poloi* Vassilcz., *T. maritima* Poir.*, T. media* Delile, *T. mesopotamica Hub.-Mor., T. spicata* Sm.*, T. spinosa* L., *T. obcordata* Benth.*, T. zaprjagaevii* Afan. & Gontsch

Source Species [*Catalogue of Life: 24th November 2014, Integrated Taxonomic Information System (IT IS)*]

9.2.7.3 Fennel

Fennel belongs to Apiaceae. It has two sub-species: *Foeniculum vulgare* sp. *Capillaceum* (garden fennel) and ssp. Piperitura (wild fennel). Sub-species *capillaceum* comprises var. *vulgare* (bitter fennel), var. *dulce* (sweet fennel or French sweet fennel or Roman fennel) and var. *panmoriwn* (Indian fennel). The oil

content ranges from 0.7 to 6 % in fennel germplasm. The oil of fennel contains mainly anethole, α-pinene, β-phellandrene, dipentene, etc. Over 629 accessions are maintained at various centers in India.

Twenty-one high-yielding fennel cultivars are released for cultivation in India (Table 9.8). These cultivars posses among themselves high yield, high quality, fruit shape and size, tolerance to leaf spot, leaf blight and sugary diseases, shattering of grains, suitability for drought, water logged and saline and alkaline conditions.

9.2.7.4 Fenugreek

Fenugreek belongs to the family Fabaceae, and is native of eastern Mediterranean. Rich diversity exists for fenugreek in Turkey. The seed is used as spice and leaf as vegetable. It has bitter taste of seeds due to alkaloid trigonelline and steroid sapogenin (diosgenin), but in appropriate quantities, it adds a special taste and flavor to culinary dishes. It also has high medicinal and nutritive value (Kakani and Anwer 2012). Over 1118 accessions are maintained at various centers in India.

Twenty-one high-yielding fenugreek cultivars are released for cultivation in India (Table 9.8). These cultivars in addition to high yield, high quality, grain size and color, dual-purpose types, with tolerance to downy mildew, powdery mildew, root rot, high diosgenin content, and medium duration types.

9.3 Genetic Erosion

Due to destruction in their natural habitats, climate change, over exploitation, preference to better yielding varieties many of the species, wild forms and primitive cultivars are slowly disappearing. Some of the important species which were classified by IUCN as rare, endangered and threatened (RET) are given in Table 9.9.

All the spices crops like any other plant follow either vegetative or sexual reproduction. While many crops show strict self or cross pollination, yet, there are no fixed borders. Because of the sampling errors, the genetic structure of the population is affected; hence, there is danger of loss of valuable alleles in the collections due to the sampling procedures. Similarly, even during the regeneration and multiplication of the samples in germplasm collections also genetic erosion sets in. Before we understand the genetic erosion in nature and in germplasm collection, it is essential that we understand the reproduction in plants and its relation to population structure.

The genetic erosion can only be monitored when we are aware of the genetic resources of the area. The selection pressure on crops for yield has resulted in the erosion of land races which may be the allelic source of adaptability to a particular region. India being the center of diversity of many spices, the genetic variability in major spices like black pepper and cardamom followed by ginger, turmeric, cinnamon and garcinia is quite reasonable. The natural variability has to be preserved

Table 9.9 Rare, endangered, and threatened (RET) species among spice crops

Genus	RET species
Piper	*Piper cordulatum* C. DC., *Piper fimbriulatum* C. DC., *Piper lucigaudens* C. DC., *Piper verrucosum* Sw., *Piper hylebates* C.DC., *P. hylophilum* C.DC., *P. lineatipilosum Yunck., P. napo-pastazanum* Trel. & Yunck., *P. nebuligaudens* Yunck., *P. schuppii* A.H. Gentry, *P. sodiroi* C. DC., *Piper subaduncum* Yunck., *Piper supernum* Trel & Yunck, *P. seychellarum* J.Gerlach, *Piper achupallasense* Yunck., *P. azuaiense* Yunck., *P. baezense* Trel., *P. begoniiforme* Yunck., *P. brachipilum* Yunck., *P. brachystylum* Trel., *P. campii* Yunck., *P. chimborazoense* Yunck., *P. coeloneurum* Diels., *P. cutucuense* Yunck., *P. densiciliatum* Yunck., *P. diffundum* Yunck., *P. disparipilum* C. DC., *P. dodsonii* Yunck., *P. eriocladum* Sodiro., *P. fallenii* A. H. Gentry., *P. huigranum* Trel. & Yunck., *P. longicaudatum* Trel. & Yunck., *P. mendezense* Yunck., *P. nanegalense* Trel. & Yunck., *P. perstrigosum* Yunck., *P. prietoi* Yunck., *P. productispicum* Yunck., *P. puyoense* Yunck., *P. regale* C. DC., *P. saloyanum* Trel. & Yunck., *P. skutchii* Trel. & Yunck., *P. valladolidense* Yunck., *P. zarumanum* Trel., *Piper angamarcanum* C.DC., *P. baezanum* C. DC., *P. bullatifolium* Sodiro., *P. clathratum* C.DC., *P. entradense* Trel & Yunck., *P. eustylum* Diels., *P. gualeanum* C.DC., *P. guayasanum* C. DC., *P. hydrolapathum* C. DC., *P. manabinum* C. DC., *P. mexiae* Trel. & Yunck., *P. molliusculum* Sodiro., *P. platylobum* Sodiro., *P. poscitum* Trel & Yunck., *P. stipulosum* Sodiro., *P. subnitidifolium* Yunck., *P. trachyphyllum* C. DC., *P. wibomii* Yunck.
Curcuma	*Curcuma alismatifolia* Gagnep., *Curcuma sparganiifolia* Gagnep, *Curcuma candida* (Wall.) Techaprasan & Škorničk., *Curcuma pseudomontana* J.Graham, *Curcuma rhabdota* Sirirugsa & M.F.Newman, *Curcuma caulina* J.Graham, *Curcuma coriacea* Mangaly & M.Sabu, *Curcuma vitellina* Škorničk. & H.Đ.Tran
Zingiber	*Zingiber fragile* S.Q.Tong., *Zingiber collinsii* Mood & Theilade, *Zingiber monophyllum* Gagnep.
Cinnamomum	*Cinnamomum japonicum* Siebold ex Nakai., *Cinnamomum capparu-coronde* Blume, *Cinnamomum litseifolium* Thw., *Cinnamomum macrostemon* Hayata, *Cinnamomum mathewsii* (Meisn.) Kostermans, *Cinnamomum mercadoi* Vid., *Cinnamomum osmophloeum* Kaneh., *Cinnamomum parviflorum* (Nees) Kosterm., *Cinnamomum perrottetii* Meissner, *Cinnamomum reticulatum* Hayata, *Cinnamomum riparium* Gamble, *Cinnamomum balansae* Lecomte, *Cinnamomum brevipedunculatum* Chang, *Cinnamomum chemungianum* Mohan & Henry, *Cinnamomum citriodorum* Thwaites, *Cinnamomum filipedicellatum* Kosterm, *Cinnamomum kanahirae* Hay., *Cinnamomum mairei* Leveille, *Cinnamomum kotoense* Kaneh. & Sas., *Cinnamomum rivulorum* Kosterm., *Cinnamomum walaiwarense* Kosterm
Syzygium	*Syzygium beddomei* (Duthie) Chithra, *Syzygium microphyllum* Gamble, *Syzygium parameswaranii* Mohanan & Henry, *Syzygium bourdillonii* (Gamble) Rathakr. & Nair, Syzygium chavaran (Bourd.) Gamble, *Syzygium caryophyllatum* (L.) Alston, *Syzygium fergusoni* Gamble, *Syzygium spathulatum* Thwaites, *Syzygium turbinatum* Alston, *Syzygium umbrosum* Thwaites, *Syzygium discophorum* (Koord. & Valet.) Amshoff, *Syzygium minus* A.C.Sm., *Syzygium myhendrae* (Beddome ex Brandis) Gamble, *Syzygium parvulum*Mildbr., *Syzygium pendulinum* J.W. Dawson, *Syzygium veillonii* J.W. Dawson, *Syzygium stocksii* (Duthie) Gamble

Table 9.9 (continued)

Genus	RET species
Myristica	*Myristica magnifica* Bedd., *Myristica teijsmannii* Miq., *Myristica yunnanensis* Y.H. Li
Garcinia	*Garcinia acutifolia* Robson, *Garcinia afzelii* Engl.,*Garcinia brevipedicellata* (Bak.G.) Hutch. & Dalz., *Garcinia clusiaefolia* Ridley, *Garcinia costata* Hemsley ex King, *Garcinia holttumii* Ridley, *Garcinia Montana* Ridley, *Garcinia decussate* Adams, *Garcinia epunctata* Stapf, *Garcinia kola* Heckel, *Garcinia quaesita* Pierre, *Garcinia rubro-echinata* Kosterm, *Garcinia travancorica* Bedd., *Garcinia semseii* B. Verdcourt, *Garcinia staudtii* Engl., *Garcinia wightii* T. Andr., *Garcinia bifasciculata* N. Robson, *Garcinia imberti* Bourd., *Garcinia kingie* Pierre ex Vesque, *Garcinia linii* C.E.Chang, *Garcinia paucinervis* Chun & How, *Garcinia thwaitesii* Pierre, *Garcinia zeylanica* Roxb.
Vanilla	*V. andamanica* Rolfe, *V. pilifera* Holtt, *V. aphylla* Blume; *Vanilla wightiana* Lindl. ex Hook. F, *Vanilla griffithii* Rchb.f.,*Vanilla calopogonRchb.f.*, *Vanilla somai*

in the place of primary origin as well as in secondary origin by conservation as to escape the risk of extinction of the genetic variability. The wild species presumably became extinct because of over collection. Owing to the strong commercial pressure of food and pharmaceutical industries of today, unregulated gatherings have led to severe genetic erosion of a range of herbs and spices. The status of genetic erosion will be likely speeded up during the process of development of economy. The forest fire causes erosion of wild species and it results in the spread of rhizomatous crops present in the forest fire-infected region, as the aerial shoots get affected by this natural calamity, underground parts escapes the disaster, and further regenerate vigorously as there will be no competition (Table 9.9).

9.4 Conservation Strategies

Many wild and related species of spices still occur in the wild and are severely affected by both natural and manmade ecological disturbances. Identifying and demarking the ecological niches as protected biosphere reserves will help in in situ conservation of these valuable genetic resources for posterity. Most of the cross-pollinated tropical spices are either vegetatively propagated or have recalcitrant and heterozygous seeds. Spices like black pepper, cardamom, ginger, turmeric, and vanilla are essentially vegetatively propagated. Although essentially seed propagated, many tree spices like nutmeg, clove, cinnamon, and garcinia have efficient vegetative propagation methods. Hence, ex situ conservation in clonal repositories or in field gene banks (Fig. 9.3) is essential if we are to conserve these valuable genetic resources especially the cultivated types.

In many crop species like seed spices, conventional seed storage can satisfy most of the conservation requirements. Seed spices, except fenugreek, are highly cross-pollinated and if sufficient population (Oka 1975) and isolation distance is not maintained, the purity of the variety will get eroded and there will be a genetic drift. In them, maintaining an individual collection in small quantities always

Fig. 9.3 Clonal repositories of **a** black pepper, **b** cardamom, **c** ginger, **d** turmeric, **e** vanilla, and **f** nutmeg germplasm

poses a problem and theoretically it will be very difficult to eliminate genetic erosion even on small scale. Another approach is to use the gene pool approach. In this, composites are synthesized so that all the genes belonging to constituent lines are conserved in at intermediate gene frequencies. This approach can be applied to annual cross-pollinated spice crops. So its important controlled pollination of minimum population is required to ensure generic purity in subsequent generations. Storing a population of seeds, depending upon the diversity and breeding behavior, in low-temperature seed storage will help in augmenting the seed storage. Because of the heterozygous and heterogeneous nature, the populations of seed spices are particularly vulnerable to changes in gene and genotype structure throughout breeding and selection. Hence, in order to maintain the proper genetic structure of a given collection, the following care should be taken (Breese 1989).

1. Avoid contamination by foreign pollen or seed through proper isolation and seed handling techniques (Fig. 9.4).

Fig. 9.4 Field gene bank of seed spices (*Inset* bagging to maintain purity of genotype)

2. Minimize the genetic drift by ensuring sufficient population size and reducing opportunities for natural selections.
3. Securing effective random mating through appropriate pollination techniques.

Ex situ conservation is ideal as it maintains the population structure and allows the evolutionary forces to modify the population for better adaptation. Farmer's fields also can be used for conservation Altieri and Merrick 1987; Brush 1991) of cultivars and varieties of seed spices, since we are dealing with cultivated species. Further, even now, farmers are still growing their traditional varieties.

However, crops with recalcitrant seeds and those having conservation needs cannot be satisfied by seed storage, which have to be stored in clonal repositories and in vitro *gene* banks. Most field gene banks are prone to high labor cost, vulnerable to hazards like natural disasters, pests, and pathogens attack (especially viruses and systemic pathogens), to which they are continuously exposed and require large areas of space. This supports in vitro and cryoconservation. In addition, other resources like continuous supply of standard stock cultures for experiments to examine physiological and biochemical processes, cell and callus lines developed for in vitro synthesis of valuable secondary products, flavors, and other important compounds will benefit strongly from in vitro cultures.

Many spices are plagued by destructive and epidemic diseases caused by viruses, bacteria, and fungi. This makes germplasm conservation in field gene bank risky. Thus in vitro (Fig. 9.5) and cryostorage system becomes important in the overall strategy of conserving genepool. Each technology should be chosen on the basis of utility, security, and complementarily to other components of the strategy. A balance needs to be struck between seed, field gene bank, in vitro and cryoconservation of propagules, tissues, pollen, cell lines, and DNA storage for overall objective of conserving gene pool (Adams 1997; Nirmal Babu et al. 2007, 2012).

Pollen storage can be considerable value supplementing the germplasm conservation strategy by facilitating hybridisation between plants with different times of flowering and to transport pollen across the globe for various crop improvement programs in addition to developing haploid or homozygous lines. Cryopreservation of pollen (Fig. 9.6) might represent an interesting alternative for the long-term conservation of problematic species (IPGRI 1996).

Consequent with the advancements in gene cloning and transfer has been the development of technology for the removal and analysis of DNA. DNAs from the nucleus, mitochondrion, and chloroplast are now routinely extracted and immobilized onto nitrocellulose sheets where the DNA can be probed with numerous cloned genes. These advances, coupled with the prospect of the loss of significant plant genetic resources throughout the world, have led to the establishment of DNA bank for the storage of genomic DNA. The advantage of storing DNA is that it is efficient and simple and overcomes many physical limitations and constraints that characterize other forms of storage (Adams et al. 1994).

Fig. 9.5 In vitro gene bank of vegetatively propagated spices (*Inset* Ginger cultures under slow growth regime)

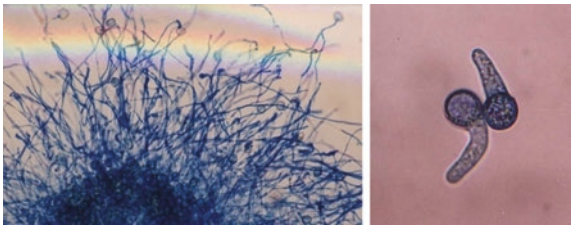

Fig. 9.6 Viability and germination of cryo-preserved pollen in vanilla

Table 9.10 Genetic resources of major spices conserved at various centers in India

Crop/Center	Indigenous accessions		Exotic accessions
	Cultivated	Wild and related sp	
Black pepper	1952	1300	13
Cardamom	900	13	–
Ginger	1200	15	40
Turmeric	2500	27	9
Clove	250	10	2
Nutmeg	475	34	–
Cinnamon	430	30	75
Coriander	1986	–	124
Cumin	577	–	13
Fennel	629	–	22
Fenugreek	1106	–	12
Garcinia	190	28	2

At present, the germplasm collection of spices available in India is the largest in the world comprising cultivars, wild relatives and genotypes having special characteristics (Table 9.10). These are maintained at various research centers. The germplasm conservation is through field gene banks, seed banks supplemented by in vitro, cryogene banks and DNA storage, where ever possible, depending upon the crop involved.

The existing germplasm available at various centers in India was effectively utilized in selection, hybridization, and mutation breeding programs and over 150 varieties of spices with high yield and resistance to biotic and abiotic stresses were released (Table 9.11).

9.5 Information System Support

The distribution of the wild species in the wild cannot be manually evaluated as it requires intensively more skilled personnel. GIS analysis of the germplasm data helps to better understand and develop new strategies for exploiting geographic diversity and to predict where species naturally occur or may be successfully introduced. Habitat loss and fragmentation are among the most common threats facing endangered species, making GIS-based evaluations an essential component of population viability analysis.

Table 9.11 Improved varieties of spices in India and their important characters

Sl. No.	Crop	Variety with salient features
1.	Black pepper	Panniyur 1(highest yield potential, Long spikes & bold berries, adopted to all regions except shade and high oleoresin); Panniyur 2 (Shade tolerant, rich in piperine); Panniyur 3. (Long spikes & bold berries;) Panniyur 4 (Stable yield); Panniyur 5 (Suitable for both mixed cropping); Panniyur 6 (Vigorous vine, rich in high piperine; Panniyur 7(wide adaptability); Panniyur 8 (tolerant to drought); Sreekara (wide adaptability and high volatile oil); Subhakara(wide adaptability and high volatile oil); Panchami (rich in oleoresin); Pournami (tolerant to root knot nematode); IISR Sakthi (Tolerant to *Phytophthora capsici);* IISR Thevam (field tolerant to *Phytophthora);* IISR Girimunda (suitable for high elevation); IISR Malabar Excel (suitable for high elevation); PLD –2 (rich in oleoresis); Arka Coorg Excel; Vijaya (tolerant to *Phytophthora*)
2.	Cardamom	Mudigere 1 (suitable for high density planting, moderately tolerant to thrips); Mudigere 2(early maturing, bold capsules); PV 1 (long, bold capsules); PV 2 (long panicle, long bold capsules, high dry recovery, field tolerant to stem borer and thrips); ICRI 1 (round extra bold dark green capsules); ICRI 2 (parrot green capsules); ICRI 3 (pubescent leaves, tolerant to rhizome rot, oblong, bold parrot green capsules); ICRI 4 (round bold capsules.); ICRI 5 (bold capsules, moderately tolerant to drought); ICRI 6(bold capsule); ICRI 7(Angular bold capsules, rich in oleoresin); IISR Suvasini (short plant type, suitable for high density planting); IISR Avinash (tolerant to rhizome rot); IISR Vijetha (tolerant to *katte*); Appangala 2 (First *katte* tolerant hybrid, high α-terpinyl acetate); S1 (PV 3) (Moderately tolerant to drought)
3.	Ginger	Suprabha (plumpy low fiber rhizomes); Suruchi (Early maturing, bold rhizomes); Suravi (Plumpy rhizomes); Himgiri (best for green ginger, less susceptible to rhizome rot); IISR Varada (Low fiber, high quality, tolerant to diseases); IISR Mahima (Plumpy extra bold rhizomes, Resistant to *M. incognita and M. javanica* pathotype 1); IISR Rejatha (High yielder, plumpy-bold rhizomes); Aswathy (suitable for green with high recovery of volatile oil and oleoresin, field tolerant to *Phyllosticta* leaf spot); Athira (High-yielding high-quality clone with high zingiberence); Karthika (High pungency clone with high gingerol, low infestation of shoot borer); Subhada (Suitable for hills and plains)
4.	Turmeric	Co.1 (Suitable for drought, water logged, saline and alkaline areas); BSR 1 (Suitable for drought prone areas); BSR 2 (High yielding, short duration, resistant to scale insects); Krishna (Plumpy rhizomes); Sugadham (Short internodes, Moderately tolerant to pest and diseases); Roma (Suitable for rainfed and irrigated condition); Suroma (field tolerance to leaf blotch, leaf spot and rhizome scales); Ranga (Moderately resistant to leaf blotch and scales); Rasmi (Bold rhizomes, suitable for early and late sown season); Rajedra Sonia (Bold and plumpy rhizome); Megha turmeric 1 (High curcumin content, bold rhizomes); Pant Peetabh (Resistant to rhizome rot); Suranjana (Tolerant to rhizome rot and leaf blotch, resistant to rhizome scales, suitable for open and shaded condition); Suvarna (Bright orange, slender fingers); Suguna (Early maturing, field tolerant to rhizome rot); Sudarsana (Early maturing, field tolerant to rhizome rot); IISR Prabha (High yielding); IISR Prathibha (High yielding); IISR

(continued)

Table 9.11 (continued)

Sl. No.	Crop	Variety with salient features
		Kedaram (Resistant to leaf blotch); IISR Alleppey Supreme (Tolerant to leaf blotch); Kanthi (Big mother rhizomes, medium bold fingers, closer internodes); Sobha (High yielding, high curcumin content—7.39 %, big mother rhizome and more territory rhizomes); Sona (Field tolerant to leaf blotch); Varna (closer internodes, field tolerant to leaf blotch); Narendra Haldi—1 (high-yield potential, high curcumin and essential oil); Duggirala Red (High yielding, long and plumpy rhizomes); Narendra Haldi-2 (High-yield potential); Narendra Haldi—3 (Root knot nematode resistant); Surangi (Suitable for hills and plains)
5.	Cinnamon	YCD 1 (Good bark recovery); PPI (C)—1 (Suitable for cultivation in high rainfall zones); Konkan Tej (Superieor quality); Sugandhini (Dense foliage, Suitable for leaf oil production); RRL (B) C-6 (High quality, sweet pungent bark); IISR Nithyashree (Good regeneration capacity, bark and leaf oleoresin content is high); IISR Navashree (Good aroma and taste, high shoot regeneration)
6.	Nutmeg	Konkan Sugandha (No incidence of major pests and diseases); Konkan Swad (Erect canopy, Warm, humid and shaded conditions are suitable); IISR Viswasree (Low incidence of fruit rot, suitable for mixed cropping); IISR Keralashree (High yield, high quality and extra bold fruit, mace and nut)
7.	Coriander	Gujarat coriander 2 (Suitable for early sowing, moderately tolerant to wilt and powdery mildew); Co. 1 (Dual-purpose variety, Small grains); Co. 2 (Dual-purpose variety, Suitable for saline, alkaline and drought prone areas); Co. 3 (Dual-purpose variety, Field tolerant to powdery mildew, wilt and grain mold); Co.4 (Early maturing, field tolerant to wilt and grain mold); Gujarat coriander 2 (Semi spreading, tolerant to powdery mildew, shattering resistant); Rajendra Swathi (Aromatic round grains, suitable for intercropping, field tolerant to aphids); Sadhana (Dual-purpose variety, field tolerance to white fly, mites and aphids, withstands moisture stress); Swathi (Field tolerant to white fly, escapes powdery mildew disease); CS 287 (Early maturing, field tolerant to wilt and grain mold); Sindhu (Tolerant to wilt, powdery mildew as well as drought condition); Hisar Anand (Dual purpose, Spreading type so resistant to lodging); Hisar Sugandh (Resistant to stem gall disease); Hisar Surabhi (Tolerant to frost, medium duration); Azad Dhania—1 (Tolerant to moisture stress, powdery mildew and aphids); Pant haritima (Dual-purpose type, Smaller seeds with high oil content, resistant to stem gall); DWA 3 (Dual-purpose variety, Moderately tolerant to powdery mildew); CIMPO S-33 (Grains small and bold, suitable for oil production); ACR-01-256 (NRCSS ACR-1) (Dual purpose, resistant to stem gall and wilt); RCr 20 (Early maturing, bold grains, moderately tolerant to stem gall); RCr 41 (Small seeded, resistant to stem gall and wilt); RCr 435 (Resistant to root knot nematode and powdery mildew); RCr 436 (Semi dwarf, bushy type, resistant to root rot and root knot nematode); RCr 446 (Moderately resistant to stem gall); RCr 684 (Bold seeds, resistant to stem gall); LCC-234 (High yielding leafy variety suitable for off season production in Andhra Pradesh); Hisar Bhoomit (Small seeded, high oil content, suitable for leaf production); UD-475 (RCr-475) (High-yield potential, suitable for grain purpose); Narendra Dhania 2 (Dual purpose)

(continued)

Table 9.11 (continued)

Sl. No.	Crop	Variety with salient features
8.	Cumin	Mc.43 (Semi spreading, withstand lodging and shattering); Gujarat cumin 1 (Bushy plants, withstand shattering and lodging, moderately tolerant to wilt, powdery mildew and blight); RZ-19 (tolerant to wilt and blight); Gujarat cumin 2 (tolerant to wilt and blight); Gujarat cumin 3 (Resistant to frost and wilt, seeds are pungent with high essential oil content); RZ-19 (Pink flowers, tolerant to wilt and blight); RZ-209 (Resistant to blight and wilt); RZ-223 (Wide adaptability, resistant to wilt); Ac-01-167 (Bold seeds resistant to wilt)
9.	Fennel	PF-35 (Moderately tolerant to leaf spot, leaf blight and sugary disease); Co-1 (Suitable for intercropping, Suitable for drought prone, water logged, saline and alkaline conditions); Gujarat Fennel – 1 (Tolerant to drought moderately tolerant to sugary disease); Gujarat Fennel 2 (Rich in volatile oil); S-7-9 (Moderately tolerant to blight); RF 125 (Tolerant to sugary disease); Hisar Sawrup (Spreading, resistant to lodging and shattering of grains); Azad Saunf 1 (Resistant to root rot and blight, early maturing so escapes attack of aphids); Pant Madhurika (Sweet in taste); RF 143 (Medium tall); HF 33 (High yielding); JF-444-1 (Compact seeds in umbellate, synchronous maturity)
10.	Fenugreek	Co.1 (Dual-purpose variety, tolerant root rot); Co. 2 (Short duration, dual purpose, field tolerant to *Rhizoctonia* root rot); Rajendra kanti (Bushy plant, suitable for intercropping, field tolerant to *cercospora* leaf spot, powdery mildew and aphids); RMt.1 (yellow colored grains, moderately resistant to root knot nematode, powdery mildew and aphids); Lam sel.1 (Dual purpose); Hisar Sonali (Dual purpose, moderately resistant to root rot and aphids); Hisar Suvarna (Dual purpose, moderately resistant to *Cercospora* and powdery mildew); Hisar Madhavi (Dual purpose, resistant to powdery mildew and Downey mildew); Hisar Muktha (Wide adaptability); RMt 303 (Yellow color seeds); RMt 305 (First determinant type, multipodant, resistant to powdery mildew and root knot nematodes); Gujarat Methi 1 (Dwarf plants); RMt 143 (Moderately resistant to powdery mildew, seeds bold yellow color, suitable for heavier soils); Pant Ragini (Dual purpose, resistant to downy mildew and root rot); NRCS-AM -1 AM-01-35 (Dual purpose, tolerant to powdery mildew); LFC-103 (Suitable for both rainfed and irrigated conditions)

(*Source* Johny et al. 2006)

9.6 Conclusion and Prospects

The genetic resources which are the reservoirs of identified and unidentified different genes are always the source for study for the breeders of all generations. The primary and secondary centers of origin are the source for different germplasms due to the natural hybridization and flow of genes throughout their existence. Detailed study on germplasm gives us the source material for resistance to biotic and abiotic stresses which can be further used in the improvement aspect.

The factors contributing to erosion due to the enormous diversity in cultivated plants, population growth, deforestation, erosion, changing land use and climate factors are major threats to the existing biodiversity of the region.

Natural productivity of any given species is always less, as the survival and continuation of a species is more important in nature than productivity. However, under domestication, the crops have shown the reverse. Due to the efforts of the human being, the productivities of all the crops have constantly raised, and in turn the survival mechanisms of the crops have been put to stake. Thus, the natural balance of maintenance of different forms has been disturbed.

The wild species presumably became extinct because of over collection. Owing to the strong commercial pressure of food and pharmaceutical industries of today, unregulated gatherings have led to severe genetic erosion of a range of herbs and spices. The status of genetic erosion will be likely speeded up during the process of development of economy. The forest fire causes erosion of wild species, and it results in the spread of rhizomatous crops present in the forest fire-infected region, as the aerial shoots get affected by this natural calamity, underground parts escapes the disaster, and further regenerate vigorously as there will be no competition. Preserving the biodiversity hot spots as natural sanctuaries will certainly help in slow in the gene erosion.

Large-scale cultivation is one practice that can take the pressure off wild stocks. This can be possible only by identifying the commercial importance of the wild species and exploring the rare information in the wild species which helps in domestication of the plant genes by the farmers which are possible. Thus it becomes a valid concern to evaluate and utilize the materials.

In many spices, conventional seed storage can satisfy most of the conservation requirements. However, crops with recalcitrant seeds and those having conservation needs cannot be satisfied by seed storage, which have to be stored in vitro. Most field gene banks are prone to high labor cost, vulnerable to hazards like natural disasters, pests and pathogens attack (especially viruses and systemic pathogens), to which they are continuously exposed and require large areas of space.

Most of the spice crops are either vegetatively propagated or have recalcitrant seeds. The spices germplasm is mostly conserved in field gene banks. Most of the spices are plagued by destructive and epidemic diseases caused by viruses, bacteria, and fungi. This makes germplasm conservation in field gene bank risky. Thus in vitro and cryostorage system becomes important in the overall strategy of conserving gene pool. Each technology should be chosen on the basis of utility, security, and complementarily to other components of the strategy. A balance needs to be struck between seed, field gene bank, in vitro and cryoconservation of propagules, tissues, pollen, cell lines, and DNA storage for overall objective of conserving gene pool. The genetic resources of black pepper, cardamom, ginger, turmeric, and vanilla are best conserved in field clonal repositories supplemented in vitro gene banks of active germplasm, while field gene banks are sufficient for perennial tree spices. However, for seed spices, field gene banks field with controlled pollination to maintain the pollution structure is essential with annual resurrection. This should be supplemented by long-term storage of base germplasm in low-temperature seed banks, which is ideal. For all these crops, DNA and pollen storage will supply the conservation methods mentioned above. Certainly, this does not mean to say that in situ conservation through protection of their natural habitats is less important. In fact, all the

native genes for crop improvement are in the wild populations and hence have to be protected under biosphere reserves for posterity.

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Chapter 10
Genetic Diversity and Erosion—A Global Perspective

Imke Thormann and Johannes M.M. Engels

Abstract Biodiversity is continually declining, according to global biodiversity indicators (Butchart et al. in Science 328:1164–1168, 2010). Population trends, habitat extent, habitat condition, and composition of species communities—indicators of the state of diversity—are declining, while at the same time pressures on biodiversity posed by resource consumption, invasive alien species, pollution, overexploitation, and climate change are increasing. The rate of current loss of species is reported to be 100–1000 times the natural background rate (Chivian and Berstein in Sustaining life on earth. How human health depends on biodiversity. Oxford University Press, New York, 2008, Chivian and Berstein in How our health depends on biodiversity. Center for Health and the global environment. Harvard medical school, Boston, 2010; Pimm et al. in Science 344, 2014). Dramatic though that figure is, it underestimates the full loss of diversity because it ignores loss at both genetic and population level (Myers in Seeds and sovereignty. The use and control of plant genetic resources. Duke University Press, Durham, 1988; Mendenhall et al. in Biol Conserv 151:32–34, 2012). One of the first publications alerting the world about the losses of genetic diversity within species, later termed "genetic erosion," was published in 1914 (Baur in Die Bedeutung der primitiven Kulturrassen und der wilden Verwandten unserer Kulturpflanzen fuer die Pflanzenzuechtung; Jahrbuch Deutsche Landwirt, 1914). The first concern about loss of diversity regarded agriculturally important species, as these are of direct and daily use to people. One hundred years later, genetic erosion is addressed at the global level in international agendas that set targets and propose actions to reduce the loss of genetic diversity, such as the Global Plan of Action (GPA) for Plant Genetic Resources for Food and Agriculture (PGRFA) of the FAO Commission on Genetic Resources for Food and Agriculture (CGRFA) and the Aichi biodiversity targets of the Convention on Biodiversity (CBD). The fact that genetic erosion today is addressed at global level implies that the crucial importance of genetic diversity for sustaining life on earth has been recognized. Strategies and

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actions to reduce the ongoing loss of genetic diversity are now in place. However, these measures have been found only partially successful as only few significant reductions in rates of decline were observed (Butchart et al. in Science 328:1164–1168, 2010), and global estimates of the extent of genetic erosion are still lacking. This chapter focuses on the importance of genetic diversity in PGRFA, how diversity of PGRFA is affected by genetic erosion, development of activities undertaken by international bodies to address genetic erosion, options to improve knowledge about the underlying processes that lead to genetic erosion, and the need for systematic monitoring of genetic diversity to better safeguard, conserve, and use PGRFA.

Keywords Genetic erosion · Genetic diversity · PGRFA · Germplasm collections · Monitoring

10.1 The Importance of Genetic Diversity in PGRFA

10.1.1 Genetic Diversity

Genetic diversity is the extent of genetic variation in a population or species, typically described by polymorphism, average heterozygosity, and allelic diversity. The genetic diversity in populations is generated by mutations or introduced by migration, and allele frequencies change due to selection, migration, and genetic drift. These forces are affected by the interaction of the plant with its biotic and physical environment and with human interventions, and by the reproductive biology of the species. Genetic diversity represents the essential raw material for species to evolve and to adapt. The amount of genetic variation determines the ability to respond to changing selection pressures (Frankham et al. 2010). A reduction in genetic diversity negatively affects the short-term viability of individuals and populations and the evolutionary potential of populations and species, and can contribute to accelerate the so-called extinction vortex, which describes the adverse interactions between human impacts, inbreeding, and demographic instability leading into a downward spiral toward extinction (Frankham et al. 2010).

Farmers and plant breeders look for genetic variation in specific traits to use in crop adaptation and improvement. The resources that provide them with the largest amount of novel genetic variation are landraces and Crop Wild Relatives (CWR) (Esquinas-Alcazar 1993; Ceccarelli and Grando 2000; Hajjar and Hodgkin 2007; Maxted et al. 2012; McCouch et al. 2013; Bertoldo et al. 2014).

10.1.2 Genetic Diversity in CWR

A CWR is defined as a wild plant taxon that has an indirect use derived from its relatively close genetic relationship to a crop (Maxted et al. 2006). Hajjar and

Hodgkin (2007) reviewed the use of CWR in crop improvement and found a steady increase in the rate of release of cultivars containing genes from CWR. They also found that the range of characteristics used has widened from a strong focus on pest and disease resistance genes to drought and salt tolerance, improved quality, and cytoplasmic male sterility. This trend is confirmed by Maxted and Kell (2009), who provide an extensive list of examples of uses of CWR in crop improvement programs for 14 major food crop gene pools. Farmers often tolerate the presence of CWR in their fields and in home gardens because they recognize the value of these species in providing beneficial traits to their crops (Engels 2001; Hughes et al. 2007; Galluzzi et al. 2010). For example, it was observed that subsistence farmers in Mexico grew cultivated corn near its wild relatives to facilitate introgression between the CWR and the crop as a means of crop improvement (Hoyt 1988). The total number of CWR existing in the world is estimated to be between 50,000 and 60,000 species, of which 700 are considered of the highest conservation priority (Maxted and Kell 2009). National, regional, and global inventories are being developed (for review see Dulloo et al. 2015), but data on the extent, distribution, and trends of genetic diversity harbored in these species are mostly unavailable.

10.1.3 Genetic Diversity in Landraces

Landraces are defined as a crop variety, often harboring genetic variability yet with a certain genetic integrity that has evolved through cultivation usually in a traditional agricultural system over long periods, and which is adapted to a specific local environment or purpose (Jarvis et al. 2016). Landraces are used in conventional and participatory plant breeding programs to contribute to a variety of traits from biotic and abiotic tolerance and resistance to, more recently, traits for improved nutrition (Newton et al. 2010; Bertoldo et al. 2014). For example, a local wheat variety collected in Turkey in 1948—initially of little interest, as it was tall with a thin stem and susceptible to leaf rust and cold weather—was later found to be resistant to several other pathogens including stripe rust. When stripe rust became a problem in Northwestern USA, this little known variety was included in major wheat breeding programs (Fowler and Mooney 1990). The genetic diversity in landraces used in traditional agricultural production systems constitute an important element for the livelihood strategies of farmers and confers resilience to agricultural production systems. Sorghum growers in West Africa use a diversity of traditional varieties with different flowering dates to minimize risks of crop failure due to climatic variability (Weltzien et al. 2006). Intra- and interspecific diversity in traditional varieties can enable the farmers' crop populations to better adapt and evolve to changing environmental and economic selection pressures, contribute to regulating and controlling pest and diseases, and sustain pollinator diversity (Brush and Meng 1998; Heal et al. 2004; Cavatassi et al. 2006; Hajjar et al. 2008; Jarvis et al. 2008a). No global estimate on numbers of landraces exists, and very few comprehensive national inventories have been established so far.

10.1.4 Loss of Genetic Diversity A Global Challenge

The increased environmental variability that is expected to result from climate change, one of the greatest development challenges, implies that in the future, farmers and plant breeders will need to access an even wider range of landrace and CWR genetic diversity than today (FAO 2010). At the same time, the genetic diversity in those species is itself threatened by global and climate change as well as other factors and continues to be lost.

Estimates for natural ecosystems indicate that the natural background rate of species extinction is one species per year for every one million species. The current rate of species loss varies, depending on the group of species, between 100 and 1000 times this natural background rate (Mendenhall et al. 2012). Global estimates of diversity loss in agricultural production systems do not exist. An estimate of the full loss of diversity would need to include also losses at genetic and population level.

While the importance of genetic diversity and the need to halt its loss is globally recognized and has become part of international agendas (see 19.2), actions to reduce the rate of loss are insufficient (Butchart et al. 2010) and need to be enhanced. Conservation, use, and monitoring need to be improved and extended to maintain the viability and evolutionary potential of landraces and CWR populations so that variation in genes and alleles can continue to be generated and provide biotic and abiotic resistance and yield enhancement traits, as well as microhabitat adaption.

10.2 History of Genetic Erosion Awareness: From the First References to Global Agendas

At the beginning of the last century, when scientific principles like Mendel's laws and new techniques started to influence agricultural development, traditional crop varieties started to be lost from farmers' fields, first observed by Baur (1914) in his article about the importance of primitive landraces and wild relatives for plant breeding, followed in the 1920s and 1930s by the work of Nikolai Vavilov and Jack Harlan (Scarascia-Mugnozza and Perrino 2002). Harlan and Martini (1936), upon return from collecting barley genetic resources in the Middle East warned of an irreplaceable loss of variation in barley grown by farmers in Ethiopia and Tibet, if it were to be replaced by modern varieties. Whitney et al. (1939) estimated that more than half of traditional taro cultivars in Hawaii were no longer in existence, and were able to record only eight of 25 cultivar groups previously recognized by Hawaiians. These observations were later described as "genetic erosion" in a report written for the Technical Conference of the FAO International Biological Programme in 1967 (Bennett 1968), when the spread of improved and more productive, genetically much less heterogeneous varieties, had gained momentum

to the point to be called "green revolution" (Wilkes and Wilkes 1972). The FAO conference had already made a call in 1959 for immediate actions in improving conservation of primitive landraces and their wild relatives. The European Society for Research and Plant Breeding (EUCARPIA), established in 1956 in the Netherlands, had highlighted the loss of genetic resources in its third general congress in 1962.

In 1970, after outbreaks of diseases that destroyed major crops such as maize in the US (NAS 1972) and coffee in Brazil (Kushalappa and Eskes 1989), the consequences of genetic vulnerability and erosion started to receive public attention. Although plant exploration activities had increased during the 1960s, they were considered not to compensate the rate of loss of diversity which was reported to rapidly increase, in particular, in many centers of diversity (Frankel and Hawkes 1975; Harlan 1975; Frankel and Bennett 1970). The first event where the loss of genetic resources attracted worldwide attention was the United Nations Conference on the Human Environment in Stockholm, 1972. Strong recommendations were made to preserve the irreplaceable genetic resources for the good of present and future generations. Subsequent actions to stem the loss of landraces and CWR and the progress of genetic erosion were to increase collecting efforts and create a global network of genetic resource centers, as recommended by a group of experts that met 1972 in Beltsville (USA), supported by the Consultative Group on International Agricultural Research (CGIAR). These developments led in 1974 to the establishment of the International Board for Plant Genetic Resources (IBPGR, now Bioversity International[1]) with the mandate to coordinate global collecting and conservation efforts.

The IBPGR coordinated a worldwide effort to collect and conserve landraces and CWR as a response to the alarming reports on genetic erosion due to variety and crop replacement, the green revolution, land use change, and modernization of agriculture (CGIAR 1972; FAO 1998). During the first two decades (1975–1995), over 200,000 samples of landraces and CWR were collected in over 1000 collecting trips in more than 130 countries. The collecting missions were conducted in areas where landrace and CWR diversity was under risk of erosion or loss, and targeted species of major importance to food security were collected. The material collected was subsequently conserved in genebanks (Thormann et al. 2015).

Ex situ conservation was the method of choice to conserve the rescued genetic diversity and maintain it for future uses. The number of genebanks grew rapidly from an initial handful of long-term storage facilities in 1975 to nearly 400 with long or medium term storage in 1996 (FAO 1998) and to about 1750 in 2006 (FAO 2010). About 10 out of 15 CGIAR centers with PGR conservation responsibilities assumed a central role in ex situ conservation of major food crops and their wild relatives (Marshall 1989).

[1]The IBPGR became the International Plant Genetic Resources Institute (IPGRI) in 1991 and in 1994 IPGRI started to operate as an independent CGIAR center, since 2006 it operates under the name Bioversity International.

The FAO conference established in 1983 the CGRFA as the first permanent intergovernmental forum in the United Nations system to address global PGRFA matters. In the same year, the International Undertaking on Plant Genetic Resources was adopted, which developed almost two decades later into the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA), adopted in 2001 and in force since 2004. The first Global Plan of Action (GPA) for the Conservation and Sustainable Use of PGRFA was agreed in 1996 and its updated second version was endorsed in 2011. The global agenda that regards biodiversity more widely without a focus only on PGRFA is provided by the Convention of Biological Diversity (CBD), in force since 1993, its Global Strategy for Plant Conservation (GSPC), which was adopted in 2002 by the world's governments as a program under the CBD (Sharrock 2012), and its corresponding Strategic Plan for Biodiversity for the period 2011–2020 with its 20 so-called Aichi Targets. While most of the targets focus on conservation of biodiversity in the widest sense, Target 13 reads as follows: "*By 2020, the genetic diversity of cultivated plants and farmed and domesticated animals and of wild relatives, including other socioeconomically as well as culturally valuable species, is maintained, and strategies have been developed and implemented for minimizing genetic erosion and safeguarding their genetic diversity*."

These global agendas recognize the crucial importance of diversity and include targets and measures to minimize genetic erosion and safeguard genetic diversity.

10.3 Definitions of Genetic Erosion and Vulnerability

The term "genetic erosion" describes the process of displacement of old, indigenous landraces and varieties by new, high-yielding varieties (HYV), which is equated to loss of genes (Qualset et al. 1997). First observations regarded the loss of local varieties from farmers' fields in terms of their numbers, not their intraspecific genetic variation or specific alleles or gene complexes. Harlan (1970) spoke about varietal diversity that humankind could not afford to lose, and the FAO Technical Conference of 1967 (Bennett 1968) refers to the displacement by HYV. This use in the broad sense of loss of varieties or of species has later been changed to explicitly include also reference to the loss of alleles. Qualset et al. (1997), considering the main unit of conservation of the gene and its allelic forms, define the broad sense meaning of the term also as gene displacement or genomic erosion, when the whole genome is lost by the substitution of one crop species by another one or by the elimination of the crop entirely. For genetic erosion in the narrow sense, they propose the term gene replacement or genic or allelic erosion when indigenous varieties are replaced by introduced ones, resulting in the substitution of alternative alleles within the same species.

The first and second State of the World Reports on PGRFA (SOW1, SOW2) (FAO 1998, 2010) define genetic erosion as "the loss of individual genes and the loss of particular combinations of genes (i.e. of gene complexes) such as those

maintained in locally adapted landraces" and explicitly state the two uses of the term, i.e., narrow as the loss of genes or alleles, as well as more broadly, referring to the loss of varieties. Genetic erosion, therefore, does not necessarily entail the extinction of a variety of subpopulation, but it does signify a loss of variation and thus a loss of the ability to evolve. The FAO technical meeting on the "Methodology of the World Information and Early Warning System on Plant Genetic Resources (WIEWS)" held in Prague in 1999 agreed on a working definition of genetic erosion taking on board the concepts of richness and evenness[2] and extending the concept to CWR: "A permanent reduction in the number, evenness and distinctness of alleles, or combinations of alleles, of actual or potential agricultural importance in a defined geographical area" (Serwinski and Faberova 1999). Maxted and Guarino (2006) further modified the definition into "permanent reduction in richness (or evenness) of common localized alleles or the loss of combination of alleles over time in a defined area." They include the aspects of local adaptation and dynamics of diversity in time. Generally, the definitive reduction in diversity needs to be distinguished from the normal addition and disappearance of genetic variability over time in a population (Brush 1999, 2004).

While the initial broad term use of the concept of genetic erosion was extended to include also the narrow sense of the concept, it remained related to agricultural diversity and in particular to cultivated diversity (Brush 2004). With the development of the CBD, the GSPC and the Aichi targets, the concept of genetic erosion has been extended to biodiversity more widely (Rogers 2004). Whether we consider genetic erosion in agricultural diversity or in natural populations, it is usually used in the context of losses in genetic diversity caused by human-driven or related activities, as these losses are faster in rate or extension than one would expect under natural conditions alone.

Often, and particularly in SOW1 and SOW2, genetic erosion is mentioned together with genetic vulnerability. Genetic vulnerability is defined in SOW1 as the condition that results when a widely planted crop is uniformly susceptible to a pest, pathogen, or environmental hazard as a result of its genetic constitution, thereby creating potential for widespread crop losses. The concept of genetic vulnerability is therefore associated with a component of space—distribution of diversity across a defined space, rather than a component of time-like genetic erosion. Given that genetic uniformity generates vulnerability, it is inversely related to the locally present genetic diversity, in particular to the extent that this local diversity possesses the capacity to adapt to new pathogens or changing environmental conditions (Brown 2008).

The largest global example of the impact of genetic vulnerability that has occurred since the SOW1 was published is the outbreak and continued spread of the Ug99 race of stem rust, to which the large majority of existing wheat varieties are susceptible (FAO 2010). Another example is the above referred disease

[2]richness and evenness are two important concepts of diversity, the first being the number of different kinds of entities and the second the relative frequency of these entities (Brown 2008).

outbreak in the US in 1970 that destroyed about 15 % of the US maize yields (up to 50 % in some southern states) (NAS 1972). This epidemic disease of southern leaf blight was caused by the fungus *Helminthosporium maydis,* which had probably always been present in maize fields but had not caused any major problems because of the variability existing in the maize crop. By 1970, nearly all farmers grew a single high-yielding hybrid. A mutant form of the fungus developed that proved ideal for this hybrid maize and favorable weather conditions supported the epidemic outbreak (NAS 1972).

Over time and with the increase in studies that attempt to quantify genetic erosion in PGRFA, the complexity of the genetic erosion concept has become evident. Although it is an accepted model, careful scrutiny reveals that neither the model nor the concept has been very clearly articulated nor extensively tested (Brush 2004). However articulated, several aspects render the concept difficult to measure. Missing data, unavailability of historical seed material, or little knowledge about the diversity existing before genetic erosion became a global issue have constrained the establishment of baselines required for assessment of changes in diversity over time. The often ambiguous naming of landraces by farmers and the role of seed systems in determining local diversity add significant complexity to the assessment of changes in landrace diversity.

10.4 Causes and Instances of Genetic Erosion In Situ and Ex Situ

Genetic erosion can occur in farmers' fields and in natural environments, but it also occurs ex situ in genebank collections and botanic gardens. An overview of instances of reported genetic erosion for each type of environment is provided and the causes to which genetic erosion has been attributed are described.

10.4.1 Causes and Instances of Genetic Erosion On-Farm and in Natural Environments

The replacement of locally distributed and traditionally diverse landraces by modern varieties has long been considered to be the main threat to diversity and main reason of genetic erosion (Baur 1914; Harlan and Martini 1936; Bennett 1968; Frankel and Bennett 1970; Wilkes and Wilkes 1972; Fernando and Thomas 1978; Hawkes 1983; Hutchinson and Weiss 1999). This was based on the hypothesis that competition between local and introduced varieties would necessarily result in a replacement of local varieties (Frankel 1970). A body of documented and quantified cases from a range of diverse environments and farming systems was lacking but some impressive reports on replacement of local diversity in certain areas were

available, such as the reduction of wheat varieties cultivated in China from 10,000 to 1000 between 1949 and 1970 (Thrupp 1998); the replacement of local maize landrace populations by hybrid maize between 1925 and 1950 in the USA corn belt or the planting of 83 % of the winter wheat area in Lebanon to modern varieties by 1997 (reported in Brush 2004). While also recent studies report replacement of local varieties by HYV, e.g., rice in China (Gao 2003), other recent studies show that: (i) many other causes led to the loss of diversity and (ii) improved varieties, even if available to farmers, were not necessarily adopted (Chambers et al. 2007; Hellin et al. 2014), or their adoption did not necessarily lead to either abandonment of local varieties (Brush 1995; Bellon 1996; Perales et al. 2003) or reduction of locally available diversity (Lohar and Rana 1998; Steele et al. 2009).

The GPA (FAO 2012), SOW1,and SOW2 (FAO 1998, 2010), addressing specifically PGRFA, summarize as the main causes of genetic erosion the replacement of landraces, overexploitation and overgrazing, environmental degradation, reduced water availability, population pressures and urbanization, changing dietary habits, changing agricultural systems, legislation and policy, civil strife and war, and pests, diseases, and weeds. Diversity losses due to pest and disease outbreaks or to absence of tolerance to abiotic stresses, such as heat, drought or frost, reported by many countries in their contributions to the SOW2, are expected to grow with the increasing number of extreme weather events and global climate change. The term genetic erosion is mainly used here in its broad sense referring to complete loss of landraces or a major reduction in the area cultivated to landraces.

The following studies illustrate the diverse range of causes to which erosion in different crops is attributed:

- Ortega-Paczka (1999) reported a heterogeneous situation in various areas in Mexico. In some areas, native diversity was threatened by genetic erosion because of state efforts to modernize agriculture (adoption of improved seeds, substitution of maize by more profitable crops) and of emigration of peasants to work in other regions or abroad, and of peasants abandoning agriculture. In other regions, farmers actively maintained their local diversity.
- In Ethiopia, changes and development in agriculture, land use change and drought are considered at the same level as displacement of landraces in causing loss of landrace diversity. Natural disasters such as drought lead to famine and force farmers to eat or sell their own seeds in order to survive. If support is provided by relief agencies, native seed stocks are often replaced by exotic seeds (Worede 1997).
- Loss of diversity within the swidden cultivation system in the Brazilian Atlantic Forest was mainly driven by restrictive environmental laws, abandonment of rural areas, increasing tourism, and loss of local agricultural skills (Peroni and Hanazaki 2002).
- Introduction of exotic crops rather than improved varieties of the same landrace crop led to abandonment of native local diversity in Peru (Ortega 1997). Niches earlier cultivated by native Andean crops were abandoned in favor of barley introduced from Europe and to a lesser extent, broad beans, peas, and other

crops. Other factors for loss of native local diversity were pressure from market forces which reduced the importance of local crops, cheap food imports, land use change (from cropping to pastures), and destruction of forests.

- Ochoa (1975) reported major losses of numbers of indigenous potato varieties in Chile, Peru, and Bolivia mainly due to pests and diseases and introduction of improved varieties.
- Several studies found that the loss of landraces was due to the abandonment of subsistence agricultural practices (Guarino et al. 1991; Negri 2003; Rocha et al. 2008), caused by changes in human population structure such as aging, a lack of skills in the younger generation to practice traditional techniques, e.g., grafting, or abandonment of rural areas.
- Traditionally cultivated vegetable diversity decreased in villages on the Varanim plains in Iran and the existing vegetable diversity is threatened by genetic erosion due to water shortage, an imbalance between selling prices and production costs, and land degradation. Changes in land use practices, interest in cash crops, inappropriate cultivation patterns, and decreased soil quality had the highest contributions to genetic erosion (Davari et al. 2013).
- Erosion in a number of fruit tree species was observed on the Italian island, Sardinia. The main causes affecting the various species to different extents were market failure, new pests and diseases, environmental degradation and disturbance, urbanization, introduction of new varieties, and competition with domestic livestock and wild fauna (Chessa and Nieddu 2005).
- Evolving agricultural systems and land use changes have led to the loss of several local lentil populations in Italy and are exposing those still in cultivation to risk of severe genetic erosion (Piergiovanni 2000).

Instances of genetic erosion are sometimes reported without providing any concrete measure of their extent, like the loss of several local rice varieties in Northern Mali (Synnevag et al. 1999), or the loss of most wheat germplasm that was widely cultivated at the beginning of the twentieth century in Italy (Figliuolo et al. 2007). In particular, einkorn is reported to be extinct and emmer subject to strong genetic erosion (Laghetti et al. 2009) because cultivated only by few farmers. Also, many country reports submitted to FAO for the SOW2 refer to general losses of landraces or landraces becoming rare, only few reports provide some quantitative assessment. Chile, for example, reports that, through modernization of agriculture, the number of potato varieties cultivated by peasant farmers on the Island of Chiloé has decreased to 270 from originally 800 to 1000 varieties. Mali reports that 60 % of local varieties of sorghum were lost in one region over the previous 20 years due to the expansion of cotton production, introduction of maize cultivation, and the saturation of the available cropping area.

The following studies provide some further examples of quantitative measures of genetic erosion in landraces in specific areas within countries:

- Ecuador: Genetic erosion in three Andean tuber species varied from 25 to 46.5 %, based on morphological and genetic comparison of tubers collected for the study for a comparison with ex situ conserved tubers, and a socio-economic survey (Tapia and Estrella 2001).

- Ethiopia: Megersa (2014) assessed erosion in barley in North Shewa zone of Ethiopia recording a loss of 65% of landraces between 1994 and 2010, attributed to introduction of improved varieties and other crops, recurrent drought, changed land use pattern, and lack of policy support. In two districts in the highlands of West Shewa, of 14 barley landraces described by farmers only four were still found in cultivation. The loss was mainly attributed to environmental degradation, in particular loss of soil fertility (Eticha et al. 2010). In several regions, losses in tetraploid wheat varieties were found to be above 70 % due to numerous reasons, most importantly expansion of improved wheat varieties and tef, difficulties in seed supply, reduction in farm sizes, changes in land use and cropping pattern, and lack of policy support for wheat landrace cultivation, decrease in soil fertility and rainfall (Teklu and Hammer 2006; Tsegaye and Berg 2007).
- India: A large number of genetically rich rice varieties, including varieties with medicinal properties, and a wide range of millet species faded out of cultivation in their native habitats. At the beginning of the last century, each region in the state of Chhattisgarh (a part of Madhya Pradesh) cultivated 19,000 rice varieties suitable to the soil, climate, and other variations. In the 1960s, almost all the local varieties were replaced by high-yielding varieties of rice (Chaudhury 2005).
- Italy: Genetic erosion measured in numbers of lost varieties is reported for several field and garden crops to correspond to 72.8 % in Southern Italy (Hammer et al. 1996). Hammer and Laghetti (2005) observed an annual genetic erosion rate of 13.2 % in wheat in Italy before 1950s, which decreased to estimated rates between 0.48 and 4 % by the 1980s. For the island of Favignana, they report an annual rate of 12.2 %.
- Japan: Starting in 1962, more than 1000 varieties were collected but are now no longer available in their original habitats (Morishima and Oka 1995).
- Republic of Congo: Average rates of landrace losses were calculated to be 32 % for cassava in 21 villages (Kombo et al. 2012).
- Taiwan: report several instances of loss of rice landraces in Asia. Some 100 landraces collected from mountain tribes in different villages in Taiwan in 1943 do not exist anymore in these villages (Morishima and Oka 1995).

Published records about genetic erosion in wild plant species and CWR are much fewer compared to studies that address genetic erosion in landraces and in cultivars:

- Several studies report the high levels of threat to and extinctions of populations of wild rice in Asia (Kiang et al. 1977; Morishima and Oka 1995; Arunachalam 1999; Akimoto et al. 1999; Gao 2003) due to invasive species and environmental changes caused by rapid population growth, new agricultural technologies, economic and cultural changes.
- Populations of African wild rice (*Oryza longistaminata*, *O. barthii*, *O. punctate*, *O. eichingeri*, *O. brachyantha*) were found threatened by land use change resulting from increasing population pressure that destroyed natural wild rice habitats converting them into agricultural land, and overgrazing (Kiambi et al. 2005).

- Wild fruit tree diversity decreased over the last two decades of the last century on the Italian island Sardinia, due to degradation of the natural environment. Frequent summer fires and intensive exploitation greatly altered vegetation cover and many genotypes of the CWR disappeared (Chessa and Nieddu 2005).
- Ipecac (*Psychotria ipecacuanha*), an endangered medicinal plant native to the Atlantic rainforest in Southeastern Brazil is mainly threatened by the short distance of plant populations from inhabited areas and poor conservation status of plant populations (De Oliveira and Martins 2002).
- Several wild *Arachis* species in Latin America were found threatened by extinction based on highly restricted distribution ranges and land use pressures (Jarvis et al. 2003).

Global assessments like the Biodiversity Outlook 2010 report habitat loss and degradation, overexploitation, pollution, invasive alien species, and climate change as the direct drivers of genetic erosion that were found constant or increasing. Like for landrace diversity, climate change is forecast to have major consequences for the adaptation and geographic distribution of wild plants and CWR (Jarvis et al. 2008b). CWR thriving in agricultural landscapes rather than pristine environments will be affected by the same threats as landraces.

One of the major impediments for studies on genetic erosion in CWR has been the availability of data about their distribution and population occurrences, as well as of historic seed material necessary to monitor genetic change over time.

The studies on landrace and CWR illustrate the complex reality of genetic erosion. Loss of diversity is often driven by multiple factors acting together. The range of causes leading to loss and the extent of loss vary, even for the same crop, by geography, national policy environment, and agricultural system. Not always is it verified if those lost varieties were only cultivated in the specific area under study and hence are definitely lost if no longer cultivated and neither conserved ex situ in genebanks, or whether simply the number of cultivated varieties in the area is reduced (loss of varietal diversity) which may not necessarily correspond to absolute loss of genetic diversity if the variety is still cultivated elsewhere. Causes and threats are dynamic; levels of threat can change rapidly and unexpectedly. Thus, a species or a natural area may, for example, suddenly come under threat of industrial development, road-building or logging.

As mentioned earlier, cases exist where improved varieties did not necessarily lead to abandonment of landraces. Studies on local maize varieties in Mexico, its center of origin, show that improved maize varieties have not been widely adopted where farmers prefer landraces due to culinary, agronomic and cultural reasons (Chambers et al. 2007; Hellin et al. 2014). Where improved seeds were introduced, landraces often persisted after the introduction (Bellon 1996; Perales et al. 2003). Similarly, in several potato farming systems in Peru, farmers who adopted higher yielding cultivars continued to cultivate landraces as they were regarded superior in flavor, they store better for home consumption, and they are used as gifts or payment for labor. Steele et al. (2009) monitored the uptake of modern rice varieties in a high-altitude region in Nepal. They found that partial

adoption of a modern variety can increase the overall genetic diversity within the agricultural system, if at least 35 % of the cultivated area continues to be planted to traditional varieties. Also Lohar and Rana (1998) found an increase in varietal richness in some villages in Nepal after the introduction of modern varieties. Several studies in Africa report that varietal diversity has remained stable over time for example sorghum and pearl millet in Niger (Bezancon et al. 2009), sorghum diversity in eastern Ethiopia (Mekbib 2008), or rice diversity in Guinea (Barry et al. 2008). Ford-Lloyd et al. (2009) report stable diversity, analyzing the traditional rice varieties collected across South- and Southeast- Asia over decades and conserved in genebanks. No loss of genetic diversity was found in a study that analyzed samples of cultivated wheat (*Triticum aestivum* L.) collected over an interval of 40–50 years in four comparable geographical regions of Europe and Asia (Khlestkina et al. 2004).

The inherent complexity of the phenomenon, the dynamism and broad range of possible threats, and the lack of comparable quantitative assessments due to widely varying assessment methods and scales, are reasons for the difficulty in producing comparable measurements and drawing a clear picture of the overall status and trends in genetic erosion. They clearly show that genetic erosion assessments need to be addressed in a more systematic way, which includes regular monitoring in agricultural and natural systems and harmonized and standardized quantification of trends and extent.

10.4.2 Instances and Causes of Genetic Erosion Ex Situ

Storing plant germplasm ex situ in genebanks has been the most significant reaction of the research community to genetic erosion occurring in CWR and in farmers' fields. Over 1700 genebank facilities store a total of approximately 7.4 million germplasm accessions today (FAO 2010). Furthermore, over 2500 botanic gardens conserve about 80,000 species (FAO 2010). However, ex situ collections are not immune to genetic erosion and accessions need to be managed properly to maintain genetic integrity of the stored material.

As early as 1975, it was noted that genetic erosion occurs in ex situ collections. Frankel (1975) reported in a survey of genetic resources that many collections had suffered genetic erosion due to hybridization, selection, genetic drift, unsuitable growing conditions, or human error during propagation.

While major threats like natural disasters, armed conflicts and outright war, or economic instability can put entire collections at risk; pest and disease outbreaks and abiotic stresses like drought and heat can lead to loss of field collections or loss of accessions during regeneration; poor quality of the original material stored, inappropriate storage conditions, incorrectly applied collection management procedures, and lack of resources (financial, personnel) and skills can lead to erosion within single accessions (Engels and Visser 2003; Gomez-Campo 2006; Rao et al. 2006). Appropriate collection management needs to avoid genetic drift,

unintentional selection, pollen contamination, seed contamination, and mislabelling, which are all factors that contribute to genetic changes in genebanks.

Regeneration backlogs are considered one of the most critical threats to genetic diversity in genebanks (CIMMYT 2007). A review of conservation strategies carried out by the Global Crop Diversity Trust (Khoury et al. 2010) reported large regeneration backlogs in a considerable number of national collections. This implies a risk of loss of viability and hence of genetic diversity in many accessions. This risk can be aggravated by the fact that the number of individuals (seeds, tissues, tubers, plants, etc.) conserved per accession is frequently below the optimum for maintaining heterogeneous populations in these collections.

Genetic erosion in genebanks has effectively been measured using molecular markers. A number of studies compare accessions of a specific crop that have been regenerated varying numbers of times within the same genebanks to assess potential losses due to regeneration practices. In a comparison of rye accessions, nearly 50 % of the alleles discovered in the original samples were not found any more in the most recent seed lots, but new alleles were detected in the most recently propagated subpopulations that were not observed in the original seed lots. These changes were attributed to selection pressure during regeneration due to severe winter damage and diseases that decreased the sizes of the populations (Chetobar et al. 2003). Genetic erosion attributed to regeneration was also observed in pea accessions (Cieslarova et al. 2011; Hagenblad et al. 2014). Comparisons of oat and wheat seed samples that were conserved in different genebanks after collecting confirm that differences in genebank management and regeneration procedures affect diversity (Steiner et al. 1997; Hirano et al. 2009). Absence of changes in genetic diversity over time, like in wheat accessions (Boerner et al. 2000) or wild potato species (Del Rio et al. 1997) is considered to confirm effective collection management. Results of these studies clearly show the importance of most careful handling of passport data, regeneration and storage to maintain genetic integrity and avoid genetic erosion.

Rare wild plant species are often conserved in botanic gardens (albeit with generally a poor representation of the existing genetic diversity within a species), but are not exempt from genetic erosion. The conservation of too small living populations in botanic gardens can affect the genetic diversity, and lead to changes and losses of genetic diversity (Enßlin et al. 2011; Rucinska and Puchalski 2011; Lauterbach et al. 2012; Bruetting et al. 2013).

10.4.3 Genetic Erosion in Modern Varieties

A large number of studies investigate the impact of breeding on genetic diversity of cultivars, in particular of wheat (Donini et al. 2000; Manifesto et al. 2001; Christiansen et al. 2002; Fu et al. 2005, 2006; Roussel et al. 2004, 2005; Khan et al. 2005; Martos et al. 2005; Figliuolo et al. 2007; Huang et al. 2007; Hysing et al. 2008; Mir et al. 2012); rice (Qi et al. 2006; Mantegazza et al. 2008; Wei

et al. 2009; Choudhary et al. 2013), barley (Koebner et al. 2003; Kolodinska-Brantestam 2004; Malysheva-Otto et al. 2007), maize (Le Clerc et al. 2005, 2006; Reif et al. 2005), as well as oats (Nersting et al. 2006) or pea (Le Clerc et al. 2006; Cieslarova et al. 2012). These studies usually group cultivars by time periods, often by decades, in which they were released. The overall time ranges studied vary from 35 to 200 years. Usually studies regard cultivars released within a country, while a few studies have wider extension, like the study of European maize (Reif et al. 2005), winter wheat (Huang et al. 2007), barley (Malysheva-Otto et al. 2007), Nordic barley (Kolodinska-Brantestram 2004), bread wheat (Hysing et al. 2008), spring wheat (Christiansen et al. 2002), and oats (Nersting et al. 2006), or two countries (Italy and Spain) (Martos et al. 2005). A few of these studies (Nordic oats, Italian durum wheat, and French bread wheat) include a comparison between landraces and cultivars, and all find a higher genetic diversity in landraces than in cultivars (Nersting et al. 2006; Roussel et al. 2004; Figliuolo et al. 2007). The findings vary, even within the same crop species, between studies that do not observe decreases in diversity, those that observe qualitative rather than quantitative changes (Donini et al. 2000) and those that observe reduction in diversity to various degrees. The majority of these studies do not report loss of genetic diversity. A meta-analysis of the impact of breeding on genetic diversity in improved cultivars could not reveal any gradual narrowing of the genetic base of released varieties (Van de Wouw et al. 2010) in the production system. It should be noted that the conclusion of the latter paper applies to the "landscape level" and not to the community or farmers' field level. In case of the lower level substantial loss of genetic diversity has been reported (see also above).

10.5 Assessment of Genetic Erosion

10.5.1 Constraints in the Measurement of Genetic Erosion

The challenges in measuring and monitoring genetic erosion are related to the fact that it describes changes in genetic variation that take place over time, and to the unit of measurement required to measure change. Genetic erosion is quantified as the proportion of richness of genetic diversity that no longer exists in current populations when compared with historic population or that is predicted to be lost in the near future if no remedial measures are taken (Brown 2008). This proportion of richness, as the definitions of genetic erosion above-outlined show, can refer to two different units—numbers of landraces or number of alleles or combination of alleles—in which both are complex units themselves. These units need to be measured in a comparable way in at least two different time points. Research on genetic erosion therefore demands a complex set of time series data which is not readily and easily available.

The first methods that aimed to provide some measures were developed to estimate the threat of genetic erosion (Goodrich (1987) and modified by Guarino

(1995) that a taxon (wild or cultivated) faces in a particular region, rather than to quantify the effective loss. A checklist of factors about the species and its environment is scored to evaluate level of threat. The model could be operated without the necessity to actually visit the region, provided that adequate background or baseline data were available on the taxon and the region. The model was further adapted for specific studies by De Oliveira and Martins (2002) to assess threat of genetic erosion in ipecac (*Psychotria ipecacuanha*) and by Keiša et al. (2008) to study wild *Vicia* species belonging to the secondary genepool of faba bean, in Syria. This approach is an indirect genetic erosion assessment. No direct comparison between populations of landraces or wild species is made, due to lack of available historical plant material and occurrence data. Other indirect measures that have regularly been employed are household surveys and farmer interviews (Peroni and Hanazaki 2002; Davari et al. 2013).

Direct genetic erosion assessment is based on the comparison between historical and extant plant material. When historical collections are available and sufficiently documented, contemporary plant material can be collected for a comparison at genetic level. If only historical data are available but no plant material, direct assessment can be made comparing numbers of subspecific units (subspecies, varieties, landraces) in the documentation with a contemporary assessment of landraces still cultivated in the same areas and collecting sites.

The difficulties in quantifying genetic erosion in landraces that have emerged over time are related to the concept of landrace itself and to the role that seed systems—that are complex and dynamic—play in determining local diversity. Local names of landraces do not necessarily reflect their genetic history. Different names may be given to identical landraces while, conversely, a single name may apply to heterogeneous material (Jarvis et al. 2008a). Names therefore do not necessarily define distinct genetic units and it has become evident that the disappearance of named varieties in regions where they used to be present is not sufficient to prove loss of diversity. Furthermore, also the effect of modern varieties on local diversity is far from obvious (van Heerwaarden et al. 2009). To explain this finding further it should be noted that the informal and formal seed systems are not mutually exclusive; in fact they frequently operate in parallel. Farmers may give local names to improved varieties, and they may become part of what farmers consider to be landrace seed. Diversity could even increase if improved germplasm is genetically more heterogeneous than local seed or if it offers traits that are not present in traditional landraces (Wood and Lenne 1997; Louette and Smale 2000).

Although a significant number of studies reporting incidences of diversity loss in landraces have accumulated over time and an overall trend of continuing erosion was confirmed by the SOW2, no clear quantitative picture of genetic erosion has yet emerged. Advances in marker technologies combined with a decrease in cost of these technologies have allowed some more quantitative assessments at molecular level (i.e., direct comparisons of historic and extant seeds) to assess temporal changes (Franks et al. 2007; Vigouroux et al. 2011; Nevo et al. 2012), but although the number of molecular studies has increased they appear still to be constraint by the difficulty in establishing an appropriate time series data set

composed of historical seed material and recollected seed samples. Possible reasons might be the challenges of identifying and assessing historical data and material as well as a lack of resources to implement recollections of contemporary material and molecular studies. The majority of studies assessing the extent of genetic erosion rely on comparisons of number of landraces, but methods used to determine current and past numbers, geographic scale, number and sizes of households assessed, and crops vary greatly. Without standardized information, no cross-country or cross-crop comparison can be made and no clear baseline established.

Improved varieties and cultivars, on the contrary, have become a more frequent object of quantitative genetic erosion studies. In contrast to landraces, they are very well defined and certified entities, documented in catalogs. They are therefore relatively easy to obtain and are not related to specific collecting sites or villages like landraces or CWR. However, considerations of comparability across species were raised also for this group of studies which is rather homogeneous compared to landrace and CWR studies. Differences in breeding methods, use of different molecular markers of unequal quality and diversity measurements of unequal accuracy would need to be considered when making generalizations (Fu et al. 2006).

CWR are the least frequently studied group. Although they have gained significant recognition as a resource of genetic diversity for crop improvement and adaptation, their genetic diversity is not yet well studied and their conservation requires increased attention and action (FAO 2010). Direct genetic erosion assessments in CWR have been constrained by the lack of historical data and material (Keiša et al. 2008) and possibly by lack of resources. Much less data and historical material exist compared to landraces. CWR for example comprise less than 10 % of germplasm conserved in ex situ collections.

10.5.2 Options for Generating Time Series Data

Improvements in the digitization of information and the development of online databases have facilitated access to data, the development of molecular marker techniques, and the associated decreased cost have been supporting a growing number of studies in various research areas, using historical biological specimens from collections conserved in natural history museums, herbaria, botanic gardens, and universities (Miller-Rushing et al. 2006; Wandeler et al. 2007; Vellend et al. 2013). Research on evolution of development is increasingly using material from genebanks, long-term experimental plantings, and botanical gardens (Dosmann and Groover 2012), for example to understand processes that led to independent domestication of beans in two geographic regions (Kwak and Gepts 2009), and the analysis of herbarium specimen collected from the same geographical region over decades has revealed the impact of climate change on flowering trends (Primack et al. 2004; Gallagher et al. 2009; Calinger et al. 2013; Li et al. 2013).

Genebanks and plant germplasm collecting missions are ideal sources for historical data and for PGRFA material. They have the added advantage that samples were collected to conserve the then existing diversity, hence samples are larger than museums, herbaria, or botanic garden collections, which conserve only one or few individuals of the taxon collected. During the actual collecting of the germplasm samples to be conserved in genebanks, important data about the location and environment from which the sample is collected were recorded in collecting reports, and provide important data associated with the samples.

As mentioned above, the IBPGR supported the collecting of over 200,000 samples of landraces and CWR in over 1000 collecting trips in more than 130 countries. The IBPGR employed professional collectors as well as some crop specialists to implement the collecting program and a systematic approach to targeting collecting sites, sampling, and passport data recording. The material collected was subsequently sent to genebanks for long-term conservation. The IBPGR/IPGRI coordinated collecting missions and the resulting germplasm material were well-documented—a necessary condition to make use of historical material today—as the collectors usually recorded passport data about each sample in standardized collecting sheets and captured additional data and information in reports about their collecting missions (Thormann et al. 2012). These original passport data have been extracted from the historical documentation and made available online through the Bioversity Collecting Database (BCD)[3] together with the original reports and collecting sheets, representing a unique historical resource of documented plant collections that can support the establishment of baselines for assessment of extent and drivers of genetic erosion.

Several features of the BCD are particularly relevant for its use for monitoring genetic erosion (Thormann et al. 2015).

1. The BCD contains high percentage of CWR data. While CWR are reported to represent rather small percentages in ex situ collections, such as 11.6 % wild species in the European Catalogue of ex situ Collections EURISCO[4] and globally estimated to average 2–6 % of ex situ collections (Maxted and Kell 2009), 27 % of the data in the BCD regard CWR. Many of the collecting expeditions specifically included or targeted CWR. In 39 % of all collecting trips at least half of all collected samples were samples of wild species and 25 % of all collecting trips were dedicated solely to the collecting of wild plants. Overall, about 60,000 CWR samples were collected in 115 countries.
2. Historical collecting information and material need to include the geographical coordinates of the collecting site or a clear description of the location from which it was collected, so that the historical sites can be located and revisited and surveyed, species can be recollected (if still growing there) and climate, geographical and geophysical data can be obtained for the sites. For 66.2 % of all samples included in the BCD the passport data available include

[3] http://bioversity.github.io/geosite/.

[4] verified on http://eurisco.ecpgr.org/search/advanced_search.html; 15 July 2014.

georeferenced data, and the percentage of all CWR samples that include georeferences is even higher (73 %).

3. The BCD contains records of collected samples for which accession numbers have been identified in the genebanks to which the samples were sent for conservation. The link between the original passport data and the accession stemming from the collecting event can be used to retrieve seed material (if the accession is still viable and available for distribution).
4. Many species have been collected from more than 20 sites within one country or a limited area within a country, and many also from different countries, which can provide a reasonable snapshot of past diversity and allow comparisons between different ecogeographical realities.

Several past studies have used the IBPGR collections and associated data for studying genetic erosion on-farm and in genebanks. Sorghum and pearl millet landraces originally collected in 1976 in Niger and rice collected in 1979 in Guinea Bissau were recollected in 2003 from the same villages to observe any changes over time. The current diversity was assessed and compared with data and material from the past collections (Barry et al. 2008; Bezancon et al. 2009; Deu et al. 2008, 2010). In both countries, varietal diversity had been maintained by farmers at a national scale. But further analysis at molecular level of the old and contemporary pearl millet genotypes from Niger showed that significant shifts in adaptive traits had occurred, such as a shorter life cycle and reduction in plant and spike size in the contemporary samples (Vigouroux et al. 2011). Hirano et al. (2009) compared wheat landraces collected by IBPGR in Pakistan in 1989 and conserved in Pakistan and Japan, after 17 years of storage. Accessions had been regenerated only once and the overall genetic diversity was found to be adequately conserved in both genebanks. However, observed allele changes pointed to possible unintentional selection pressure during regeneration, indicating the need for increased attention to conservation practices in one genebank and for further research into the maintenance of genetic integrity in genebanks.

The original sample passport data and documentation from the BCD have recently been used to implement several genetic erosion studies. Wild barley and barley landraces were recollected in Jordan after 31 years (Thormann and Dulloo 2015) and the material collected in 1981 could be identified in genebank collections. The accessions were requested from the genebanks and the newly collected seed samples are being compared with original seed samples from the first collecting trip to assess genetic erosion in Jordan, the center of origin and domestication of barley (pers com Thormann). Barley landrace samples collected in Libya in 1983 and in Morocco in 1985, conserved after collecting in different genebanks, could be identified and are being studied to assess maintenance of genetic integrity in genebanks (pers com Thormann).

The high potential for using historical collections in assessing and monitoring genetic erosion has been confirmed by a recent study that tested genetic metrics and sampling protocols for monitoring (Hoban et al. 2014).

10.6 Current Status and Knowledge on Amount of PGRFA Diversity in Conservation

Considerable progress has been made since the 1970s in systematically conserving diversity in crops and CWR in ex situ germplasm collections. Over 7.4 million accessions are conserved in genebanks and many botanic garden collections also include wild plant species of economic, cultural, and social value. Safe ex situ conservation requires stable, long-term funding to maintain genebank operations. The foundation of the Global Crop Diversity Trust in 2004[5] was a significant step taken to help raise funds for ex situ collections. In addition, with the support of the government of Norway, the Svalbard Seed Vault was established to provide a tertiary safety backup of existing collections. Ex situ conservation standards and guidelines for genebanks have been updated (FAO 2013), and guidelines, manuals, and best practices for genebank management and ex situ conservation procedures have been compiled and published in the Crop Genebank Knowledge Base website[6] (Jorge et al. 2010; SGRP 2010; Thormann et al. 2013), with the aim to reduce loss of genetic integrity in ex situ collections through better collection management. Despite these advances, human capacity, funds, and/or facilities in many countries are inadequate to carry out conservation work to the required standards. Suboptimal management and storage remain an important cause of genetic erosion in genebank collections (FAO 2010).

Ex situ collections are far from representing the full spectrum of genetic diversity in PGRFA. With very few exceptions among the major crops such as potato, whose genepool is relatively well covered (FAO 2010), gaps exist in collections for most crops. The global banana collection was reported to lack about 300–400 cultivars including over 100 wild types (INIBAP 2006). Geographical gaps (i.e., areas not- or not sufficiently collected) and gaps in coverage of landraces or CWR, taxonomic gaps of species not appropriate for ex situ storage, with unknown storage behavior, or neglected by research, exist in collections; so a significant proportion of CWR and landrace diversity remains in situ and in farmers' fields (i.e., under less or not controlled conditions from a conservation perspective). In contrast to ex situ collections, which represent snapshots of diversity existing at the moment of collecting and which are largely "frozen" from an evolutionary point of view, these landraces and CWR growing in their natural environments continue to be exposed to changing environmental conditions. But global and climate change are putting these same resources at risk of increasing genetic erosion and extinction (Alsos et al. 2012) and critically important components of PGRFA such as CWR and landraces adapted to specific climatic and geographical conditions and farming practices could be permanently lost if not properly managed and conserved. With the development of the CBD, ITPGRFA, and GPA, it has

[5]The Global Crop Diversity Trust was founded in 2004 in Rome, Italy by FAO and Bioversity International on behalf of CGIAR.

[6]http://cropgenebank.sgrp.cgiar.org/ or http://cgkb.cgiar.croptrust.org/.

been recognized that ex situ conservation alone is insufficient and complementary in situ conservation and on-farm management are necessary to conserve the full extent of genetic diversity within and among populations. These conservation strategies have the distinct advantage over ex situ conservation in that they allow evolutionary processes to continue, as the target species are continuously exposed to a changing natural environment that allows new diversity to be generated.

The SOW2 indicates that activities such as the number of participatory on-farm management projects and the selection of priority sites for CWR conservation have increased. Protected areas increased by 30 % over the SOW2 reporting period, which however offers only indirect additional protection to CWR and CWR conservation outside protected areas has not made much progress. Methodologies and guidelines for in situ conservation of CWR (Heywood and Dulloo 2006; Hunter and Heywood 2011) and on-farm management (Maxted et al. 2002; Jarvis et al. 2011; Jarvis et al. 2006) are now available. FAO has published a resource book for the preparation of national plans for the conservation of CWR and landraces (Maxted et al. 2013), which has recently supported the development of strategies in several countries in Europe (i.e., landrace conservation strategies in Finland, Italy, and UK and CWR conservation strategies in Finland, Italy, and Spain), and is currently being used to develop CWR conservation strategies in Mauritius, South Africa, and Zambia. A regional strategy for conservation of PGRFA in Mesoamerica was recently published by Bioversity International (2014). A global strategy for CWR conservation was developed (Heywood et al. 2008) and the status and needs for a global network for CWR in situ conservation were analyzed (Maxted and Kell 2009). FAO, together with the University of Birmingham and Bioversity International, and on behalf of the CGRFA, is currently investigating whether the current fragmented and isolated efforts at national and regional level in landrace and CWR in situ and on-farm conservation should be coordinated and aligned through the establishment of a global network for in situ conservation and on-farm management (FAO 2014). Despite the increase in in situ conservation and on-farm management activities and in the number of surveys and inventories made of landraces and CWR, no current estimates of diversity–neither at species nor at intraspecific level–managed on-farm and conserved in situ exist.

10.7 Conclusions and Prospects

A major shortcoming for the assessment of genetic erosion is the lack of data about existing PGRFA diversity in situ, and of standardized methods to assess and monitor trends in it. Current data are insufficient and it is not possible to make comparisons between studies across species and geographies, to globally evaluate the status of diversity and to establish baselines upon which to monitor changes in genetic diversity as well as progress of conservation efforts (Tittensor et al. 2014; Thormann et al. 2015). A recent analysis of progress toward the Aichi Biodiversity Targets was not able to include an assessment of progress toward the maintenance of genetic diversity and reduction of genetic erosion in PGRFA—addressed in Aichi Target 13—due to lack of time series data sources (Tittensor et al. 2014).

A more coherent and complete baseline of the current amount and status of in situ diversity of PGRFA is necessary to describe the extent of diversity at risk of being lost, and to inform and assess conservation actions. This requires further inventories and surveys to fill data gaps. Advances in molecular technologies, accompanied by a parallel significant reduction in their cost, will hopefully lead to a growing number of surveys that include diversity assessments at the genetic level. Molecular markers are increasingly used to assess diversity and genetic erosion in ex situ collections.

Assessments of genetic diversity need to be carried out in a standardized way that allows their repetition across space and at future time points in order to enable monitoring and comparison and to establish trends in diversity change and assess the quality of conservation efforts. As discussed throughout this chapter, losses have been difficult to quantify, data have been fragmentary, sometimes contradictory, and studies have been based on varying methodologies.

A methodology for monitoring the threat of extinction at the species-level is well-developed for wild species, and thus applicable also to CWR (Mora et al. 2009). The IUCN red list of threatened species provides a tool to assess the degree of threat to wild species. To date, 74,000 species have been assessed, of which over 19,000 are plants (http://www.iucnredlist.org/about/summary-statistics#Tables_1_2). The European red list of vascular plants, which focuses on 1826 selected species of vascular plants native to Europe or naturalized before the year 1500, includes specifically CWR of priority crops (Bilz et al. 2011). Proposals for the development of a red list for cultivated plants have been made and discussed at an international conference on "On-farm conservation of neglected and underutilized species: status, trends and novel approaches to cope with climate change" (Padulosi et al. 2012).

Although conserving genetic diversity and minimizing genetic erosion are part of international policies, effective implementation at genetic level of global agendas still needs to be addressed (Laikre 2010). Existing assessment and monitoring efforts often do not include measurements of genetic diversity, and consensus is missing on what exactly to monitor (Feld et al. 2009; Pereira et al. 2013). Several sets of indicators that include genetic level monitoring have recently been proposed such as, a new integrative indicator to assess the crop genetic diversity (Bonneuil et al. 2012) or the Essential Biodiversity Variables (EBVs) whose development was supported by the CBD (Pereira et al. 2013). A global system of harmonized observations based on agreed indicators that integrate advances in ecology and conservation genetics is urgently needed. Consistent monitoring, supported by enhanced mining of existing and historical data, is required to identify trends in genetic erosion.

Studies at genetic level are not only important to quantify genetic losses. More research is required to better understand the processes and causes leading to genetic erosion and the effects on contemporary evolution (Franks et al. 2008), which in turn can inform monitoring and more effective conservation of our dwindling genetic resources.

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Chapter 11
Diversity Arrays Technology (DArT) Markers for Genetic Diversity

Dariusz Grzebelus

Abstract Diversity Arrays Technology (DArT) is a hybridization-based high-throughput genotyping technology that was proposed in the beginning of the twenty-first century as an efficient and cost-effective alternative to existing genotyping systems, and since then it has been used extensively to explore genetic diversity in many plant species. In this chapter, we describe the principles behind DArT genotyping, summarize the research on plant genetic diversity utilizing the DArT system, discuss advantages, limitations, and perspectives of the technology.

Keywords Genetic diversity · Genotyping · High-throughput · Cost-effective · DArT system · Genetic erosion · Crop domestication

11.1 Introduction

Numerous marker technologies facilitating studies and management of plant genetic diversity have been developed over the past few decades. On one hand, marker-based strategies help in investigating species diversity, genetic erosion, crop domestication, etc. On the other hand, they are widely used in crop improvement, allowing more effective utilization of genetic diversity. Historically, the most popular systems were (1) restriction fragment length polymorphisms (RFLP, Botstein et al. 1980), (2) randomly amplified polymorphic DNA (RAPD, Williams et al. 1990), (3) amplified fragment length polymorphism (AFLP, Vos et al. 1995), (4) microsatellites (simple sequence repeats; SSR, Powell et al. 1996), and (5) single nucleotide polymorphisms (SNP, Rafalski 2002). They have been extensively used to genotype plants. RAPD and AFLP systems do not require any prior information on the sequence of

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polymorphic sites. They were widely used in the last decade of the twentieth century, as no cost-efficient DNA sequencing technologies were available at that time. In contrast, development of SSR, and particularly SNP markers, requires that sequences of polymorphic sites are known, which allowed their wider introduction only after data from numerous plant genome and transcriptome sequencing projects begun to accumulate in the past decade (Zalapa et al. 2012). Nevertheless, for many crops financial resources are still too low to initiate NGS-based marker discovery and arbitrary markers remain an option of interest for investigation of species genetic diversity.

The genotyping systems described above varied with respect to their capability for rapid identification of large numbers of markers. Most systems provided low- to medium-throughput efficiency, as they relied on sequential identification of polymorphisms, typically by means of agarose or polyacrylamide electrophoresis. Only SNP markers can be identified with several commercially available high-throughput genotyping platforms (reviewed by Gupta et al. 2008). Diversity Arrays Technology (DArT) markers provided a unique option of cost-efficient parallel genotyping with a set of hundreds to thousands of arbitrary markers in a single assay utilizing microarrays. By scoring presence or absence of arbitrary restriction fragments in genomic representations, DArT produces reproducible whole-genome fingerprints (Jaccoud et al. 2001). Here, we describe the principles of the DArT system and present an overview of its applications for assessment of genetic diversity in plants.

11.2 Principles of the Diversity Arrays Technology

Diversity Array Technology (DArT) is a microarray-based molecular marker system allowing cost-efficient (per data point) high-throughput genotyping of any organism. It was developed as a hybridization-based alternative to existing genotyping technologies. Importantly, DArT genotyping does not require any prior knowledge of the genome sequence (Jaccoud et al. 2001). It has been widely applied in plant science and proven to perform well for many species (Kilian et al. 2005). An updated list of reports using DArT markers for evaluating genetic diversity in plants is shown in Table 11.1.

Generally, 100 ng of genomic DNA is enough to genotype more than 7000 genomic loci in parallel in a single-reaction assay. DArT markers are strictly biallelic and are usually scored as presence versus absence variants, where the ‘present’ state is dominant over the ‘absent’ state. However, they may also be scored as hemi-dominant taking into account signal intensity as a reflection of the dosage effect (double dose vs. single dose vs. absence). The observed polymorphisms usually result from single nucleotide substitutions within restriction sites or InDels including restriction sites, but they can also be caused by differences in the methylation status (see below). Nevertheless, the structural polymorphisms account for more than 90 % of the identified variability (Wittenberg et al. 2005) and are inherited in a simple Mendelian fashion.

Table 11.1 Research projects on plant genetic diversity utilizing the Diversity Arrays Technology (DArT) platform

Species	Objective of the study	No. of DArT markers	Reference
Aegilops tauschii	Population structure and diversity	4449	Sohail et al. (2012)
Asplenium viride	Evolution of substrate specificity	444	James et al. (2008)
Banana (*Musa* sp.)	Fingerprinting and diversity	836	Risterucci et al. (2009)
Banana (*Musa* sp.)	Genetic diversity	653	Amorim et al. (2009)
Barley (*Hordeum vulgare*)	Genetic variability assessment	271	Ovesná et al. (2013)
Carrot (*Daucus carota*)	Genetic diversity, wild versus cultivated	900	Grzebelus et al. (2014)
Cassava (*Manihot esculenta*)	Genetic diversity	1000	Xia et al. (2005), Hurtado et al. (2008)
Common beans (*Phaseolus vulgaris*)	Germplasm genetic diversity	2501	Briñez et al. (2012)
Eucalyptus grandis	Fingerprinting	104	Lezar et al. (2004)
Garovagliaelegans	Phylogeography	905	James et al. (2008)
Hop (*Humulus lupulus*)	Fingerprinting and diversity	730	Howard et al. (2011)
Lesquerella (*Physaria* sp.)	Genetic diversity	2833	Cruz et al. (2013)
Mungbean (*Vigna radiata*)	Genetic diversity	1125	Hang Vu et al. (2012)
Oat (*Avena sativa*)	Genetic diversity	1295	Tinker et al. (2009)
Olive (*Olea europaea*)	Genetic diversity	2031	Domínguez-García et al. (2012); Atienza et al. (2013)
Pigeonpea (*Cajanus cajan*)	Genetic diversity, wild versus cultivated	700	Yang et al. (2006)
Rapeseed (*Brassica napus*)	Genetic diversity	1547	Raman et al. (2012)
Rice (*Oryza sativa*)	Fingerprinting and diversity	1152	Xie et al. (2006)
Rye (*Secale cereale*)	Genetic diversity	1022	Bolibok-Brągoszewska et al. (2009)
Sorghum bicolor	Genetic diversity	508	Mace et al. (2008)
Sugarcane (*Saccharum sp.*)	Genetic diversity	667	Heller-Uszynska et al. (2011)
Tall fescue (*Festuca arundinacea*)	Genetic diversity	190	Baird et al. (2012)
Triticum monococcum	Genetic diversity	846	Jing et al. (2009)
Wheat (*Triticum aestivum*)	Genetic diversity	411	Akbari et al. (2006), White et al. (2008)

(continued)

Table 11.1 (continued)

Species	Objective of the study	No. of DArT markers	Reference
Wheat (*Triticum aestivum*)	Population structure and association mapping	318	Crossa et al. (2007)
Wheat (*Triticum aestivum*)	Genetic diversity, resistance to fusarium head blight	409	Badea et al. (2008)
Wild potatoes (*Solanum bulbocastanum, S. commersonii*)	Inter-species diversity	1423	Traini et al. (2013)

11.2.1 The DArT System

The first step in the development of the DArT genotyping platform for a species of interest is the assembly of a set of arbitrary genomic DNA fragments representative of the germplasm under investigation using a procedure called 'complexity reduction' (Fig. 11.1). The fragments are derived from a collection of individuals representing the primary gene pool of the species. A few complexity reduction strategies have been applied by different authors. Here we present the most widely implemented strategy in which the fragments are obtained by double restriction digestion of pooled genomic DNAs of plants comprising the collection with *Pst*I (6-cutter) and a frequently cutting restriction enzyme (4-cutter, e.g. *Taq*I, *Bst*NI, *Apo*I, etc.). *Pst*I is used because of its methylation sensitivity—it does not cut in methylated regions and thus it allows getting rid of the heavily methylated highly repetitive fraction of the genome. It is essential to carefully select the most suitable frequently cutting restriction enzyme, as it was shown that their ability to reveal polymorphisms may differ significantly, especially in larger genomes comprising more repetitive DNA (Wenzl et al. 2004). Subsequently, *Pst*I- and 4-cutter-restriction site-specific adaptors are ligated to the ends of the restriction fragments and adaptor-specific primers are used to amplify them.

Additional modifications of complexity reduction methods, used mostly for analyses of more complex genomes, include the use of fragments developed from amplification of regions adjacent to insertion sites of miniature inverted-repeat transposable elements (MITEs) and application of suppression subtractive hybridization (SSH, Diatchenko et al. 1996) to enrich genomic representations with polymorphic clones (James et al. 2008; Mace et al. 2008; Heller-Uszynska 2011).

The amplicons are then ligated into a plasmid and cloned in *Escherichia coli*. Individual *E. coli* colonies carrying inserts are arrayed on 384-well plates. The set of inserts comprising the library is called 'genomic representation' and can be characterized by the level of complexity depending on the size of the studied genome, number of fragments in the library and size of fragments, which usually is in the range of 300–700 bp. Typically, the genomic representation of a plant genome contains no more than a few percent of the whole genome. The library

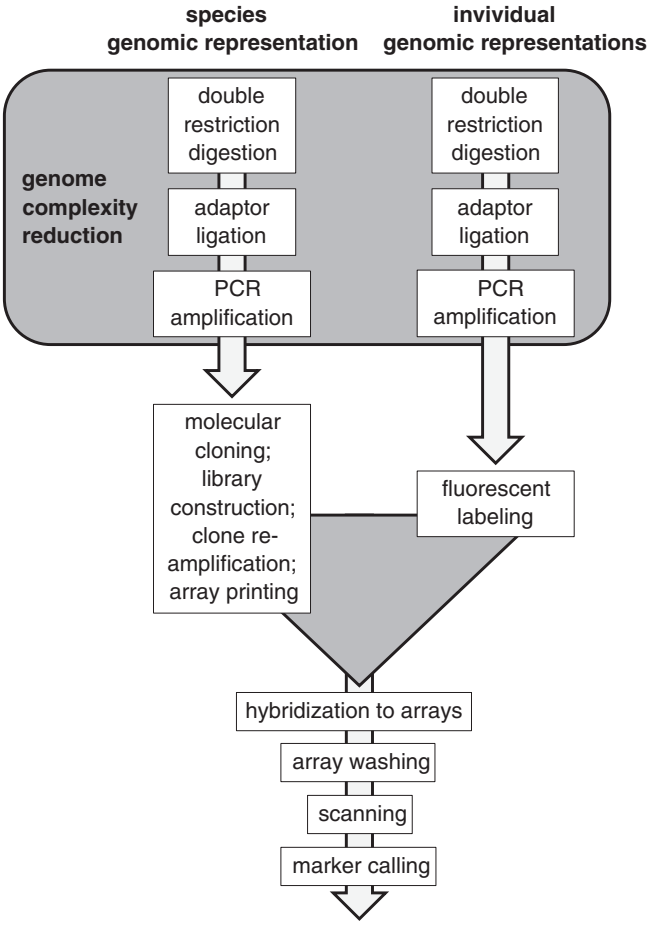

Fig. 11.1 A schematic diagram presenting steps of the Diversity Array Technology genotyping platform

is used to prepare spotted glass microarrays for routine assays. For this purpose, inserts are reamplified from plasmids using a pair of universal vector-specific primers, so that each amplicon carries a segment derived from the genomic DNA of interest and vector segments adjacent to the multiple cloning site, the latter being present in all spotted DNA fragments.

Genomic representations of individuals subject to genotyping (called 'targets') are obtained from single genomic DNA isolations using the above strategy (Fig. 11.1). They are fluorescently labeled and hybridized to the glass microarrays on which the genomic representation of the species was spotted. A multiple cloning site of the vector (called 'reference') fluorescently labeled with a dye different from that used for the genomic representation is also used for hybridization, in parallel with the target. The reference provides quality control for each spot

as it allows measurement of the signal-to-noise ratio. Following hybridization, the microarrays are washed, scanned with a confocal laser scanner, and analyzed with a dedicated software called DArT soft, performing image analysis, marker discovery, and marker scoring (Kilian et al. 2005).

11.2.2 Limitations of DArT Markers

Three major issues, i.e., low level of polymorphism, redundancy, and sensitivity to methylation, may affect optimal implementation of the DArT genotyping platform. Typically, only from 5 to 30 % of all spotted fragments allow identification of polymorphisms. In order to making the DArT genotyping more effective, it is possible to rearrange the initial array to remove all nonpolymorphic and unreliable clones. In a number of more advanced DArT genotyping programs, a strategy is used which involves initial development of 'discovery arrays', identification of the most informative DArT markers, subsequent re-arraying, and assembly of a final 'genotyping array' (Gupta et al. 2008).

Redundancy is caused by the presence of multiple clones in the genomic representation library that were derived from the same genomic region. Grzebelus et al. (2014) estimated that a very high fraction of DArT clones, reaching 50 %, were redundant in the carrot discovery array, while only 11 and 16 % redundancy was reported in *Asplenium* and *Garovaglia* arrays, respectively (James et al. 2008). There are two possible causes of the observed redundancy; (1) the redundant fragments originated from repetitive regions and (2) the redundant fragments were preferentially PCR-amplified. While the presence of repetitive fragments can be limited by careful selection of the combination of restriction enzymes, the amplification issues can at least in part be solved by optimization of cycling parameters, including primer annealing temperatures and limiting the number of PCR cycles.

As *Pst*I restriction enzyme routinely used for preparation of genomic representations is methylation-sensitive, a fraction of observed polymorphisms can originate from different methylation status of the same sequence. It was reported that for less than 10 % of DArT markers in *Arabidopsis* no sequence polymorphism could have been detected, implying that they represented methylation variants (Wittenberg et al. 2005). Interestingly, at least one of DArT markers showing strong signature for selection in the cultivated carrot was apparently a result of a systematic difference of the methylation status rather than sequence variability (D. Grzebelus, unpublished). Thus, even if sensitivity to methylation is generally undesired, in particular cases it can be viewed as an additional advantage of the technology, depending on the research objectives.

Bolibok-Brągoszewska et al. (2009) stressed the fact that the dominant character of DArT markers may limit their usefulness for the assessment of genetic diversity in highly heterozygous obligatory outcrossing species. However, other authors postulated that the high number of DArT markers identified per assay combined with the use of the most appropriate strategy for inferring population

structure provided satisfactory results. Also, it is possible to score DArT markers in a hemi-dominant (dosage-dependent) manner to identify the heterozygote state (Kilian et al. 2005).

11.3 Application of the DArT Marker System for Evaluation of Genetic Diversity

The technology was originally developed for rice, a diploid crop with a small genome of 430 Mb. In the proof-of-concept paper presenting capability of the DArT system to capture genetic variability, Jaccoud et al. (2001) demonstrated that it could be used to investigate genetic diversity of rice cultivars of different origin. Xia et al. (2006) developed a general purpose rice DArT platform and used it to study genetic diversity in 24 rice cultivars originating from the Yunnan province, concluding that the level of genetic diversity in rice hybrid cultivars was low, while it was higher in a set of investigated landraces. Recently, Courtois et al. (2013) developed a japonica rice genotyping panel employing an NGS-based variant of DArT called DArT seq (see Perspectives section) and used it to analyze 167 accessions of *O. sativa* var. *japonica* with the purpose of association mapping of root traits. With respect to genetic diversity, they revealed diversity structure comprising six subpopulations, reflecting geographic origin and breeding history. A large number of admixed accessions confirmed gene exchange among subpopulations.

DArT has been extensively to study genetic diversity in other cereal crops. Ovesná et al. (2013) analyzed genetic diversity in 94 Czech malting barley cultivars. They reported that the level of genetic diversity remained roughly unchanged, but significant shifts in allelic frequency occurred over time, likely resulting from the impact of breeding practices. Old barley cultivars grouped separately from the remaining accessions. As the DArT similarity matrices correlated well with similarity matrices based on agronomical and chemical data, the authors concluded that the DArT method accurately reflected the genetic basis of traits of the investigated barley cultivars. Thirty-one varieties and breeding lines were used to evaluate genetic diversity in rye (*Secale cereale*). All varieties clustered together, while more diversity was observed among breeding lines (Bolibok-Bragoszewska et al. 2009). Mace et al. (2008) developed a DArT platform to investigate genetic diversity in sorghum (*Sorghum bicolor*). They analyzed 90 accessions representing a significant portion of genetic variation in sorghum and showed that they were well separated upon DArT genotyping. Thirteen main clusters were revealed, reflecting the race and origin of accessions grouped in the clusters, as well as their status as B (maintainer female) or R (male parental restorer). Research on wheat and oat is outlined in the section devoted to polyploid species.

One of the early projects aiming at the development of a microarray-based platform was carried out in eucalyptus (Lezar et al. 2004). Twenty-three *Eucalyptus grandis* trees were fingerprinted with a set of 384 arbitrary clones, of which 104

identified polymorphisms. Seventeen full-sib trees could be unequivocally identified on the basis of the assay.

Xia et al. (2005) developed and validated a DArT platform for cassava (*Manihot esculenta*) and investigated genetic diversity among 38 accessions, including wild relatives. It successfully revealed genetic diversity and separated wild accessions from cultivars. Subsequently, Hurtado et al. (2008) used the above-described cassava DArT array to analyze genetic diversity of 436 cassava accessions of African and Latin American origin. While the separation of groups of accessions originating from different continents was revealed with 251 DArT polymorphisms, the expected within-continent genetic diversity could not have been precisely defined.

Several projects on genetic diversity utilizing DArT markers were carried out in legumes. Hang Vu et al. (2012) developed DArT platforms for soybean (*Glycine max*) and mungbean (*Vigna radiata*). The mungbean array was used to elucidate genetic relationships within the genus *Vigna*. Eleven *Vigna* accession were grouped into three clusters, corresponding with *Vigna* sub-genera. Interestingly, a possibility of marker transferability between the *Vigna*- and *Glycine*-specific arrays was reported, allowing their potential use for comparative genomic studies. Briñez et al. (2012) used the DArT system to study genetic diversity in 89 accessions of common beans (*Phaseolus vulgaris*). The two major gene pools of common beans were distinguished and the accessions were classified as either Andean or Mesoamerican.

Application of DArT markers allowed differentiation of 92 hop accessions into two genetically differentiated groups comprising European and North American accessions and a separate group of hybrid cultivars derived from crossings between representatives of the former two groups. Genetic diversity in both geographic groups was similar, while the hybrids showed greater diversity (Howard et al. 2011).

Risterucci et al. (2009) developed a DArT platform for two *Musa* species, *Musa acuminata* and *Musa balbisiana*, donors of A and B genomes, respectively, for cultivated sweet and cooking bananas, most of which are triploids. They analyzed a panel of 168 genotypes and found clear differentiation between the two genomes with further differentiation of *M. acuminata* into two groups, one including mostly wild and the other—mostly cultivated accessions. Grouping of the triploid cultivated forms depended on their constitution; separate groups comprising AAA, AAB, and ABB genomes were revealed. Sub-clusters representing breeding histories and geographic origin were also observed. In another study using DArT markers in *Musa*, Amorim et al. (2009) investigated genetic diversity in a group of 42 carotenoid-rich diploid, triploid, and tetraploid banana accessions. They were divided into two major clusters which did not differentiate diploid and polyploid accessions. Also, no relationship between grouping and carotenoid content was observed.

Domínguez-Garcia et al. (2012) used a collection of 87 olive (*Olea europaea*) accessions representing genetic diversity of the species to develop a DArT platform. In order to validate the array they evaluated genetic diversity in a subset

of 62 accessions, and subsequently Atienza et al. (2013) used the same tool for a large-scale study comprising 323 olive cultivars. Both studies showed the utility of the DArT platform for fingerprinting olive genetic resources. High level of genetic diversity in olive genetic resources is revealed and several duplicated accessions were identified. It was possible to use the olive array to analyze genetic diversity in 42 accessions of wild olive.

Following development of a DArT platform constructed from 107 accessions of *Brassica napus* var. *oleifera* and *Brassica rapa*, Raman et al. (2012) investigated genetic diversity in 89 accessions of rapeseed and 32 accessions of other diploid and tetraploid brassicas, i.e., *B. rapa* (AA), *Brassica juncea* (AABB), and *Brassica carinata* (BBCC). Rapeseed cultivars of the same origin or pedigree tended to form separate groupings within three main clusters. The array was also useful for differentiating species, separating also winter and spring types in the *B. napus* cluster.

A DArT array for carrot was developed by Grzebelus et al. (2014) and used to evaluate genetic diversity in a collection of wild and cultivated accessions of *Daucus carota*. Three major clusters were differentiated, grouping wild, Eastern cultivated, and Western cultivated accessions, which reflected domestication and breeding history of the species. In addition, a subset of DArT markers showing signatures for selection upon domestication was identified.

11.3.1 Performance of the DArT System in Complex Polyploid genomes

The presence of multiple copies of genes in polyploids is prohibitive for many genotyping systems. It was shown that DArT markers can efficiently genotype large polyploid species. DArT markers were effectively applied to genotype the 16Gb hexaploid genome of bread wheat and to analyze intraspecific diversity in *Triticum aestivum* (Akbari et al. 2006). Two separate groupings of European and Australian cultivars were observed in a collection of 62 wheat cultivars, the latter groups being more diverse and having a broader range of adaptation. Crossa et al. (2007) used the wheat DArT array developed by Akbari et al. (2006) to study associations with several traits of agronomic importance. They used two collections of 76 and 94 accessions and revealed a fine population structure of 17 and 15 subpopulations, respectively. The research allowed identification of many new chromosome regions for disease resistance and grain yield in the wheat genome. Badea et al. (2008) evaluated a collection of 87 spring and winter wheat accessions for diversity with respect to resistance to fusarium head blight. They identified six clusters which generally agreed with the origin, growth habit, and pedigree of the studied accessions. White et al. (2008) performed a detailed analysis of spatial and temporal changes of genetic diversity in a collection of 240 wheat varieties of UK, US, and Australian origin. The country of origin accounted for ca. 20 % of the total variation revealed by DArT markers. The highest diversity was observed

in the Australian subset, while the lowest was reported for the UK subset. The D genome occurred to be slightly less diverse than the A and B genomes. Moreover, an upward trend in diversity in the US was noticed, while diversity in Australian and UK varieties remained relatively constant.

Genetic diversity in oat (*Avena sativa*) was analyzed with a set of 182 accessions collected worldwide. Two major groups were observed, comprising spring and winter cultivars, while a finer structure of genetic diversity was attributed to geographic origin and breeding history, with subgroups related to known pedigree structure (Tinker et al. 2009). Baird et al. (2012) investigated genetic diversity in another allohexaploid species of Poaceae, tall fescue (*Festuca arundinacea*). By comparing 97 accessions of turf-type tall fescue with 14 accessions of forage type they concluded that genetic diversity in the turf type was very low and should urgently be broadened.

The DArT system was used to study diversity in sugarcane (*Saccharum* sp.) carrying a polyploid, very complex, and particularly challenging genome. The investigation of 16 genotypes of different pedigree and two modifications of the complexity reduction method revealed high genetic differentiation of sugarcane. The ancestral species of *Saccharum spontaneum* and *Saccharum officinarum* were separated from the rest of the samples (Heller-Uszynska et al. 2011).

11.3.2 Applications of the DArT System to Minor Crops, Wild Crop Relatives and Wild Species

The fact that the DArT platform, unlike other high-throughput genotyping technologies, does not rely on any prior sequence information, facilitates its use in species of little or no agronomic importance. Research on lesquerella (*Physaria* spp.), an alternative oil crop, is an example of the successful application of DArT markers for investigating genetic diversity in the group of novel crops. The DArT platform allowed differentiation of 89 accessions with respect to their species, geographic origin, and breeding status. It also revealed that a substantial genetic diversity was present in *Physaria fendleri* from which several breeding lines have been produced and could be commercialized (Cruz et al. 2013).

Pigeon pea (*Cajanus cajan*) is a representative of a group called ‘orphan’ crops, i.e., a domesticated species of low economic value and limited financial resources allocated to its breeding and conservation, which requires careful calculation of ‘per data point’ genotyping costs. Yang et al. (2006) developed a DArT platform for pigeon pea and evaluated genetic diversity in a set of 232 accessions of *C. cajan* and its wild relatives. Genetic diversity among the cultivated accessions was very low, with only 64 of nearly 700 markers being polymorphic in the cultivated germplasm, indicating a very narrow genetic base. No clear genetic diversity structure was observed in the cultivated group. In contrast, higher diversity was revealed in the group of wild accessions which were grouped according to the

species. The authors concluded that the DArT system is an inexpensive genome profiling technology that is likely to contribute significantly to the effective utilization of genetic diversity in 'orphan' crops, such as pigeon pea.

Studies on wild crop relatives can be based on existing DArT platforms developed for the related crop. Genetic diversity in *Aegilops tauschii*, a wild species and a donor of the D genome of wheat, was investigated. Sohail et al. (2012) used 5500 preselected clones from a DArT array developed for wheat, and added 2000 clones obtained de novo from 81 accessions of *A. tauschii*. Almost 70 % markers from the wheat DArT array were polymorphic, while only 34 % of the newly developed *A. tauschii*-specific clones revealed polymorphisms in the diversity collection. A relatively high level of intraspecific genetic diversity was observed. Three groups were observed, generally reflecting their geographic origin and also, at least to some extent, their classification into subspecies. The research allowed identification of accessions that could contribute tolerance to abiotic stresses for wheat breeding. A DArT platform was also developed for einkorn wheat (*Triticum monococcum*) closely related to *Triticum urartu*, a donor of the A genome of the hexaploid wheat. Genetic diversity of 16 *T. monococcum* accessions revealed population structure partially correlating with their genetic and geographic origin (Jing et al. 2009).

Wild *Solanum* species, *Solanum bulbocastanum* and *Solanum commersonii*, close relatives of potatoes and tomatoes, were investigated using the DArT system and revealed a fine microscale genome structural divergence between wild and cultivated species in Solanaceae (Traini et al. 2013).

Applicability of the DArT system is not limited to higher plants. James et al. (2008) developed DArT platforms for genotyping a diploid fern *Asplenium viride* and a haploid moss *Garovaglia elegans*. Sixteen accessions representing each species were investigated for genetic diversity with respect to substrate specificity and geography, respectively. It was shown that intraspecific diversity structure revealed by DArT markers could have been explained by substrate specificity and phylogeographic patterns. The authors indicated possible applications of DArTs in evolutionary investigations, e.g., adaptive radiations, population dynamics, hybridization, introgression, ecological differentiation, and phylogeography.

11.4 Perspectives

The DArT system effectively complements existing technologies in breeding and genomics, especially for crops with limited resources. Diversity Array Technology markers have been developed for a substantial number of plant species. For some species, projects utilizing DArT markers initially aimed at the development of an efficient tool for genetic mapping and the resulting platforms have not yet been used to study genetic diversity. For the list of available species-specific DArT platforms see www.diversityarrays.com.

With respect to genetic diversity investigations, many authors reported that DArT markers provided information on genetic diversity comparable to or exceeding that achievable with other marker systems, often showing greater discriminatory power which likely could have been attributed to a relatively high number of identified polymorphisms, compared to low throughput systems. However, a few exceptions from this general trend should be mentioned. Hurtado et al. (2008) compared performance of 36 SSR markers, with that of ca. 1000 DArT markers and concluded that the former were relatively better at detecting genetic differentiation in cassava germplasm collections. Generally, DArT markers were reported as having relatively high polymorphism information content (PIC) values, however, they were usually slightly less effective compared to SSR markers, e.g., in hop, DArT markers were less polymorphic and had lower PIC than other marker systems (Howard et al. 2011).

In recent years, several novel high-throughput genotyping strategies were developed. They are based on advantages provided by next-generation sequencing (NGS) platforms (reviewed by Davey et al. 2011) and are highly competitive with respect to 'per data point' cost efficiency. Recently, a modification of the DArT system utilizing NGS rather than hybridization to microarrays for polymorphism detection, called DArTseq™, was proposed. It combines the efficient protocol for genomic complexity reduction employed in the conventional DArT system and the power of genotyping-by-sequencing (GBS) approach based on Illumina short read sequencing. As a result, two score tables are produced, comprising DArT and SNP polymorphisms. It proved to be highly efficient in two recent reports on lesquerella (Cruz et al. 2013) and rice (Courtois et al. 2013), resulting in almost 28,000 and almost 17,000 revealed polymorphisms, respectively. In rice, it was shown that the markers covered the genome relatively evenly (Courtois et al. 2013).

On the other hand, a simple assay for site-specific genotyping may be required for only a few sites of special interest, identified as polymorphic using the DArT system. In principle, any DArT clone can be readily sequenced and used to develop a codominant site-specific marker. An example of the DArT marker conversion protocol was recently reported by Macko-Podgórni et al. (2014) who converted one of several DArT markers differentiating wild and cultivated carrots. A general strategy for the development of DArT marker-derived cleaved amplified polymorphic sequence (CAPS) markers involves the following steps: (1) clone sequencing, (2) mapping on the reference sequence and identification of *Pst*I restriction sites flanking the clone, (3) PCR amplification of fragments comprising both restriction sites with pairs of site-specific primers, (4) digestion of PCR products with *Pst*I, and (5) separation by gel electrophoresis (Fig. 11.2). Upon identification of the restriction site comprising the causative polymorphism, the same protocol can be used for routine site-specific genotyping.

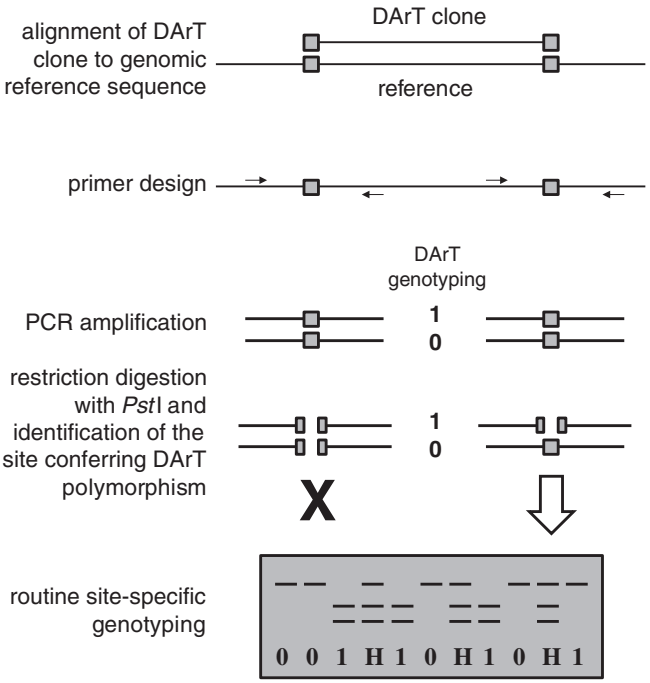

Fig. 11.2 A strategy for conversion of DArT markers into PCR-based codominant site-specific CAPS markers typed by means of gel electrophoresis. *Gray boxes* represent *Pst*I restriction sites, *arrows* represent primers, '*0*', '*1*', and '*H*' indicates genotyping scores

11.5 Conclusions

Diversity Arrays Technology was the first high-throughput genotyping platform allowing for parallel detection of hundreds to thousands of polymorphisms in a single assay. It facilitated investigations on genetic diversity in many plant species, representing both major and minor crops, and utilization of genetic resources in breeding programs. Despite the fact that DArT markers are binary (i.e., scored as 'present' vs. 'absent') and dominant, they can be identified in large numbers, resulting in the high discriminatory power. DArT remains a method of choice, in particular for researchers and breeders working with less-studied crops, e.g., those minor on a global scale, but important for local food security (Varshney et al. 2010). Recent technical advances based on the incorporation of the genotyping-by-sequencing approach into the DArT system (DArTseq™) broaden the possibilities of the technology in the era of NGS.

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Chapter 12
Exploring the Potential of Genetic Diversity via Proteomics: Past, Present, and Future Perspectives for Banana

S. Carpentier

Abstract KU Leuven is hosting the Global Collection of banana (*Musa* spp.) managed by Bioversity International for safe storage and distribution. Our mandate is to secure the crop's gene pool and encourage its use. The latter, however, requires an in-depth knowledge of the variability among the varieties and their potential. Most edible varieties are sterile and triploid involving the parental A genome of *Musa acuminata* and/or the parental B genome of *Musa balbisiana*, with hybrid genomes (AAA, AAB or ABB). A very efficient way of characterising the genetic diversity in search of interesting traits is analysing the different genomes via next generation sequencing (NGS) techniques. However, population-based associations to the genome are challenging in banana and need to fall back on crossing fertile inedible diploids. Moreover, proteins and metabolites are the main determinants of a trait/phenotype and finding correlations between the genome or transcriptome and a phenotype can be quite challenging. Therefore, proteomics is quite complementary to characterize the biodiversity and find correlations between a phenotype and the genotype. To characterize and evaluate *Musa* varieties belonging to different genomic groups and exploring their potential, we have been optimizing proteomics techniques over the years. This chapter gives a brief overview of what proteomics is, its challenges and recent improvements, and applications of proteomics approaches used in banana research.

Keywords Genetic diversity · Proteomics · Hybrid genomes · *Musa balbisiana* · *Musa acuminate* · A genome B genome

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12.1 Introduction

Bananas and plantains (*Musa* spp., collectively called bananas) are staple foods for millions of people living in the humid tropics. The cultivated banana is a sterile, parthenocarpic plant selected by early farmers in Southeast Asia, and thereafter maintained by vegetative propagation (Heslop-Harrison et al. 2007). Most cultivated banana varieties are triploid and originated from intra- and interspecific hybridizations between seed-bearing parents of *Musa acuminata* (A genome donor) and *M. balbisiana* (B genome donor) (Simmonds and Sheppard 1955). Bananas are classified on the basis of their presumed genome constitution. The morphology of many varieties is biased toward either the A or the B phenotype. The most common types are classified as AA, BB, AB (diploids) and AAA, AAB, ABB (triploids) but often this does not conform to predictions based on these genomic formulae. Many hybrid banana genomes are unbalanced with respect to the parental donor ones (De Langhe et al. 2010). Inter-genome translocation of chromosomes and epigenetic silencing are thought to be quite common in *Musa*. A very efficient way of characterising the genetic diversity is analysing the different genomes via next generation sequencing (NGS) techniques. However, population-based associations to the genome are challenging in banana and need to fall back on crossing fertile inedible diploids. Moreover, proteins and metabolites are the main determinants of the phenotype and finding correlations between the genome or transcriptome and a phenotype can be quite challenging. Therefore, proteomics is quite complementary to the NGS techniques to characterize and evaluate the biodiversity. We (at KU Leuven) are hosting the global collection of banana (>1400 accessions) managed by Bioversity International for safe storage and distribution as an in vitro and cryopreserved collection (Van den houwe et al. 1995; Panis et al. 1996). Our mandate is to secure the crop's gene pool and encourage its use. The latter, however, requires an in-depth knowledge of the variability among the varieties and their agricultural potential. We, at KU Leuven, are engaged to characterize and evaluate the biodiversity with a current focus toward drought tolerance. It is assumed that the contribution of the B genome leads to greater drought tolerance (Ekanayake et al. 1994; Thomas et al. 1998; Vanhove et al. 2012). This is attributed to the fact that *M. balbisiana* originated from more drought-prone monsoon regions in Southeast Asia unlike *M.acuminata* that originated from Asia's humid forest zones. To characterize and evaluate the different *Musa* varieties belonging to different genomic groups and exploring their potential toward drought tolerance, we have been optimizing proteomics techniques over the years. Biological research has mainly focused in the past on model organisms and most of the functional genomics studies in the field of plant sciences are still performed on models or reference species that are characterized to a great extent. The power of -omics tends to be lost in such orphan species due to the lack of genomic information, the complexities of the genome are due to the sequence divergence to a related sequenced reference variety or to a related model organism. This chapter gives a brief introduction to proteomics, provides an overview

of recent applications of proteomics approaches used in banana research and discusses the challenges of applying such an omics approach to a non-model crop.

12.1.1 Understanding Biodiversity, Gene Function, and the Underlying Molecular Processes

Understanding gene function and gene expression profiling can be approached via several techniques: genomics, transcriptomics (messenger, structural, and regulatory RNA's), proteomics (proteins and peptides), and metabolomics (primary and secondary metabolites). Each technique focuses on a subset of the biological interaction network and each technique has its strong and weak points. In prokaryotes, gene finding is essentially a matter of identifying open reading frames. As genomes get larger, it becomes increasingly complicated. Several sophisticated software algorithms have been designed to handle gene prediction in eukaryotic genomes. Despite considerable progress, gene prediction, entirely based on DNA analysis, is cumbersome and needs support from "functional genomics". Indeed, genomics focuses on the static aspects of genome information. Gene prediction and annotation in a reference variety (D'Hont et al. 2012; Davey et al. 2013) was for banana a giant step forward but by far not sufficient to get insight into the agricultural potential of the biodiversity. Functional genomics (transcriptomics, proteomics, and metabolomics) deals with dynamic aspects and describe the functions and interactions of genes that may cast a view in the agricultural potential of a variety.

12.1.2 Proteomics

"Proteomics is the endeavour to understand gene function and to characterize the molecular processes of the living cell through the large-scale study of proteins found in a specific biological/physiological context" (Liska and Shevchenko 2003). The word "proteome" was for the first time used by Marc R. Wilkins in 1994 at the Siena 2-D Electrophoresis meeting and indicates the PROTEins expressed by a genOME. The proteome is the complete set of proteins present in a cell, tissue or organism at a specific time point under specific conditions. Electrophoresis and protein analysis emerged both from biochemistry and have a long history. The basic principles for the electrophoresis methodology were established by the Nobel Price winner Tiselius (Tiselius 1937). By 1975, procedures were developed to separate complex protein mixtures from whole cells or tissues on the basis of charge in the first dimension followed by molecular mass in the second dimension (Klose 1975; O'Farrell 1975; Scheele 1975). Contrary

to genomics and transcriptomics, proteomics has to fight the prejudice of being a slow and cumbersome art. The discovery of the soft ionization techniques for mass spectrometry by the Nobel Prize winners J. Fenn and K. Tanaka, the coupling of mass spectrometry to liquid chromatography and the genomic and computational advances (Karas and Hillenkamp 1988; Fenn et al. 1989; Henzel et al. 1993; McCormack et al. 1997) made high-throughput large-scale analysis of proteins feasible. Finally, after a significant lag phase, proteomics became an important research tool for model organisms.

Two approaches are generally distinguished in the field of proteome analysis: a protein-based approach (in general referred to as gel-based approach), and a peptide-based approach (in general referred to gel-free or as shot gun approach). In the protein based approach, proteins are separated and quantified. The proteins of interest are then digested and the resulting peptides are identified via mass spectrometry. In the peptide based approach, protein digestion precedes the separation and quantification of peptides. After separation through two-dimensional gel electrophoresis (2DE) several hundreds of individual protein abundances can be quantified in a cell population or sample tissue (Fig. 12.1). Unfortunately the technique has some major drawbacks, i.e., it has a very poor performance regarding the analysis of hydrophobic and basic proteins and is limited in throughput. Yates and colleagues were one of the pioneers to explore the use of liquid chromatography

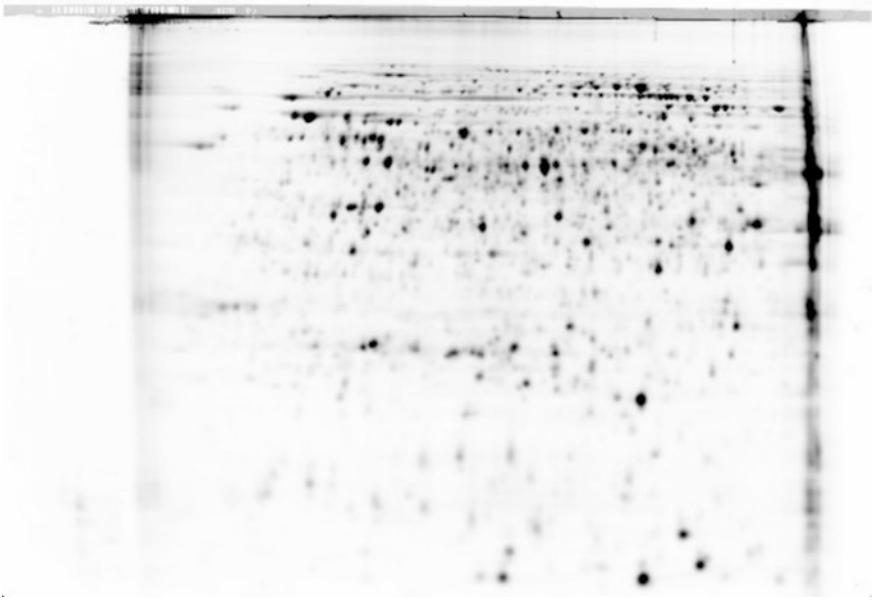

Fig. 12.1 Representative 2DE gel of banana proteins. Proteins were labeled with CY3 and separated on a 24 cm p*I* 4–7 cm IPG strip. Proteins are separated according to their isoelectric point (*pI*) *left* to *right* (*pI* 4–7) and molecular mass top–down (150–15 kDa) and are here recognized as spots

coupled to electrospray ionization tandem mass spectrometry (LC/MS/MS) to realize automated high-throughput proteomics (McCormack et al. 1997; Ducret et al. 1998; Link et al. 1999). In general, most approaches use a bottom-up strategy where proteins are first digested with a proteolytic enzyme and the obtained complex peptide mixture is then separated via reversed-phase (RP) chromatography coupled to a tandem mass spectrometer. The vast amount of acquired tandem mass spectra is then used to search protein databases and to link the peptides to the original proteins. However, this concept was only successful when identifying proteins in simple mixtures. The problem of resolution was anticipated by multidimensional chromatography (Link et al. 1999; Washburn et al. 2001). Although this was a great improvement, proteolytic digests of a higher eukaryotic proteome exceeded the analytical capacity of most mass spectrometers. During recent years, mass spectrometers have developed toward higher mass accuracy, resolving power, sensitivity, scan speed, reproducibility, and lower detection limits (Domon and Aebersold 2006; Mann and Kelleher 2008). For example, the use of hybrid LTQ-Orbitrap devices (Makarov et al. 2006; Olsen et al. 2009), high energy C-trap dissociation (Olsen et al. 2007), parallel reaction monitoring (Peterson et al. 2012), coupling of a quadrupole mass filter to an Orbitrap analyzer (Michalski et al. 2011; Kelstrup et al. 2012), UPLC combined with MSE (Plumb et al. 2006), and combining quadrupole, Orbitrap and ion trap mass analysis (Lebedev et al. 2014), all contributed to improvements in proteomics experiments, and in particular towards better peptide identifications and quantifications. While 2DE is in sequenced organisms currently no longer the tool of choice in high-throughput differential proteomics, it is still very effective to identify and quantify protein species caused by genetic variations, alternative splicing, and/or PTMs.

12.1.2.1 Banana General Proteomics

Large-scale gene expression profiling based on transcripts in non-model plants like banana were quite challenging at the time there was no reference genome. The power of those techniques, i.e., high-throughput identification of candidate genes, was lost due to the lack of genomic information. Gene sequences are rarely identical from one species to another and orthologous genes are normally riddled with nucleotide substitutions. A welcome alternative for examining gene expression at that time was 2DE (Carpentier et al. 2008a). Functional protein domains are well conserved making the identification of non-model gene products with comparison to well-known orthologous proteins quite efficient. Intact proteins are essential for a good and reliable identification, making high-throughput shot gun proteomics difficult. An approach based on the isolation and analysis of individual proteins prior to identification, has a much higher success rate and was at that time our approach to understand gene function and the underlying molecular processes of acclimation in non-model plants such as different banana varieties (Carpentier et al. 2008b). Protein separation and analysis via 2DE prior to MS analysis ensures the connectivity between the peptides and significantly reduces the complexity.

Irrespective of the proteomics method, protein sample preparation is a critical step and is absolutely essential for obtaining good results. Most plant tissues are not a ready source and need specific precautions. Most common interfering substances are phenolic compounds, proteolytic and oxidative enzymes, terpenes, pigments, organic acids, inhibitory ions, and carbohydrates. Banana contains extremely high levels of oxidative enzymes (polyphenol oxidase), phenol compounds (simple phenols (dopa), flavonoids, condensed tannins, lignin), and carbohydrates and high levels of terpenes, suberin, and waxes (Gooding et al. 2001). Moreover, the vascular bundles of a banana plant are associated with latex vessels that are also linked with the presence of polyphenols. A first step to optimize was the protein extraction. Extraction and analysis protocols were optimized for 2DE, LCMSMS, and for membrane proteins (Carpentier et al. 2005, 2007, 2009; Vertommen et al. 2010). Second step was the optimization of the protein identification via de novo identification, cross species peptide connection, and spectral library searching (Samyn et al. 2007; Carpentier et al. 2011a; Vertommen et al. 2011a, b; Buts et al. 2014; Carpentier and America 2014).

12.1.2.2 Characterization of the Banana Biodiversity: Detection of Paralogous and Homeologous Peptides via 2DE and MS/MS

As indicated above, our strategy to characterize the banana biodiversity and to detect protein isoforms at the time that the banana genome was not sequenced, was to start from a protein-based approach. We combined two-dimensional electrophoresis (2DE) and 2D DIGE with the derivatization of peptides for easy de novo MS/MS sequence determination (Carpentier et al. 2011a). Variety-specific proteins can only be distinguished if they differ in a manner that affects gel migration being molecular mass or isoelectric point. Two-dimensional difference gel electrophoresis (2D DIGE) has the advantage to use fluorophores that have a different absorption optimum, making it possible to run two samples simultaneously in the same gel (Unlu et al. 1997). Thanks to the principle of the internal standard (Alban et al. 2003) (being a mixture of all samples) all Cy2 images contain both isoform types. To characterize a range of triploid varieties and to detect typical acuminata and balbisiana encoded isoforms and possible deletions or silencing, a 2DE analysis has been performed on different triploid varieties classified as AAA, AAB, ABB, and a presumed BBB. Through Principal Component Analysis (PCA) and hierarchical clustering, we were able to blindly classify the different varieties according to their presumed genome constitution. To obtain an insight into the origin of the different protein polymorphisms, we also characterized the diploid wild type parental varieties acuminata (AA) and balbisiana (BB). Using this simple and cheap technique it was immediately clear that some of the A specific proteins were missing in the ABB variety pointing toward gene deletion or gene silencing (Henry et al. 2011) and that the BBB variety contained some A specific proteins, suggesting that backcrossing with an acuminata ancestor might have taken place. An example of a B specific protein is given in Fig. 12.2. We could conclude that

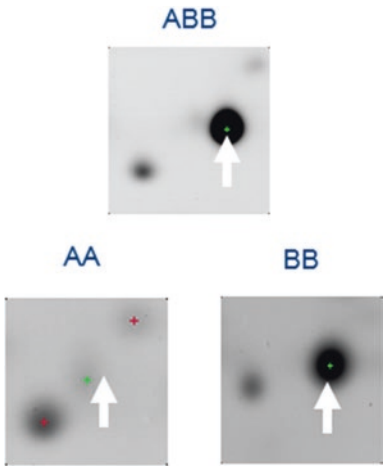

Fig. 12.2 Illustration of a stress-related protein encoded from the balbisiana allele. The protein shown is Abscisic acid-Specific Ripening (*ASR*) protein in the ABB variety and its presumed diploid parents AA and BB

the proteome does not necessarily correspond to the presumed genome formulae. The observations at the protein level provide additional indication for a more complex genome structure and genomic rearrangement in some banana varieties as we expected (De Langhe et al. 2010). This approach was very useful to recognize variety-specific proteins. To identify those proteins, the unique peptides need to be identified via de novo sequencing. Using an automated approach for the derivatization of peptides for MS/MS de novo identification we were able to speed up this process and we reported to our knowledge for the first time the use of proteomics to characterize the genetic diversity and discover possible genome rearrangements (Carpentier et al. 2011b).

12.1.2.3 The Importance of Genomic Information

The NGS techniques have high throughput and are currently affordable. But NGS produce short sequences, which is a major bottleneck for de novo assembly of the reads (Schatz et al. 2012). The double haploid *Musa acuminata* reference genome Pahang, a *Musa acuminata* ssp. malaccensis genotype (AA) was published in 2012 (D'Hont et al. 2012). A year later, the publication of a draft *Musa balbisiana* genome followed (Davey et al. 2013). This corresponding B genome was obtained by sequencing a wild diploid *Musa balbisiana* genotype, 'Pisang Klutuk Wulung', and mapping the reads to the A genome. Those reference genomes are extremely important for the analysis of the genetic diversity. It allows that an analysis can be made on the paralog and allele level. 2D gels often contain multiple spots that are functionally annotated identically. Without a sequenced genome, it is almost impossible to go beyond this general annotation. Different protein isoforms expressed from the same genome are called paralogs as they may have arisen from gene or segmental duplication. While comparing gene isoforms at the same

location but on different genomes, in this case A and B, the term allelic variants are used. After construction of an in-house database composed of non-redundant A and B encoded proteins, we went beyond the level of simply "identifying" the proteins of interest during osmotic stress experiments in an ABB variety and focused on an important stress family: the cytoplasmic HSP70s. (Vanhove et al. 2015). Using a 2D-DIGE LC MS/MS approach, we were able to measure a proteotypic peptide for each paralog and have identified a particular paralog that specifically reacted to the osmotic stress in roots and meristems. The paralog is located on chromosome 2 and the promoter region contains a paralog unique ABRE element.

Gel-free differential proteomics relies on mass spectrometry for both quantification and identification. As indicated above, the major disadvantage of this approach lies in the disconnection between the protein and its peptides. A protein sample containing several thousands of proteins is digested and all these peptides are analysed at once. This leads to both identification and quantification problems especially in the case of higher eukaryotes such as banana with complex polyploid genomes and big protein families. Peptides shared between several proteins do not contribute to the conclusive identification of a particular protein. This is the so-called protein inference problem (Nesvizhskii and Aebersold 2005). Unique peptides need to be measured and identified for final protein identification and quantification. So, the gel-free approach for banana became only practicable;

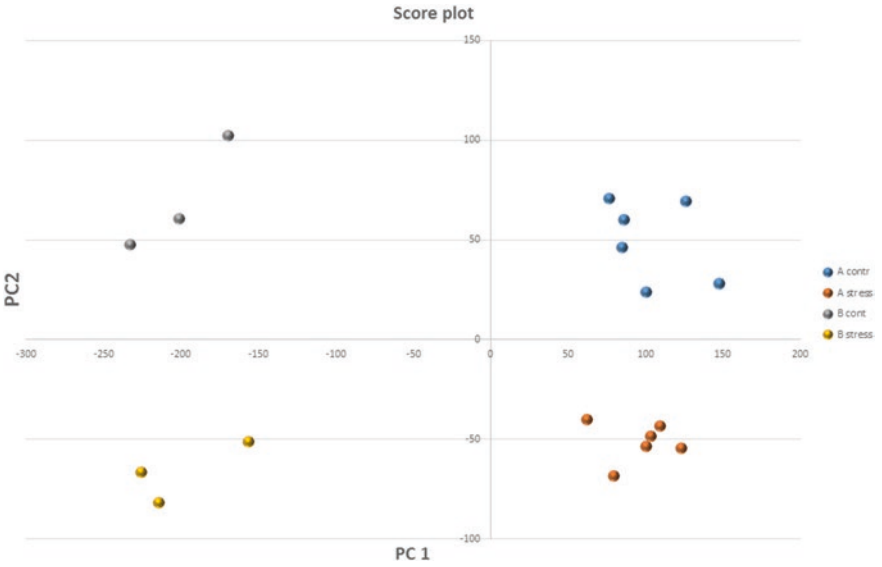

Fig. 12.3 Principal Component Analysis (*PCA*) score plot. Analysis of two AAA varieties (*blue* and *red*) and one ABB (*gray* and *yellow*) variety in triplicates under control (*gray* and *blue*), and stressed (*yellow* and *red*) conditions. Principle Component (*PC*) *1* separates the ABB variety from the *two* AAA varieties. PC 2 separates the control samples from the stressed samples. This enables us to select the peptides that are specific to the balbisiana allele and acuminata allele that specifically are correlated to control or stress

thanks to the publishing of the reference genomes. While 2DE may no longer be the tool of choice in high-throughput differential banana proteomics in the future, it is still very effective to identify and quantify protein species caused by genetic variations, alternative splicing and/or PTMs and it will give a complementary view on the proteome. Protein families and isoforms/PTMs have many common peptides when digested but often have small detectable differences in pI and/or mass. The complexity in a single spot is much lower than in a gel-free blind high-throughput approach where finding the low-abundant differential peptides is still challenging in the case of large protein families.

12.1.2.4 Characterization of the Banana Biodiversity: Detection of Genome-Specific Peptides via LCMSMS

As indicated above, the sequencing of the reference varieties was a huge step forward and make gel free proteomics quite efficient also now in banana. In the gel-free approach, proteins are digested into smaller peptides that are separated, analysed, and reconstructed into proteins based on the genome prediction. Absolute or relative peptide quantification can be performed using one of the available labeling approaches (Gygi et al. 1999; Ong et al. 2002; Gerber et al. 2003; Thompson et al. 2003). Label-free techniques either rely on spectral

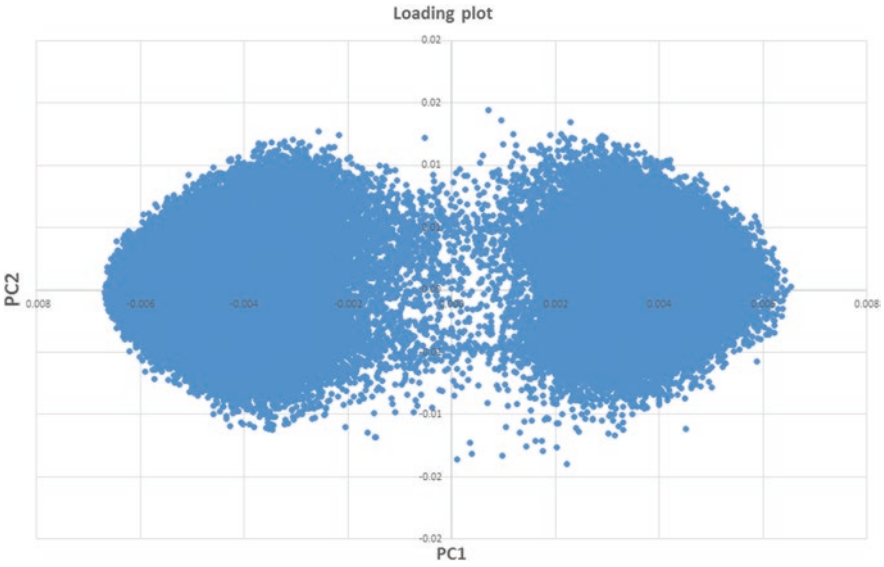

Fig. 12.4 PCA score plot. The score plot shows the four quadrants with peptides that are correlated to the AAA types and control (first quadrant upper *right*), to the ABB type and control (second quadrant upper *left*), to the ABB type and stress (third quadrant lower *left*), and to the AAA types and stress (fourth quadrant lower *right*)

counting (Liu et al. 2007), or the use of averaged and normalized ion intensities (Chelius and Bondarenko 2002). To discover allele-specific differences that can be correlated to stress in a high throughput and cheap manner, we have separated the digested proteins of an ABB, an AAA Cavendish type, and an AAA highland type under control and stressed conditions via liquid chromatography and have quantified the peptides label free based on averaged, normalized ion intensities. Principle component analysis is able to separate the ABB variety from the two AAA varieties and separates the control samples from the stressed samples (Fig. 12.3). This enables us to select the peptides that are specific to the balbisiana or acuminata allele that specifically are correlated to control or stress (Fig. 12.4). This is an excellent alternative for Quantitative Trait Locus (QTL) or Genome Wide Association Studies (GWAS). Almost all edible bananas are parthenocarpic and sterile and are clonally propagated, making those studies very challenging.

12.2 Conclusions

We conclude that proteomics has a great potential to explore the genetic diversity and to discover genotype-specific alleles that can be correlated to a certain trait/phenotype. It is an excellent alternative for the studies carried out at the genomic level such as QTL or GWAS. In the past, 2DE was the cornerstone of proteomics; in the current situation with two reference genomes sequenced and the future with variety-specific mRNA sequence libraries becoming more available, the gel-free peptide-based LC MSMS is the most powerful way to go. 2DE will still be a powerful technique if one wants to focus on particular large gene families and specific posttranslational modifications.

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