Chapter 7
Genetic Differentiation and Crop Evolution of Foxtail Millet

Kenji Fukunaga

Abstract Several studies on genetic differentiation and crop evolution based on intraspecific hybrid pollen semi-sterility, isozymes, ribosomal DNA (rDNA) RFLP, nuclear RFLP, mitochondrial DNA (mtDNA) RFLP, RAPD, AFLP, transposon display (TD) markers, and single nucleotide polymorphisms (SNPs) have been carried out to elucidate genetic relationships of foxtail millet accessions, mainly from Eurasia. Most of the studies suggest that China is the center of diversity of foxtail millet and that landraces are grouped in geographical groups. Evolution of two genes, *waxy* gene controlling amylose content in endosperm and *polyphenol oxidase* (*PPO*) gene for phenol color reaction (Phr) in grains, was also reviewed. These analyses showed that multiple independent loss-of-function mutations occurred in each of the two genes under human/natural selection.

Keywords Center of diversity • Crop evolution • Domestication • DNA markers • Geographical variation • Genetic differentiation • Phylogeny • *Polyphenol oxidase* (*PPO*) gene • *Waxy* gene • Foxtail millet • Green foxtail

7.1 Hypotheses on Geographical Origin of Foxtail Millet and Crop Evolution of Foxtail Millet

Foxtail millet, *Setaria italica* (L.) P. Beauv., is one the oldest domesticated cereals in the Old World. Archaeological remains of foxtail millet were found in sites of Peiligang and Cishan near the Yellow River, dating back to ca. 5000–6000 BC (Li and Wu 1996), in prehistoric sites in Europe (Kűster 1984) and in Transcaucasus (Lisitsina 1976). Foxtail millet has been utilized in various ways peculiar to each area of Eurasia (Sakamoto 1987), and it is thought to have played an important role in early agriculture in the Old World.

The geographical origin of foxtail millet is still a controversial issue. Cytological studies indicate that the wild ancestor of foxtail millet is green foxtail (*S. italica* ssp.

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viridis = *S. viridis*) (Kihara and Kishimoto 1942; Li et al. 1945). However, the geographical origin of domesticated foxtail millet cannot be determined from the distribution of ssp. *viridis*, since it is found commonly in various areas in Europe and Asia (and also currently in the New World). Vavilov (1926) stated that the principal center of diversity for foxtail millet is East Asia, including China and Japan. Harlan (1975) suggested independent domestication in China and Europe based on archaeological evidence. The archaeological, isozyme, and morphological evidences (de Wet et al. 1979; Jusuf and Pernes 1985; Li et al. 1995a, b) suggest that China is the center of diversity and origin of foxtail millet but independent origins in other regions of this millet cannot be excluded. Further, Li et al. (1995b) stated that landraces in Afghanistan and Lebanon had been domesticated independently in relatively recent times because they had primitive morphological characters such as several tillers with small panicles and look like ssp.*viridis* but have non-shattering large grains. Molecular analyses support the view that foxtail millet landraces have differentiated into local landrace groups and that China is the center of diversity (isozymes: Jusuf and Pernes (1985); prolamin: Nakayama et al. (1999); rDNA: Fukunaga et al. (1997a, 2006), Schontz and Rether (1998), Eda et al. (2013); RAPD: Li et al. (1998), Schontz and Rether (1999); AFLP: Le Thierry d'Ennequin et al. (2000); genomic RFLP: Fukunaga et al. 2002b, mitochondrial DNA RFLP: Fukunaga and Kato 2003, transposon display; Hirano et al. 2011). Recent archaeological evidence also supports the domestication of foxtail millet in China (Nasu et al. 2007; Hunt et al. 2008). In contrast to the hypothesis of Chinese origin and multiple origin, Sakamoto (1987) suggested that foxtail millet originated somewhere in Central Asia–Afghanistan–Pakistan–India because accessions with less compatibility (Kawase and Sakamoto 1987) and with primitive morphological traits are found there. This hypothesis, which excludes China as a center of origin of foxtail millet, is very different from the others.

In this chapter, we review studies on genetic differentiation of foxtail millet landraces from various parts of Europe and Asia in morphological/agronomic characters, biochemical markers, intraspecific pollen sterility, and DNA markers. We also review evolution of two genes, *Waxy* and *Polyphenol oxidase* (*PPO*), which were selected during domestication and dispersal of foxtail millet.

7.2 Variation in Morphological Characters

Variation in morphological and agronomic characters involved in domestication and diversification of foxtail millet has been described and analyzed: (Dekaprelevich and Kasparian 1928; de Wet et al. 1979; Kawase 1986; Takei and Sakamoto 1987, 1989; Prasada Rao et al. 1987; Ochiai et al. 1994; Li et al. 1995a, b; Ochiai 1996; Fukunaga et al. 1997b; Hammer and Khoshbakht 2007). Some researchers classify foxtail millet landraces into two to four subspecies/races such as moharia, maxima, indica, and nana (de Wet et al. 1979; Prasada Rao et al. 1987; Li et al. 1995b), but criteria for the classification are ambiguous. Kawase (1986) and Ochiai et al. (1994) investigated

variation in morphological/agronomic characters such as plant height, number of tillers, panicle length, and number of days to heading of foxtail millet landraces from Europe and Asia. Nguyen and Pernes (1985) and Li et al. (1995b) also investigated variation of morphological/agronomic characters of foxtail millet landraces by multivariate analyses. These works indicate that morphologically primitive landraces characterized by several tillers with small panicles, which look like ssp.*viridis* but have non-shattering large grains, are distributed in Afghanistan, northwestern Pakistan, Central Asia, and Lebanon whereas most of accessions from other regions such as East Asia have no or a few tillers with large panicle(s). Hammer and Khoshbakht (2007) also reported cultivation of morphologically primitive landraces in Northern Iran. A few researchers insist that foxtail millet was domesticated in Central Asia–Pakistan–Afghanistan–northwest India because the morphologically primitive type was cultivated there (Sakamoto 1987; Ochiai 1996), whereas Li et al. (1995b) insist that the morphologically primitive type with several tillers and small panicles, which look like ssp. *viridis,* was domesticated independently. Description and analyses of morphological variation are important but not sufficient to address questions on geographical origin(s) and phylogeny of foxtail millet landraces.

7.3 Genetic Differentiation of Foxtail Millet Landraces, Revealed by Biochemical and Genetic Markers and Intraspecific Hybrid Pollen Sterility

Several studies have been carried out to clarify the genetic relationships of foxtail millet from Europe and Asia (and partly from Africa) such as (1) biochemical markers (isozymes and prolamin), (2) intraspecific hybrid pollen sterility, and (3) DNA markers (nuclear RFLP, mitochondrial RFLP, RAPD, AFLP, transposon display (TD) markers, and single nucleotide polymorphisms (SNPs)). As summarized in Table 7.1, these studies revealed that foxtail millet landraces differentiated into local geographical groups and that East Asian landraces (in particular Chinese landraces) are the most diverse.

These genetic studies are described as follows:

1. Variation in biochemical markers (isozymes and prolamin)

   Kawase and Sakamoto (1984) investigated polymorphism in two loci, *Est-1* and *Est-2* of the esterase isozymes of 432 accessions of foxtail millet collected from different areas throughout Eurasia by gel isoelectric focusing. On locus *Est-1*, most of accessions had *Est-1 a*, which was widely distributed throughout Eurasia, 9% of accessions had *Est-1 b* which was distributed in China and Korea. On locus *Est-2*, most of accessions had *Est-2 a*, but nine had *Est-2 b*, which is found in all of the accessions from the western part of Europe and in one of the Indian accessions. Six had the *Est-2 c* allele, which was found in Japan and China. They concluded that the distribution of *Est-2 a* and *-2 b* might indicate some degree of phylogenetic differentiation between the Asian and the European accessions and that Chinese accessions showed polymorphisms in both loci.

Table 7.1 Genetic studies on genetic differentiation of foxtail millet landraces and geographical groups and centers of diversity

Genetic markers/ intraspecific hybridity/ pollen sterility	Geographical groups	Center of diversity	References
Esterase isozymes	East Asia vs. Europe	East Asia	Kawase and Sakamoto (1984)
Ten isozymes	China–Korea–Japan, Okinawa (Nansei Islands of Japan)–Taiwan, India–Kenya, Europe		Jusuf and Pernes (1985)
Prolamine	Europe, Tropical Groups	China	Nakayama et al. (1999)
Hybrid sterility	China–Korea–Japan, Okinawa (Nansei Islands of Japan)–Taiwan, Lan-Hsű-Batan Islands India–Afghanistan, Europe		Kawase and Sakamoto (1987), Kawase et al. (1997) and Kawase and Fukunaga (1999)
rDNA	China–Korea–Japan, Okinawa (Nansei Islands of Japan)–Taiwan–the Philippines, India, Afghanistan–Northern Pakistan	China	Fukunaga et al. (1997a, 2006, 2011) and Eda et al.(2013)
Nuclear RFLP	East Asia, Nansei Islands–Taiwan–the Philippines, India, Afghanistan–Central Asia–Europe	China	Fukunaga et al. (2002b)
mtDNA	Not clear	China	Fukunaga and Kato (2003)
RAPD	Central Europe and two Asiatic groups (north and south)		Schontz and Rether (1999)
AFLP	Not clear	China	Le Thierry d'Ennequin et al. (2000)
TD	East Asia, Nansei Islands–Taiwan–the Philippines, India, Central Asia, Europe	China	Hirano et al. (2011)
SNPs	North China, Central–South China, South Asia, Central Asia, Europe, etc.	China	Jia et al. (2013)

Jusuf and Pernes (1985) investigated the genetic diversity of a world collection of foxtail millet accessions and some samples of wild populations (ssp. *viridis*) by means of electrophoresis on five enzymes (ten loci) *Est*, *Acph*, *Got*, *Mdh*, and *Pgd*. They found some genetic groups of foxtail millet in China–Korea–Japan, Okinawa (Nansei Islands of Japan)–Taiwan, India–Kenya, and Europe. They also investigated wild populations collected in France and China and concluded that it is possible that there were independent domestications in both Europe and China because foxtail millet and *S. viridis* accessions were

more closely related in isozyme alleles in each of Europe and China. Nakayama et al. (1999) investigated allelic variation at the two prolamin loci (*Pro1* and *Pro2*) and their geographical distribution in 560 local cultivars of foxtail millet collected mainly from Eurasia and studied using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Two alleles (*Pro1a* and *Pro1null*) at the *Pro1* locus and six alleles (*Pro2a*, *Pro2b*, *Pro2c*, *Pro2d*, *Pro2e*, and *Pro2f*) at the *Pro2* locus were detected among the cultivars examined. No apparent trend in *Pro1* was observed in geographical distribution. In contrast, two common alleles at the *Pro2* locus, *Pro2b* and *Pro2f*, had clear differential geographical distribution. The *Pro2b* allele was most frequent in Europe and decreased in frequency eastwards. The *Pro2f* allele occurred frequently in subtropical and tropical regions including the Nansei islands of Japan, the Philippines, Nepal, India, Pakistan, and Africa. All eight alleles at the *Pro1* and *Pro2* loci occurred in China, suggesting China is a center of diversity. They also found a "tropical group" characterized by the *Pro2f* allele and other genes.

2. Classification by mean of intraspecific hybrid pollen sterility

   Intraspecific hybrid pollen sterility can be a genetic indicator of differences. In rice, classification based on hybrid sterility was carried out (Kato et al. 1928), and Asian rice varieties were classified into two main groups, japonica, and indica types. Kawase and Sakamoto (1987) crossed 83 accessions of *Setaria italica* collected from various areas throughout Eurasia with three tester strains from Japan (tester A), Lan Hsü Island of Taiwan (B) and Belgium (C). The accessions could be clearly classified into six types, designated as types A, B, C, AC, BC, and X. They regarded pollen fertility of more than 75 % as normal. The accessions of types A, B, and C were those that produced F1 hybrids having normal pollen fertility when crossed with testers A, B, and C, respectively. When both F1 hybrids from the crosses with two testers, A and C, or B and C, showed normal pollen fertility, the accession was classified as type AC or BC. The accessions whose F1 hybrids always showed pollen fertility of less than 75 % in all three cross combinations were designated as type X. Kawase et al. (1997) further investigated collections from northern Pakistan. Kawase and Fukunaga (1999) also used a landrace from Lan Hsü Island of Taiwan, which was classified into type X in Kawase and Sakamoto (1987), as tester D, and crossed it with landraces and reclassified type X in Kawase and Sakamoto (1987). Geographical distribution of these different types is shown in Fig. 7.1a. Most type A accessions were distributed in East Asia including Japan, Korea, and China. Type B accessions were narrowly found in Taiwan and in the southwestern part of the Nansei Islands of Japan. Most European accessions were found to be type C. Type D is distributed in Lan Hsü Island of Taiwan and Batan Islands of the Philippines. Accessions of types AC and BC were distributed in Afghanistan and India, respectively. Kawase and Sakamoto (1987) and Sakamoto (1987) concluded that Types AC and BC were less specialized genetically than A, B, or C and that the geographical distribution of these landrace groups suggests that *S. italica* was first domesticated in an area ranging from Afghanistan to India, and then dispersed both eastward and westward from there.

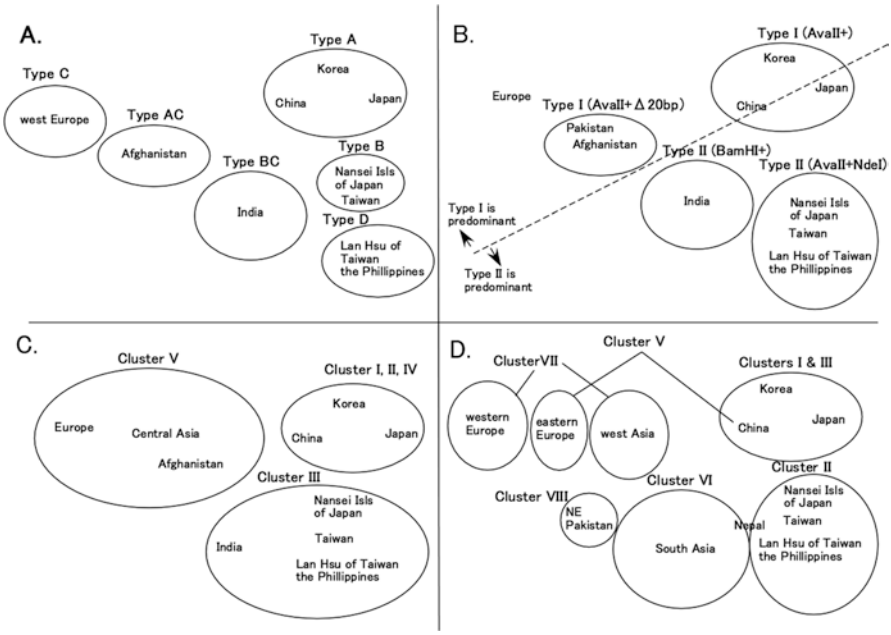

Fig. 7.1 Schematic drawings of geographical distribution of different phylogenetic groups of foxtail millet based on four different analyses such as intraspecific hybrid pollen semi-sterility, rDNA PCR-RFLP, nuclear RFLP markers, and transposon diplay (TD markers). (**a**) Geographical distribution of intraspecific hybrid pollen semi-sterility types (Kawase and Sakamoto 1987; Kawase and Fukunaga 1999). (**b**) Geographical distribution of specific rDNA PCR-RFLP types (Eda et al. 2013). (**c**) Geographical distribution of clusters of a dendrogram showing genetic similarity between 62 accessions of foxtail millet based on 16 nuclear RFLP markers (Fukunaga et al. 2002b). (**d**) Geographical distribution of clusters of a dendrogram based on transposon display (TD) markers (Hirano et al. 2011)

3. DNA markers

(a) Ribosomal DNA (rDNA)

Fukunaga et al. (1997a) investigated restriction fragment length polymorphism (RFLP) and the structure of ribosomal RNA genes (rDNA) in 117 landraces of foxtail millet. Five RFLP phenotypes were found when the genomic DNA was digested with *Bam*HI; these were named types I–V. Of these types I, II, and III were the most frequent. Type I was mainly distributed in the temperate zone, type II in the Taiwan–Philippines Islands, and type III in South Asia. Restriction mapping of the cloned rDNA and comparison with RFLP phenotypes showed that the different types originated from a length polymorphism within the intergenic spacer (IGS) and *Bam*HI site changes within the IGS. Schontz and Rether (1998) also investigated rDNA in a world collection (43 accessions) for variation in repeat unit length and restriction enzyme site variability. They detected two lengths of repeat units of about 7.9 or 7.6 kb; the central European accessions and

most western European accessions have only the 7.6 kb repeat unit and most Asiatic lines have a 7.9 kb repeat unit, while lines originating from the north or the south of Asia showed different numbers of *Bam* HI fragments. These types correspond to types I–III in Fukunaga et al. (1997a). They concluded that the fact that difference between the Asiatic and European pool is not continuous (7.9 or 7.6 kb) excludes the hypothesis of domestication being based on the spread of an initial population over Eurasia. Fukunaga et al. (1997a) suggested that foxtail millet landraces differentiated into mainly two geographical groups, 7.6 kb repeat unit (=type I) from the temperate region and 7.9 kb repeat unit (=types II and III) from the subtropical–tropical region, whereas Schontz and Rether (1998) insisted that foxtail millet landraces differentiated into 7.9 kb repeat unit from Asia and 7.6 kb repeat unit from Europe. The difference in conclusions between these two studies are likely due to difference in number of Asian accessions used. Fukunaga et al. (2005) also determined the sequence of ribosomal DNA (rDNA) intergenic spacer (IGS) of foxtail millet isolated in the previous study and identified subrepeats in the polymorphic region. Fukunaga et al. (2006) sequenced ribosomal DNA intergenic spacer subrepeats and their flanking regions of foxtail millet landraces from various regions in Europe and Asia, as well as its wild ancestor green foxtail, to elucidate phylogenetic differentiation within each of types I–III found in the previous work and to elucidate relationships among these three types. Type I was classified into seven subtypes designated as Ia–Ig based on subrepeat sequences; C repeats downstream of those subrepeats were also polymorphic. Of these, subtypes Ia–Id and Ig were found in foxtail millet landraces. Subtypes Ia and Ib were distributed broadly throughout Asia and Europe. Subtype Ic was distributed in China, Korea, and Japan. Subtype Id has a 20-bp deletion in subrepeat 3 and has a unique C repeat sequence. This subtype was found in a morphologically primitive landrace group from Afghanistan and northwestern Pakistan and differed greatly from other type I subtypes, implying that these landraces were domesticated independently. Subtypes Ig was found in a landrace from Pakistan and Ia and Ie–Ig were in six wild ancestor accessions. Type II was also highly polymorphic and four subtypes were found and designated as subtypes IIa–IId, but sequence analyses indicated type III as monomorphic. This work indicates that type III should be classified as a subtype of type II (subtype IIe). Sequence polymorphism of subrepeats of types I–III indicated that subrepeats of subtype IIa are greatly divergent from others. Relationships among types I–III were much more complicated than anticipated based on previous RFLP work.

Ribosomal DNA spacer length polymorphisms were also studied in foxtail millet landraces from Pakistan and Afghanistan and in its wild ancestor (*S. viridis*) from Pakistan by PCR-based methods (Fukunaga et al. 2011). Sequence polymorphisms were investigated for accessions selected based on the observed length polymorphism. The PCR-based length and sequence polymorphisms of rDNA IGS clearly demonstrated genetic differentiation

between cultivated and wild forms in the region. Genetic differentiation was observed between different areas to some extent in the cultivated form and between different regions in the wild form from northern Pakistan. Recently, the rDNA PCR–RFLP of foxtail millet germplasm (480 accessions) collected throughout Eurasia and from a part of Africa was investigated with five restriction enzymes (Eda et al. 2013). Foxtail millet germplasm was classified by length of the rDNA IGS and RFLP, and clear geographical differentiation was observed between East Asia, the Nansei Islands of Japan–Taiwan–the Philippines area, South Asia, and Afghanistan–Pakistan (Fig. 7.1b). Evidence of migration of foxtail millet landraces between the areas was also found. Diversity indices (D) for each region were calculated, and it was concluded that the center of diversity of this millet is East Asia, including China, Korea, and Japan.

(b) Random Amplified Polymorphic DNA (RAPD) markers

Li et al. (1998) investigated random amplified polymorphic DNA (RAPD) in foxtail millet and wild relatives and confirmed that foxtail millet had been domesticated from *S. viridis*. Schontz and Rether (1999) investigated RAPDs in 37 accessions of cultivated *Setaria italica*, representative of Eurasian accessions. By using four 10-mer primers, they obtained 25 polymorphic bands and identified 33 different genotypes. A factorial analysis of correspondence was performed on the presence–absence data and three genetic groups were identified. These genetic groups were closely related to the geographical origin of the different accessions: one central European and two Asiatic groups (the first Asiatic accessions originating in latitudes below 35°N and the second comprising the Asiatic accessions originating in latitudes above 35° N).

(c) Nuclear genomic RFLP

Fukunaga et al. (2002b) investigated 16 RFLP loci in 62 landraces to study genetic differentiation in foxtail millet. Among 52 bands, 47 were polymorphic among foxtail millet landraces. A dendrogram based on RFLPs was divided into five major clusters (cluster I–V: Fig. 7.1c). Clusters I and II contained mainly accessions from East Asia. Cluster III consisted of accessions from subtropical and tropical regions in Asia, such as Nansei Islands of Japan, Taiwan, the Philippines, and India, and cluster IV consisted of some accessions from East Asia, an accession from Nepal and an accession from Myanmar. Cluster V contained accessions from central and western regions of Eurasia such as Afghanistan, Central Asia, and Europe. Chinese landraces were classified into four clusters. These results indicate that foxtail millet landraces have differentiated genetically between different regions and that Chinese landraces are highly variable.

(d) Mitochondrial DNA (mtDNA)

Mitochondrial DNA (mtDNA) was characterized by RFLPs in 94 accessions (Fukunaga and Kato 2003). Three RFLP patterns were observed by using rice *atp6* as a probe and were designated as types I–III. Difference between types I and II seem to be due to recombination between two *atp6* genes. In East and Southeast Asia and Afghanistan, both types I and II were found, while type I was predominant in India, Central Asia and Europe. In

China, type III was also found. Chinese accessions showed higher gene diversity than those from other regions. This result supported the previous studies on isozymes and nuclear RFLPs.

(e) AFLP

Le Thierry d'Ennequin et al. (2000) investigated AFLP markers to assess genetic diversity and patterns of geographic variation among 39 accessions of foxtail millet and 22 accessions of its wild progenitor. A high level of polymorphism was observed. Dendrograms based on Nei and Li distances from a neighbor joining procedure were constructed using 160 polymorphic bands. In contrast to other molecular marker studies, no specific geographic structure could be extracted from the data. The high level of diversity among Chinese accessions was consistent with the hypothesis of a center of domestication in China.

(f) Transposon Display (TD) markers

Hirano et al. (2011) investigated genetic structure by transposon display (TD) using 425 accessions of foxtail millet and 12 of the wild ancestor green foxtail. They used three recently active transposons (*TSI-1*, *TSI-7*, and *TSI-10*) as genome-wide markers and succeeded in demonstrating geographical structure for foxtail millet. A neighbor-joining dendrogram based on TD grouped the foxtail millet accessions into eight major clusters, each of which consisted of accessions collected from adjacent geographical areas (Fig. 7.1d). Eleven out of 12 green foxtail accessions were grouped separately from the clusters of foxtail millet. These results indicated strong regional differentiations and a long history of cultivation in each region. They also suggest a monophyletic origin of foxtail millet domestication.

(g) Single nucleotide polymorphisms (SNPs)

Recently, a large-scale analysis of whole genome single nucleotide polymorphism (SNP) in 916 accessions (mainly from China but also including accessions from other regions such as Japan and Korea, Southeast Asia, South Asia, Central Asia, Europe, Africa, and the USA) was carried out by Jia et al. (2013). They found that the 916 varieties can be clearly classified into two divergent groups, spring-sown form (type 1) with 292 varieties and summer sown form (type 2) with 624 varieties, and there was a clear geographical distribution of these two groups in the Chinese accessions—the majority of type 1 accessions were from northern China and high altitude areas of northwest China, whereas most of the type 2 accessions originated from central and southern China, which have warmer climates (see Chap. 2). As for accessions from other countries, they found that varieties from the same geographical regions tended to belong to the same clades in phylogenetic trees. They concluded that foxtail millet may have a single origin of domestication but that a deep investigation of the wild ancestor is needed. Their results that foxtail millet accessions can be divided into northern and southern groups may correspond with distribution of rDNA types I and II and results of other genetic studies although most of the materials that Jia et al. (2013) used were from China. Further analysis using more accessions from other countries will be also required.

7.4 Evolution of Two Genes (*Waxy* and *PPO*) Under Human/Natural Selection

In cereals, several genes involved in domestication and diversification have been studied in rice (e.g., Konishi et al. 2006; Ishii et al. 2013) and maize (e.g., Doebley et al. 1997; Wang et al. 2005) and six row and naked grains in barley (Komatsuda et al. 2007; Taketa et al. 2008). In foxtail millet, the *waxy* gene controlling amylose content in endosperm and the *polyphenol oxidase* (*PPO*) gene for phenol color reaction (Phr) in grains have been investigated, as it is known that these genes have evolved under human/natural selection in other cereals.

7.4.1 Evolution of Waxy

Endosperm starch of cereals consists of amylose and amylopectin. Wild type (non-waxy) of endosperm starch consists of ca. 20 % or more of amylose and ca. 80 % of amylopectin, whereas the waxy type consists of ca. 100 % amylopectin and lacks amylose. The non-waxy type (*Wx*) is genetically dominant to the waxy type (*wx*). Endosperm starch with the recessive genotype, waxy type, has a stickier texture than the normally dominant non-waxy type. Both of these endosperm types are found among landraces of sorghum, rice, foxtail millet, maize, common millet, barley, and Job's tears (Sakamoto 1996). The waxy types of these cereals are found in East and Southeast Asia, but are rare in India and further westward. A core area where people show a strong ethnobotanical preference for waxy cereals, which extends from Southern China through Northern Thailand to Assam, has been identified (Sakamoto 1996; Yoshida 2002). In adjacent areas like Taiwan, Japan, and Korea, waxy cereals are grown mainly on upland soils, and are used in traditional rituals, or eaten only on special occasions. This trait is apparently associated with ethnological preferences in the areas (e.g., Fogg 1983; Takei 1994).

Waxy endosperm arises through the disrupted expression or loss of function of the *waxy* (*GBSS1*) gene that encodes granule-bound starch synthase I (GBSS I) (Sano 1984). Waxy-type cereals are characterized by little or no starch amylose, which constitutes about 20 % or more of the total starch in the non-waxy endosperm. This character has often been neglected in other regions, although waxy maize, which was first reported (Collins 1909) in Chinese landraces, is now globally used for the production of waxy corn starch. The molecular basis of artificial and spontaneous waxy mutants has also been clarified (Wessler and Varagona 1985). Several mutants arose by insertion of transposable elements into this gene. The molecular genetics of GBSS I has also been studied in rice (Hirano and Sano 1991; Hirano et al. 1998; Isshiki et al. 1998; Olsen and Purugganan 2002), barley (Domon et al. 2002a, b), and sorghum (McIntyre et al. 2008; Sattler et al. 2009). In rice, two dominant waxy alleles, Wx^a and Wx^b were observed mainly in indica and japonica rice groups, respectively. It was reported that Wx^b arose by a point

mutation of the 5′ end of intron 1, resulting in aberrant splicing of the intron (Hirano et al. 1998; Isshiki et al. 1998; Olsen and Purugganan 2002). It was also reported that waxy rice originated by a 23-bp duplication of exon 2 (Wanchana et al. 2003). In barley, deletion in the 5′ region of the gene was found in wx genotype (Domon et al. 2002a, b; Patron et al. 2002). In sorghum, two different mutations were found for wx alleles; one resulting from a transposable element being inserted into the gene and the other from a point mutation resulting in amino acid substitution (McIntyre et al. 2008; Sattler et al. 2009). In hexaploid wheat, waxy wheat was artificially synthesized (Nakamura 1995), and it was concluded that deletion of the gene was responsible for the phenotype (Vrinten and Nakamura 1999).

Waxy phenotypes have also been observed in millets. In foxtail millet, the molecular basis of naturally occurring *wx* mutants has been well characterized (Nakayama et al. 1998; Fukunaga et al. 2002a; Kawase et al. 2005; Van et al. 2008). Waxy foxtail millet probably evolved from the non-waxy type after domestication, since the wild ancestor has a non-waxy endosperm (Nakayama et al. 1998). In addition to those two types, an intermediate or low-amylose type of this crop has been reported (Sakamoto 1987). Amylose content is positively correlated with amounts of GBSS 1 protein among the three phenotypes (Afzal et al. 1996) and is genetically controlled by waxy (GBSS 1) alleles (Nakayama et al. 1998). No other genes that regulate amylose content, such as *du* in rice (Okuno et al. 1983), are known in *S. italica*. The sequence of the full-length cDNA and the genomic structure of the *waxy* (*GBSS 1)* gene in foxtail millet was determined, and a preliminary diversity analysis indicated multiple origins of the waxy endosperm types (Fukunaga et al. 2002a). Kawase et al. (2005) analyzed 841 landraces of foxtail millet and classified 11 types by PCR-based methods. They concluded that waxy foxtail millet originated four times independently and low-amylose foxtail millet three times by insertions of transposable elements (Fig. 7.2). Van et al. (2008) found that the foxtail millet *waxy* gene has several SNPs and small indels. Recently, Hachiken et al. (2013) also investigated sequence variation of

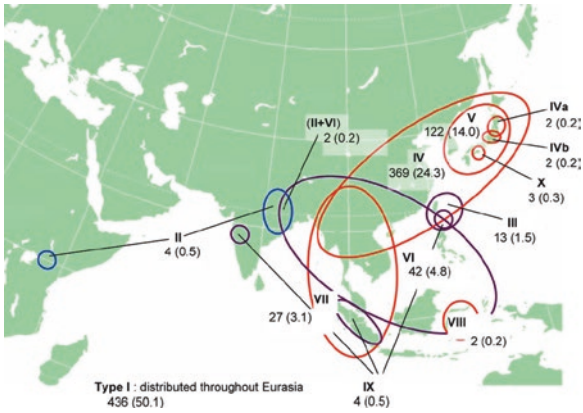

Fig. 7.2 Summary of the geographical distribution of waxy and low amylose types of foxtail millet in Asia, Europe, and Africa (Kawase et al. 2005)

the *waxy* gene and revealed that *waxy* alleles of non-waxy accessions are more polymorphic than those of waxy and low-amylose accessions at the sequence level. This supports the hypothesis that waxy and low-amylose types originated from a non-waxy type.

7.4.2 *Variation in Phenol Color Reaction (Phr) and Evolution of the* Polyphenol Oxidase *(*PPO*) Gene*

Phenol color reaction (Phr) is a coloration of hulls/lemma and palea (grains) of cereals after soaking in phenol solution, as reported for rice (Oka 1953) and barley (Takeda and Chang 1996). The positive Phr type shows a black coloration after soaking in phenol solution, whereas the negative Phr type does not show coloration. Variation of Phr and geographical distribution of Phr phenotypes for foxtail millet have been reported (Kawase and Sakamoto 1982). It was shown in that study that Phr in foxtail millet is controlled by a single gene (positive Phr being dominant and negative Phr being recessive) and that the negative Phr type is predominant in Eurasia, whereas the positive Phr type generally has a skewed distribution toward subtropical and tropical regions including Nansei Islands of Japan, Taiwan, the Philippines, Nepal, and India (21–100 %).

The molecular mechanism of this trait has been investigated recently (Inoue et al. 2015). The *polyphenol oxidase* (*PPO*) gene responsible for Phr was isolated and molecular genetic basis of negative Phr and crop evolution of foxtail millet was investigated. Firstly, they searched for *PPO* gene homologs in a foxtail millet genome database (Bennetzen et al. 2012) using a rice *PPO* gene as a query, and successfully found three copies of the *PPO* gene. One of the *PPO* gene homologs on chromosome 7 showed the highest similarity with *PPO* genes expressed in hulls (grains) of other cereal species including rice, wheat, and barley and was designated as *Si7PPO*. Phr phenotypes and *Si7PPO* genotypes completely co-segregated in a segregating population. They also analyzed the genetic variation conferring negative Phr reaction. Of 480 accessions of the landraces investigated, 87 (18.1 %) showed positive Phr and 393 (81.9 %) showed negative Phr. In the 393 Phr negative accessions, three types of loss-of-function *Si7PPO* genes were predominant, with mutations found in various locations. One of them has an SNP in exon 1 resulting in a premature stop codon and was designated as stop codon type, another has an insertion of a transposon (*Si7PPO-TE1*) in intron 2 and was designated as TE1-insertion type, and the other has a 6-bp duplication in exon 3 resulting in the duplication of two amino acids and was designated as 6-bp duplication type. As a rare variant of the stop codon type, one accession additionally has an insertion of a transposon, *Si7PPO-TE2*, in intron 2 and was designated as "stop codon + TE2 insertion type." The geographical distribution of accessions with positive Phr and those with three major types of negative Phr was also investigated (Fig. 7.3). Accessions with positive Phr were found in subtropical

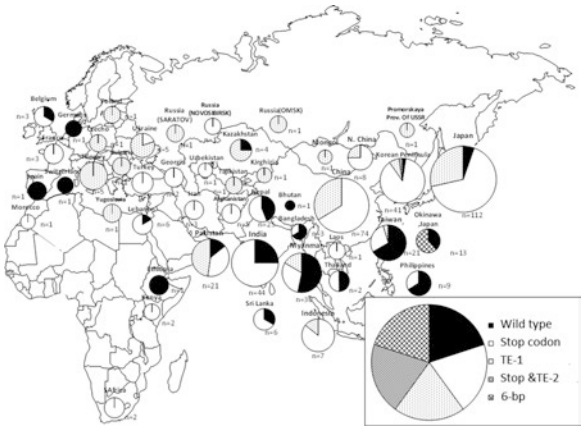

Fig. 7.3 Geographical distribution of positive Phr (wild type) and four different genotypes of Phr, stop codon type, TE1 insertion type, 6-bp duplication type, and stop codon and TE2 insertion type (Inoue et al. 2015)

and tropical regions at frequencies of ca. 25–67 % and those with negative Phr were broadly found in Europe and Asia. The stop codon type was found in 285 accessions and was broadly distributed in Europe and Asia, whereas the TE1-insertion type was found in 99 accessions from Europe and Asia but was not found in India. The 6-bp duplication type was found in only eight accessions from Nansei Islands (Okinawa Prefecture) of Japan. They also analyzed Phr in the wild ancestor and concluded that the negative Phr type was likely to have originated after domestication of foxtail millet. Their study also suggested that the negative Phr of foxtail millet arose by multiple independent loss of function of *PPO* gene, that proved advantageous under some environmental conditions and under human selection, as also seen in rice (Yu et al. 2008) and barley (Taketa et al. 2010).

7.5 Perspective

Recent studies in phylogeny and association mapping using Next-Generation Sequencing (NGS) technology (Jia et al. 2013) have updated relationships in foxtail millet and revealed several candidate genes involved in domestication and diversification of landraces in foxtail millet. Genetic mapping of a gene involved in panicle morphology (Sato et al. 2013) and QTLs for inflorescence structure, branching and height (Doust et al. 2004; Doust et al. 2005; Mauro-Herrera and Doust 2016), and flowering time (Mauro-Herrera et al. 2013) also have been carried out. Some of these studies on association mapping and QTLs are described in chapter 12. Further analyses of candidate genes in the context of domestication and crop evolution of foxtail millet by using foxtail millet landraces from broad area of Europe and Asia will be interesting. Currently, we are focused on genes involved in panicle morphology, which will be helpful to understand crop evolution and diversification of foxtail millet.

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