Chapter 14
An Ancient Medicinal Plant at the Crossroads of Modern Horticulture and Genetics: Genetic Resources and Biotechnology of Sea Buckthorn (*Hippophae* L., Elaeagnaceae)

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Abstract Sea buckthorn (*Hippophae* L., Elaeagnaceae) has been exploited by humans for thousands of years on the Quinghai–Tibetan Plateau (QTP) and nearby areas. However, the considerable modern economic potential of this plant has started to receive full appreciation only recently. Expanding its traditional use in harsh climatic zones as important source of nutrients, vitamins, and as wood in treeless areas, today this plant is used also on large scales as landscape protection tools against corrosion of soil, and as a source of wide range of products in pharmaceutic, cosmetic, and nutritional supplement industries. This review aims to provide the latest insights from studies on the evolutionary history and biogeography of the genus, structure, and phylogeography of genetic diversity within its species. Understanding the genic and genomic interactions among populations and phylogenetically distant lineages within species of *Hippophae* should help to improve the efficiency of exploitation of genetic resources in this crop. Research efforts in the past century in breeding, systematics, cytogenetics, biochemistry, and genetics of *Hippophae* have created a solid background for advances in modern biotechnology of this crop. Recent studies reported application of next-generation sequencing (NGS) technologies and identification of thousands of genes in transcriptomes of sea buckthorn. Analyses of the transcriptomes provided better understanding of gene expression in biochemical pathways of unsaturated fatty acids, some other secondary metabolites, and regulation of gene complexes responsible for adaptation to different categories of abiotic stress. Further studies should focus on the creation of genetic maps of breeding populations; identification of quantitative trait loci, biochemical pathways of synthesis of bioactive secondary

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metabolites and correspondent genes, molecular mechanisms of tolerance and resistance to abiotic stress, diseases, and pests; and cloning of genes of agricultural importance. Advances in these research areas can lead to genetic engineering of plants with a combination of traits of high horticultural, medicinal, or nutrient value, adapted to specific environments of areas of their cultivation.

Keywords Biogeography · Genetic diversity · Genetic engineering · *Hippophae rhamnoides* · Homoploid hybridization · Marker-assisted selection · Molecular breeding · Molecular cloning · Phylogeography · Population structure

14.1 General Introduction

Several reviews on sea buckthorn have been published in the past two decades on horticulture (Li and Schroeder 1996; Li 2003), biochemistry (Kalia et al. 2011), medicinal and nutritional properties (Warnock and Miskin 2009; Singh and Ahmed 2010; Suryakumar and Gupta 2011; Wang et al. 2011a; Kanayama et al. 2012), causes and effects of dried-shrink disease and susceptibility to insect pests (Ruan et al. 2013); and biotechnological advances in the development of this crop (Kalia et al. 2011; Ruan et al. 2013). However, three important factors make it necessary to continue summarizing information on studies of this plant. First, all these reviews focused on only one species in the genus *Hippophae*, namely *H. rhamnoides*, leaving discussions of genetic diversity and evolution in the whole genus mostly untreated. Second, it is hard to overview all aspects of research on this wonderful plant in one paper. Directions of these studies cover a wide range of research areas: taxonomy and systematics, evolution and biogeography, population genetics, biochemistry, ecology; horticultural, ecological, and medicinal applications; ethnobotany, ethnopharmacology, and its impact on economy of indigenous people; conventional breeding, different aspects of biotechnology (in vitro cultivation, molecular breeding, cloning of genes, genetic transformation), etc. Inevitably each review can focus only on some of these areas, while treating others in a cursory way or leaving them out completely. Third, research work on *Hippophae* and *H. rhamnoides* has considerably intensified in the past two decades. This leads to a fast increase in the amount of information published each year, making each review partly outdated soon after publication. This study is aimed to provide an overview of results in those areas that have not been reflected in sufficient detail in earlier reviews, such as evolution, biogeography, phylogeography, and population genetics of *Hippophae* in different geographical regions. Besides, promising advances have been made in recent years in the biotechnology of this plant, and a brief summary of these results is also provided.

14.2 Characterization of the Genus

14.2.1 Systematics and Taxonomy

Sea buckthorn is a common English name for plants from the genus *Hippophae*. This genus belongs to Elaeagnaceae, a small family of three genera (two other genera are *Shepherdia* and *Elaeagnus*). Circumscription of species and subspecies of *Hippophae* has varied over the past century, mainly due to the homoplastic morphology and different opinions as to what constitute species, subspecies, or hybrids. In the first monograph of the genus, Servettaz (1908) recognized one species with three subspecies (Table 14.1). In the second major revision of the genus, Rousi (1971) raised these subspecies to species level and described seven new subspecies of *H.rhamnoides*. Lian et al. (1998) recognized six species in two sections with some additional new subspecies described by themselves, i.e., *H. goniocarpa* subsp. *goniocarpa*, *H. goniocarpa* subsp. *litangensis*, and *H. neurocarpa* subsp. *stellatopilosa* (Lian et al. 1995). In one of the latest treatments of the genus, Swenson and Bartish (2002) mostly accepted suggestions of Rousi (1971) and Lian et al. (1998) and recognized eight subspecies within *H. rhamnoides*, two subspecies within *H. neurocarpa*, and three other non-hybrid and currently monotypic species (*H. gyantsensis*, *H. salicifolia*, and *H. tibetana*). However, they also raised *H. litangensis* to the species level, which together with *H. goniocarpa* created a group of two species of hybridogeneous origin and therefore unclear taxonomic status (for a more detailed discussion on interspecific hybridization and hypotheses of hybrid origin for different taxa within the genus see chapter "Homoploid hybridization in *Hippophae*"). Swenson and Bartish (2002) did not recognize sections *Hippophae* and *Gyantsensis*, suggested by Lian and Chen (1993). Circumscription of the sections was based on differences in the morphology of seed coat among species, but molecular markers did not support these groups (Bartish et al. 2000a, 2002; Sun et al. 2002; Jia et al. 2012; Jia 2013). On the other hand, all five currently recognized non-hybrid species in the genus can be clearly defined by morphological characters (Rousi 1971; Lian et al. 1998; Swenson and Bartish 2002).

Several additional systematic modifications have been suggested for *Hippophae* soon after the publication of the taxonomic synopsis of the genus by Swenson and Bartish (2002). Lian et al. (2003a) described *H. rhamnoides* subsp. *wolongensis*. Analysis of random amplified polymorphic DNA (RAPD) markers suggested a close relationship between subsp. *wolongensis* and subsp. *sinensis* (Sheng et al. 2006). However, analyses of sequences of *trn*L-*trn*F and *trn*S-*trn*D genes of chloroplast DNA (cpDNA) and internal transcribed spacer (ITS) sequences of ribosomal DNA indicated instead that this taxon is a part of *H. rhamnoides* subsp. *yunnanensis* (Jia et al. 2012). The status of *H. rhamnoides* subsp. *wolongensis* is therefore not clear yet, and it has to be confirmed in further analyses of

Table 14.1 Overview of the main systematic treatments of *Hippophae* L

Servettaz (1908)	Rousi (1971)	Lian et al. (1998)	Swenson and Bartish (2002)	Tsvelev (2002)
Sect. Hippophae				
H. rhamnoides subsp. *rhamnoides*	*H. rhamnoides*	*H. rhamnoides*	*H. rhamnoides*	*H. rhamnoides*
	subsp. *carpatica*	subsp. *carpatica*	subsp. *carpatica*	
	subsp. *caucasica*	subsp. *caucasica*	subsp. *caucasica*	*H. caucasica*
	subsp. *fluviatilis*	subsp. *fluviatilis*	subsp. *fluviatilis*	*H. caucasica* subsp. *fluviatilis* (?)
	subsp. *gyantsensis*			
	subsp. *mongolica*	subsp. *mongolica*	subsp. *mongolica*	*H. mongolica*
	subsp. *rhamnoides*	subsp. *rhamnoides*	subsp. *rhamnoides*	
	subsp. *sinensis*	subsp. *sinensis*	subsp. *sinensis*	*H. sinensis*
	subsp. *turkestanica*	subsp. *turkestanica*	subsp. *turkestanica*	*H. turkestanica*
	subsp. *yunnanensis*	subsp. *yunnanensis*	subsp. *yunnanensis*	*H. yunnanensis*
subsp. *salicifolia*	*H. salicifolia*	*H. salicifolia*	*H. salicifolia*	*H. salicifolia*
Sect. Gyantsensis				
		H. gyantsensis	*H. gyantsensis*	*H. gyantsensis*
		H. goniocarpa	*H. goniocarpa*	
		subsp. *goniocarpa*		
		subsp. *litangensis*	*H. litangensis*	
		H. neurocarpa	*H. neurocarpa*	*H. neurocarpa*
		subsp. *neurocarpa*	subsp. *neurocarpa*	
		subsp. *stellatopilosa*	subsp. *stellatopilosa*	
subsp. *tibetana*	*H. tibetana*	*H. tibetana*	*H. tibetana*	*H. tibetana*

morphological, ecological, and genetic differentiations of a comprehensive sample of populations including *H. rhamnoides* subsp. *yunnanensis* and *sinensis*. Lian et al. (2003b) also did not recognize a species status of *H. litangensis* and suggested instead to treat it as a subspecies of *H. goniocarpa*, arguing that the putative parents of *H. litangensis* are subspecies of *H. rhamnoides* and *H. neurocarpa*. In another taxonomic study, Tsvelev (2002) analyzed three subspecies of *Hippophae rhamnoides* from Russia (subsp. *caucasica*, *mongolica*, and *turkestanica*) and suggested raising these and two Chinese taxa (subsp. *sinensis* and *yunnanensis*) to species level. He was not certain regarding the treatment of *H. rhamnoides* subsp. *fluviatilis*. Noting that it is morphologically distinct from the other European taxa, he suggested a possible position for this taxon as a subspecies of *H. caucasica*. Besides, Tsvelev (2002) considered subsp. *carpatica* to be indistinguishable from subsp. *caucasica*. These taxonomic suggestions are summarized in Table 14.1. However, molecular data does not support close relations between subsp. *caucasica* and *carpatica* (Bartish et al. 2000a; Bartish 2006), or treatment of subsp. *fluviatilis* as one of taxa within *H. caucasica* (Bartish et al. 2002; Bartish 2006; Jia et al. 2012; Jia 2013).

Several molecular analyses (Bartish et al. 2000a, 2006; Jia et al. 2012; Jia 2013) recovered almost all subspecies of *H. rhamnoides* as monophyletic with respect to each other (reciprocally monophyletic). These studies could thus provide support for the proposition of Tsvelev (2002) to raise several subspecies of *H. rhamnoides* to species level. However, a clearly identified set of optical morphological characters to differentiate these taxa from each other in the field has not yet been developed (Swenson and Bartish 2002). The ability to distinguish taxa in the field using morphological characters is required for robust and stable taxonomic decisions in addition to strongly supported monophyly of correspondent clades (Backlund and Bremer 1998). A comprehensive analysis of a representative set of morphological traits of all subspecies of *H. rhamnoides* in a large sample of populations (including numerous adult individuals within these populations) across the whole range of the species have not been published yet. Rousi (1965, 1971) reported a variation in leaf and seed traits across all subspecies of *H. rhamnoides* and the whole genus. Unfortunately, with the exception of subsp. *fluviatilis* and *rhamnoides*, his samples in these two studies were restricted to few individuals within most of the populations and most of the scored traits, thus preventing any robust comparisons of variability within and between populations from different taxa he recognized. A clear understanding of distribution of diversity within and among populations and taxa of *H. rhamnoides* is therefore still lacking for most morphological traits. Careful statistical analyses of morphological diversity in combination with correspondent analyses of molecular markers on the same sample of multiple populations across the whole range of the species are required to advance discussions on taxonomical status of subspecies from this species. The main task of these analyses would be identification of morphological traits, in which variation among subspecies is clearly higher, than variation within all of them.

14.2.2 Morphological Characters

In addition to taxonomical applications, carefully scored morphological characters can provide important insight into the species' biology and ecology, and into agricultural potential of different regional genetic resources. Differences in the morphology of shoots and thorns can potentially reflect variable intensities of browsing by mammals in different regions, and also variable potential of local populations in breeding programs for thornless cultivars. Variations in seed and fruit sizes and yield can characterize variable levels of investment of individual plants into sexual and clonal reproduction, and also variable potential of local populations in breeding programs for yield and quality of fruits. Numerous studies have focused on analyses of fruit and seed morphologies, physiology, and biochemical composition, mostly in cultivated plant varieties of sea buckthorn. This literature, however, needs a special review and is not discussed here. On the other hand, as already mentioned above, there is currently a lack of standardized and well-documented statistical analyses on variations within the natural populations of multiple morphological characters across the whole range of the genus. This situation probably reflects insufficient appreciation by taxonomists of variation within populations and taxa of *Hippophae* in most of the studied morphological characters.

Despite the lack of systematic analyses of morphological traits in representative samples of populations across the genus, several studies did provide some insights into morphological variations (or its lack) in *Hippophae*. An analysis of seeds and seedlings across 46 natural populations (between 2 and 43 seedlings per population) of six western subspecies of *H. rhamnoides* (Rousi 1965), and analysis of 2264 herbarium specimens across the whole genus (Rousi 1971), are still probably the most advanced attempts to study morphological variations in both the species and the genus. These analyses revealed significant differences among all taxa recognized by Rousi (1971) in at least one of several quantitative biometric characters: leaf length, breadth, and length/breadth ratio; seed length, width, thickness, length/width ratio, width/thickness ratio, and weight. The only exception was the absence of significant differences in any of these characters between *H. rhamnoides* subsp. *mongolica* and *rhamnoides* (Rousi 1971). Pollen morphology was analyzed by Sorsa (1971) across several taxa of the genus including those recognized by Rousi (1971), but no traits to differentiate these taxa could be identified.

Yao and Tigerstedt (1995) studied variations in hardiness and plant height among populations of three subspecies of *Hippophae rhamnoides* (subsp. *rhamnoides*, *sinensis,* and *turkestanica*). They found strong correlation between ecological (hardiness) and morphological (plant height) traits. This study supports the idea of Rousi (1971) that particular morphological characters in different species of the genus can reflect their adaptation to variable environments and ecological challenges. However, different morphological characters can be associated with environmental factors in different ways. Unlike tree height, fruit and seed characters of *Hippophae* can be unrelated to climatic conditions of localities of their sampling (Aras et al. 2007). It is also not clear how adaptations to different environments,

possibly expressed in variation and differentiation of relevant morphological characters, are associated with genetic differentiation between populations within the species of this genus.

In conclusion, the lack of standardized and statistically sound data on variation within and between individual plants, populations, and taxa for multiple morphological characters across the whole range of the genus prevents (i) reliable differentiation between subspecies of *H. rhamnoides* and *H. neurocarpa*; (ii) clear identification of hybrids between different sympatric taxa in the field; (iii) analyses of potential association of morphology of particular traits with environments of specific localities; (iv) efficient identification of perspective regional resources of variable agricultural traits for breeding programs; and (v) analyses of genetic linkage groups of characters and search for molecular markers of quantitative trait loci (QTL), controlling morphological traits in natural populations of the genus.

14.2.3 Evolution and Biogeography

14.2.3.1 Molecular Phylogenies of Elaeagnaceae and Hippophae

According to APG III, Elaeagnaceae are included into Rosales, an order of nine families (APG III 2009). In the latest phylogenetic reconstructions Elaeagnaceae are sisters to a small clade of Barbeyaceae and Dirachmaceae, but this relationship received only moderate statistical support (Wang et al. 2009; Zhang et al. 2011). Phylogenetic relationships within Elaeagnaceae have been clearly resolved (Jia and Bartish, unpubished) and species from *Hippophae* formed a clade, which was strongly supported by molecular markers from chloroplast and nuclear genome by different statistical methods, further supporting the findings of Bartish et al. (2002). The genus was sister to *Shepherdia* and this relationship was strongly supported by molecular markers from both chloroplast and nuclear genomes.

Phylogenetic relationships within *Hippophae* have been subject to several molecular studies. In analyses of RAPD markers, Bartish et al. (2000a) obtained a clade of all subspecies of *H. rhamnoides*, in which, however, only six western subspecies received strong statistical support, while placement of subsp. *sinensis* and *yunnanensis* was not supported. Topology of relationships among other taxa did not receive any robust support in these analyses. Bartish et al. (2002) analyzed variations in restriction sites from several genes of cpDNA and a set of morphological characters, and found strong statistical support for monophyly of the genus. They reconstructed a (weakly supported) clade of eight subspecies of *H. rhamnoides*, and a clade of four other (non-hybrid) species, which also received only weak statistical support. Sun et al. (2002) added described by Lian et al. (2003a) a Chinese subspecies *H. rhamnoides* subsp. *wolongensis* to their sample. They used in their analyses ITS sequences and mostly confirmed results of the earlier RAPD- and cpDNA-based studies. The exceptions were placement of *H. tibetana* in strict consensus tree as sister to the clade of all other taxa, and high statistical support

(97 and 91 %, respectively) for sister clades of nine subspecies of *H. rhamnoides*, and three remaining non-hybrid species (*H. gyantsensis*, *H. neurocarpa*, and *H. salicifolia*).

In a recent analysis of all non-hybrid taxa in the genus (with the exception of *H. rhamnoides* subsp. *wolongensis*), Jia (2013) obtained two sets of sequences from chloroplast and nuclear genomes (five genes in each). All taxa in this study were represented by three populations across the whole range of each taxon. This study indicated that the current systematics of the genus by Swenson and Bartish (2002) is relatively robust. However, together with phylogenetic reconstructions by Jia et al. (2012), it also revealed the problems to be resolved in further studies. These include (i) the uncertain status of *H. rhamnoides* subsp. *yunnanensis* and *wolongensis*, (ii) possibility of raising the status of all subspecies to species level, provided these species can be identified and circumscribed by morphological characters, which can be easily identified in the field; (iii) uncertain status of hybrid taxa (see also chapter on hybridization in the genus).

14.2.3.2 *Biogeography of* Hippophae

Bobrov (1962) was probably the first to suggest east to west migrations along the mountain ranges of Eurasia currently occupied by *H. rhamnoides* as the main biogeographic hypothesis for this genus. Based only on paleobotanical data, he suggested the late Miocene as the most likely geological epoch of the putative migrations. Hyvönen (1996) conducted the first phylogenetic analysis in *Hippophae* using morphological characters. Contrary to Bobrov (1962), he suggested the western part of the genus' range as its most likely ancestral area, and an ancient range fragmentation in the genus. The first molecular phylogenies (Bartish et al. 2000a, 2002; Sun et al. 2002) mostly supported the hypothesis of East Asian origin of the genus. This hypothesis received further support in the explicit tests of probability of ancestral area for several taxa in *Hippophae* by Jia et al. (2012), who identified an area to the east of Qinghai–Tibetan Plateau (QTP) as the most likely ancestral area of the genus and suggested a likely route for migration of *H. rhamnoides* from East Asia to Europe. These authors also suggested that diversification in the genus started in the late Miocene to Pliocene. However, sampling of populations in this study was not well-balanced taxonomically and geographically. It focused mainly on two taxa from East Asia (*H. rhamnoides* subsp. *sinensis* and *yunnanensis*), which might bias the results. Moreover, molecular phylogenies in this study were dated using secondary calibrations, which can be misleading (Ho and Phillips 2009; Sauquet et al. 2012).

Therefore, Jia (2013) carried out biogeographic analyses of a well-balanced sample of populations across ranges of all five non-hybrid species and ten subspecies in the genus. He calibrated his tree using a set of several carefully selected fossil records across Rosales. He also tested his dating by a recently published record of *Hippophae* from the late Miocene (about seven million annuals [Ma] before present) of Anatolia and Greece (Biltekin 2010). Results of this study

provide strong support for the hypothesis of Bobrov (1962) and suggest also a detailed set of biogeographic scenarios for evolutionary processes in the genus during a period of considerable orogeneses and climatic changes in Eurasia (the Miocene, the Pliocene, and the Quaternary).

14.2.3.3 Genetic Diversity of Populations and Phylogeography

Genetic Diversity of Natural Populations from Different Taxa of Hippophae

Molecular studies in the past two decades provide a relatively detailed picture of the population genetic structure within most taxa of *Hippophae* (Table 14.2). Yao and Tigerstedt (1993) used isozyme loci in the first application of molecular markers for analysis of genetic structure in three species of *Hippophae* (*H. neurocarpa*, *H. rhamnoides*, and *H. tibetana*) and three subspecies of *H. rhamnoides* (*rhamnoides*, *sinensis*, and *turkestanica*). They found that most of the genetic variations in *H. rhamnoides* were allocated within populations (53.9 %) and between subspecies (41.6 %), while differentiation between populations within subspecies can be very low (4.5 %; Dsp = 0.011). This pattern was further confirmed in general in the first study of DNA markers (RAPDs) in the genus, an analysis of population genetic structure of *H. rhamnoides* subsp. *rhamnoides* (Bartish et al. 1999), and also in analyses of a group of western subspecies of this species (Bartish et al. 2000a).

Fixation indices (Gst) varied considerably (0.068–0.470) in four studies of *H. rhamnoides* subsp. *sinensis*, even though the marker system used in these studies was the same (inter-simple sequence repeats, ISSRs), and sample sizes of populations were similar (Table 14.2). Sun et al. (2006) reported intermediate values of Gst (0.183) in analyses of RAPD markers in this subspecies. The unusually high differentiation among populations reported by Wang et al. (2011b) probably reflects the sampling strategy of these authors. They selected populations from three regions across the whole range of the taxon with contrasting vegetations and environments.

High differentiation among populations was reported for *Hippophae rhamnoides* subsp. *turkestanica* (Gst = 0.457) for amplified fragment length polymorphisms (AFLPs) by Raina et al. (2012) and for subsp. *yunnanensis* (Gst = 0.459) for RAPDs by Chen et al. (2010). The authors of both the studies attributed this unusually high for the genus and dominant markers differentiation among populations to climatic heterogeneity and complex landscape characteristics of localities of their sampling. Raina et al. (2012) found also relatively low differentiation among populations in two other species, *H. salicifolia* and *H. tibetan*a (0.291 and 0.194, respectively). These estimates were similar to those observed in *H. rhamnoides* subsp. *rhamnoides* by Bartish et al. (1999). It should be noted, however, that analyses of *H. salicifolia* and *H. tibetan*a were based on a relatively low number of populations (three in each species), so they should be considered as preliminary. In recently reported analyses of genetic variation in *H. rhamnoides* subsp. *turkestanica,* Srihari et al. (2013) applied several molecular marker systems (RAPD, ISSR, simple sequence repeats [SSR], two MAD-box loci and ITS) to

Table 14.2 Analyses of population genetic structure in different taxa of *Hippophae* using molecular markers

Taxon	Marker system	Region	No. of populations	No. of plants	No. of loci	Gst	Reference
Dominant markers							
H. rhamnoides ssp. *rhamnoides*	RAPD	N. Europe	10	106	174	0.151[a]	Bartish et al. (1999)
H. rhamnoides ssp. *sinensis*	RAPD	E. Asia	13	232	107	0.183	Sun et al. (2006)
H. rhamnoides ssp. *sinensis*	RAPD	E. Asia	5	–	151	0.406	Zhao et al. (2007)
H. rhamnoides ssp. *sinensis*	ISSR	E. Asia	10	145	326	0.182	Chen et al. (2008)
H. rhamnoides ssp. *sinensis*	ISSR	E. Asia	11	220	207	0.068	Tian et al. (2004a)
H. rhamnoides ssp. *sinensis*	ISSR	E. Asia	7	140	288	0.418	Tian et al. (2004b)
H. rhamnoides ssp. *sinensis*	ISSR	E. Asia	12	218	252	0.470	Wang et al. (2011b)
H. rhamnoides ssp. *turkestanica*	AFLP	C. Asia	32	348	163	0.457[b]	Raina et al. (2012)
H. rhamnoides ssp. *turkestanica*	SAMPL[c]	C. Asia	32	348	99	0.552[b]	Raina et al. (2012)
H. rhamnoides ssp. *yunnanensis*	ISSR	E. Asia	7	140	288	0.279	Tian et al. (2004b)
H. rhamnoides ssp. *yunnanensis*	RAPD	E. Asia	6	113	112	0.459	Chen et al. (2010)
Co-dominant markers							
H. rhamnoides ssp. *rhamnoides*	Isozymes	N. Europe	9	1825	6	0.045[d]	Yao and Tigerstedt (1993)
H. rhamnoides ssp. *sinensis*	cpSSR[e]	E. Asia	12	218	6	0.602	Wang et al. (2011b)
Sequences of chloroplast genes							
H. gyantsensis	trnL-trnF trnS-trnG	E. Asia	8	63	2	0.730	Cheng et al. (2009)
H. neurocarpa ssp. *neurocarpa*	trnL-trnF	E. Asia	7	35	1	0.535	Meng et al. (2008)
H. neurocarpa ssp. *stellatopilosa*	trnL-trnF	E. Asia	7	35	1	0.606	Meng et al. (2008)
H. rhamnoides ssp. *yunnanensis*	trnL-trnF trnS-trnG	E. Asia	6	46	2	0.627	Cheng et al. (2009)

(continued)

Table 14.2 (continued)

Taxon	Marker system	Region	No. of populations	No. of plants	No. of loci	Gst	Reference
H. tibetana	trnL-trnF trnS-trnG	E. Asia	21	183	2	0.798	Jia et al. (2011)
H. tibetana	trnT-trnF	E. Asia	37	891	1	0.611	Wang et al. (2010)

In most cases differentiation among populations within each taxon is reported as Gst (coefficient of genetic variation among all populations in the sample belonging to the same taxon). N. Europe—North Europe; E. Asia—East Asia; C. Asia—Central Asia

[a]The estimate is based on the proportion of total variance in the sample attributed to variation among populations

[b]These estimates are means of two groups of populations from Jammu and Kashmir and Himachal Pradesh. Ten populations from Uttaranchal were excluded from calculations because authors of this study were not certain about their taxonomic affiliation

[c]SAMPL—selective amplification of microsatellite polymorphic loci

[d]The estimate is the proportion of the total gene diversity in all isozymes attributed to variation between the geographical populations within subspecies

[e]cpSSR—chloroplast simple sequence repeats

evaluate genetic relationships among 15 populations of this taxon from the same regions and approximately the same localities, as in the study of Raina et al. (2012), and in one population of *H. salicifolia*. However, these authors used only three individual plants per each of the populations, combined populations of *H. rhamnoides* and *H. salicifolia*, and combined different marker systems in their analyses of molecular variance (AMOVA). It is therefore not possible to infer partitioning of genetic variation within *H. rhamnoides* subsp. *turkestanica* by different marker systems from these analyses.

Several reports of partitioning of genetic variations within the species from QTP were based on analyses of variation in haplotypes of chloroplast DNA (chlorotypes). Meng et al. (2008) sequenced *trn*L-F gene in 70 individuals from 14 populations of two subspecies of *Hippophae neurocarpa*. They found moderate relatively to other studies of chlorotypes levels of differentiation among populations (0.535 and 0.606 for *H. neurocarpa* subsp. *neurocarpa* and *stellatopilosa*, respectively). Cheng et al. (2009) studied diversity and differentiation of chlorotypes of *trn*L-*trn*F and *trn*S-*trn*D genes in 109 individual plants from 14 populations of *H. gyantsensis* and *H. rhamnoides* subsp *yunnanensis*. They found stronger differentiation among populations in these two taxa (0.730 and 0.627 for *H. gyantsensis* and *H. rhamnoides* subsp *yunnanensis*, respectively). However, it should be noted that reconstructed in this study lineages did not correlate with taxonomic circumscriptions of the sampled material. The incongruence between taxonomic identifications of populations and reported cladogenic reconstructions might result from (i) sampling of hybrid populations, (ii) incomplete lineage sorting

among recently differentiated taxa, or (iii) incorrect taxonomic identifications (no morphological data were reported in this paper). In all of these cases estimates of differentiation among populations within species will be inflated, so these results should be treated with a certain degree of precaution. Wang et al. (2010) found similarly high levels of differentiation (0.611) in a sample of 37 populations of *H. tibetana*, representing most of the range of this species. Finally, Jia et al. (2011) analyzed genetic variation in chlorotypes from a sample of 21 populations of *H. tibetana* across the main areas of distribution of the species and reported even higher levels of differentiation among populations (0.798).

Results on partitioning of genetic variation within and among populations should not be directly compared and discussed, if genetic estimates were based on different molecular marker systems. Nuclear and chloroplast genomes are transmitted between generations in a different way: only nuclear genome information is carried by pollen, while both nuclear and chloroplast genomes can be dispersed via seeds in *Hippophae* (Bartish et al. 2002) as in most other angiosperms. The difference in transmission routes from parent to offspring generations for cpDNA and nuclear DNA (nDNA) results in differences in effective population sizes between exclusively maternally (via seeds) transmitted cpDNA and both maternally and paternally (via pollen) transmitted nDNA. In populations of strictly outcrossing dioecious *Hippophae,* this theoretically means that (all else being equal) effective population size of chloroplast genes is one-quarter of nuclear genes (Birky et al. 1983). Besides, anonymous dominant markers (AFLPs, ISSRs, RAPDs), which are amplified mostly from nuclear genomes, can represent different rates of mutation comparatively to sequences of individual genes of cpDNA (Wolfe et al. 1987). These differences are a likely reason for variable levels of resolution in estimates of genetic differentiation between the two types of molecular marker systems.

Although results of the sequencing analyses of particular cpDNA genes thus are not comparable directly with results based on dominant markers, they nevertheless indicate relatively low levels of gene flow and high isolation of maternally transmitted lineages in the samples of populations of all six studied taxa with ranges on QTP and around it (*Hippophae gyantsensis*, *H. neurocarpa* subsp. *neurocarpa* and *stellatopilosa*, *H. rhamnoides* subsp. *turkestanica* and *yunnanensis*, *H. tibetana*). When fixation indices are compared for similar types of markers between different taxa, a contrast between high levels of differentiation among populations from QTP and neighboring areas and relatively low levels of differentiation among populations from lowlands (*H. rhamnoides* subsp. *rhamnoides* and *sinensis*), is indicated. Further analyses should test explicitly if this contrast can be explained by the landscape complexity and climatic gradients of the QTP and surrounding areas, as has been suggested by some authors (Chen et al. 2010; Wang et al. 2010; Raina et al. 2012), by different ages and demography of taxa and populations, by different ecological adaptations, or by a combination of any of these factors. Comprehensive analyses of population genetic structure are so far lacking for *H. rhamnoides* subsp. *carpatica*, *caucasica*, *fluviatilis*, and *mongolica*, as well as for *H. salicifolia.*

Phylogeography of Different Species of Hippophae

Phylogeographic studies focused so far on two of several main regions from the range of *Hippophae*, namely Europe and QTP (Table 14.3). Long before the advances of molecular techniques and based exclusively on analyses of palinological data, Gams (1943) suggested recolonization of Central Europe and Scandinavia by *H. rhamnoides* following deglaciation of these regions after the Last Glacial Maximum (LGM). According to his inferences, recolonization likely originated from a glacial refugium in southeastern Europe. This was one of the first explicitly formulated hypotheses regarding the late Quaternary phylogeography in the genus. This hypothesis has been tested in a study of four taxa from Europe and Asia Minor (*H. rhamnoides* subsp. *carpatica*, *caucasica*, *fluviatilis*, and *rhamnoides*) by Bartish et al. (2006). These authors analyzed DNA sequences of chalcone synthase intron (*Chs*i) and carried out a restriction fragment length polymorphism (RFLP) analyses of variation in cpDNA within the sample of 26 populations. This study supported the hypothesis of Gams (1943) and revealed a detailed picture of demographic and evolutionary processes in *H. rhamnoides* from the region. In particular, Bartish et al. (2006) (i) identified southeastern Europe as the most likely source area of recolonization into central Europe and Scandinavia after LGM, (ii) revealed multiple lineages of *Chs*i and possibility of existence of several microrefugia in the area; (iii) identified northern Alps as a contact zone between populations from the Alps and the East/Central European–Scandinavian clade; (iv) detected at least four episodes of population growth, all within about the last 40 ky (thousand years); and (v) found nearly synchronized timing of population expansions in the area of sampling, most likely correlating with the Younger Dryas Stadial shortly after LGM. This study reported the highest levels of nucleotide diversity within European populations of *H. rhamnoides* in the area close to northern and northeastern Alps. The observed pattern of genetic diversity in this species likely reflects the admixture of haplotypes after secondary contact of lineages from different glacial refugia, following a general trend of widespread European trees and shrubs (Petit et al. 2003). Surprisingly high levels of genetic diversity in the two most northern populations from Sweden and Norway suggest the possibility of an additional hybrid zone in northern Scandinavia. Genetic structure of populations from Asia Minor indicated demographic trends, which were different from European, and possibility of variable evolutionary responses to global climatic processes in different regions.

The first phylogeographic study of *Hippophae* from QTP was reported by Meng et al. (2008). These authors analyzed phylogeographic patterns in *H. neurocarpa* subsp. *neurocarpa* and *stellatopilosa* and found weak genealogical concordance in the sequenced fragment of cpDNA (*trn*L-*trn*F) with morphological differentiation between these two taxa. Their nested cladogram was based on eight haplotypes and classified these haplotypes into three lineages: one consisted of endemic haplotypes of subsp. *neurocarpa* and the other two included haplotypes from both subspecies. Several unique haplotypes were recovered in the high altitudes, suggesting that *H. neurocarpa* might have survived in these arid habitats at the LGM or even earlier in

Table 14.3 Phylogeographic analyses of different taxa of *Hippophae*

Taxa	Marker system	Region	N of pops	N of plants	Main patterns revealed	Reference
H. gyantsensis	*trn*L-*trn*F *trn*S-*trn*G	S QTP	8	63	Allopatric divergence between lineages from different parts of QTP	Cheng et al. (2009)
H. neurocarpa ssp. *neurocarpa* and *stellatopilosa*	*trn*L-*trn*F	E and N QTP	14	70	Allopatric divergence and CRE in E and N QTP	Meng et al. (2008)
H. rhamnoides ssp. *carpatica, caucasica, fluvialtilis,* and *rhamnoides*	Chsi, *trn*C-*trn*D, *trn*D-*trn*T, *trn*S-*trn*fM	Europe; Asia Minor	27	128	Allopatric divergence between lineages from different mountain ranges in the Pleistocene; CRE from SE to NW Eur after LGM; recent hybridization in NA	Bartish et al. (2006)
H. rhamnoides ssp. *sinensis*	*trn*L-*trn*F; *trn*S-*trn*G; ITS	E QTP; NEC	26	295	HDE in NEC in the LIP or FG	Jia et al. (2012)
H. rhamnoides ssp. *yunnanensis*	*trn*L-*trn*F *trn*S-*trn*G	E QTP	6	46	Allopatric divergence between lineages from different parts of QTP	Cheng et al. (2009)
H. rhamnoides ssp. *yunnanensis*	*trn*L-*trn*F; *trn*S-*trn*G; ITS	E QTP	15	171	Allopatric divergence in the Pliocene/Pleistocene; no range expansion	Jia et al. (2012)
H. tibetana	*trn*T-*trn*F	E, S QTP	37	891	Allopatric divergence in the Pliocene/Pleistocene; range expansion in S QTP in the Pleistocene	Wang et al. (2010)
H. tibetana	*trn*L-*trn*F; *trn*S-*trn*G; ITS	E, S QTP	21	183	Allopatric divergence between lineages from different parts of QTP; range expansion in E QTP in FG; range expansion in E and S QTP after LGM	Jia et al. (2011)

Chsi—chalcon synthase intron (nuclear gene). E, N, S, QTP—east, north, and south Qinghai–Tibetan Plateau; SE, NW Eur—southeast, northwest Europe; NA—northern Alps; NEC—northeast China. CRE—contiguous range expansion; HDE—historical demographic expansion. EG—Early Glacial (114-74 ky); FG—Full Glacial (74-14.6 ky); LGM—Last Glacial Maximum (24-14.6 ky), LIP—Last Interglacial Period (125-114 ky). Dates of geological epochs are according to Tzedakis et al. (2013)

multiple refugia. The authors of this study also report contiguous range expansion from three main putative glacial refugia identified in their analyses.

Similar to the study of Meng et al. (2008), analyses of phylogeographic patterns in chlorotypes of *H. gyantsensis* and *H. rhamnoides* subsp. *yunnanensis* by Cheng et al. (2009) did not reconstruct clades of haplotypes, which would be concordant with morphological taxa. Their data could not therefore distinguish between two alterative hypotheses regarding origin of *H. gyantsensis*: through homoploid hybridization or allopatric speciation. These authors explained their results by complex maternal lineage sorting between the studied taxa. They also argued that the unique haplotypes recovered in separate populations from each of the taxa they sampled might indicate maintainance of both these taxa in multiple refugia across their ranges during the LGM.

Wang et al. (2010) studied phylogeography of *H. tibetana* across most of the range of the species and revealed three main clades of chlorotypes, reconstructed from sequences of cpDNA *trn*T-*trn*F region. These clades were geographically structured within eastern, southeastern, and western groups of populations along respective edges of QTP. Results of this study suggested existence of multiple microrefugia of the species across its geographical range during the LGM and even earlier glaciations. Besides, the putative LGM microrefugia of *H. tibetana* may have been maintained at 4000 m above sea level and higher, the highest of all known and reported so far refugia at the global scale. Results of this study further supported the theory of the recent and rapid uplift of the QTP. The support was based on estimates of divergence times among and within the three lineages (from the problematic secondary calibrations, however), distinct geographic structures of the lineages, and general co-occurrence of these lineages with landscape-determined dispersal barriers. The authors concluded that uplift of the plateau, which likely was especially rapid starting from the late Pliocene (about 3.5 Ma), and the associated with this process climatic changes may have affected the dispersal and differentiation of *H. tibetana* and shaped its phylogeographic structure.

Populations of *H. tibetana* from approximately the same areas as in the study of Wang et al. (2010) were sampled by Jia et al. (2011), but these authors sequenced different genes in their analyses (*trn*S-*trn*D and *trn*L-*trn*F from cpDNA; ITS from nDNA; Table 14.3). They found two main clades with strong geographical structure. For most populations in both eastern and western regions they found a single widespread chlorotype. This pattern indicated a recent postglacial expansion within each region, while mismatch analyses of all chlorotypes within the eastern group of populations suggested an earlier regional expansion before the LGM. Existence of different chlorotypes across populations indicated possibility of multiple refugia in both regions. Coalescent tests rejected the hypothesis of origin of extant populations from a single refugium during the LGM, but supported hypothesis of diversification of the two main lineages before the late Pleistocene. Similarly to Wang et al. (2010), Jia et al. (2011) concluded that the patterns they found indicated evolutionary response in the species to orogenic processes and the climate changes on QTP and around it in the Quaternary.

Finally, Jia et al. (2012) collected populations across most of the ranges of *H. rhamnoides* focusing their sampling on east QTP and two most eastern subspecies of this species, subsp. *sinensis* and *yunnanensis* (41 populations from these two taxa). Applying phylogeographic analyses of both cpDNA (*trn*L-F and *trn*S-D) and nDNA (ITS) genomic sequences simultaneously, they identified (i) the most likely ancestral areas for *H. rhamnoides* subsp. *sinensis* and *yunnanensis*; (ii) a range expansion in subsp. *sinensis* (dated by a secondary calibration for mean substitution rate, it was estimated to have occurred before the LGM); and (iii) possible hybrid origin of subsp. *mongolica* from a putative cross between subsp. *sinensis* (as a seed parent) and subsp. *turkestanica* (as a pollen parent). These authors further argued that the Quaternary climatic oscillations are likely to have fragmented the distribution of *H. rhamnoides* and triggered allopatric divergence and the formation of strongly differentiated clades within the species.

In conclusion, the published phylogeographic studies of different species of *Hippophae* at two extremes of the range of this genus (Europe and QTP with surrounding areas) revealed several main patterns, which likely reflect responses of these species to the Quaternary climatic fluctuations (Table 14.3). Both allopatric lineage divergence, likely during periods of climatic deterioration, and population range expansion, likely during periods of favorable for *Hippophae* climate, have been indicated by phylogenetic and demographic analyses. Throughout periods of unfavorable climate, populations of *Hippophae* could be maintained in multiple isolated microrefugia some of them at very high altitudes across QTP. Although nearly synchronized timing of population expansions has been identified in Europe (Bartish et al. 2006), it is not clear if population expansions were synchronized in the late Quaternary across different mountain ranges of Eurasia. Currently the data, which could allow comparisons between demographic and population genetic processes in taxa from different regions, are not available, are from different molecular markers, or lack resolution.

14.2.4 Homoploid Interspecific Hybridization in the Genus

Table 14.4 provides a summary of all reported so far putative hybridizations in the genus. As has been discussed above (see "Systematics and Taxonomy"), Lian et al. (1995) introduced new names: *Hippophae goniocarpa* subsp. *goniocarpa* and *H. goniocarpa* subsp. *litangensis*. They further suggested, based on morphological characters and unpublished isozyme data, that these two taxa had their origin through hybridization between *H. rhamnoides* subsp. *sinensis* /*H. neurocarpa*, and *H. rhamnoides* subsp. *yunnanensis/H. neurocarpa* subsp. *stellatopilosa*, respectively. This hypothesis was supported in analyses of RAPD markers by Bartish et al. (2000a) and ITS sequences by Sun et al. (2002). However, these studies were unable to specify direction of the crosses. Bartish et al. (2002) used a cladistic approach and cpDNA RFLPs in their phylogenetic analyses of all taxa in the genus, including two subspecies of *H. goniocarpa*, described by Lian et al. (1995).

Table 14.4 Taxa of putative hybrid origin and hybridization events, suggested by analyses of molecular markers

Taxon	Seed parents	Pollen parent	Source of data	References
H. goniocarpa	*H. rhamnoides* ssp. *sinensis* or *H. neurocarpa* ssp. *neurocarpa*	*H. rhamnoides* ssp. *sinensis* or *H. neurocarpa* ssp. *neurocarpa*	Morphology and isozymes	Lian et al. (1995)
	H. rhamnoides ssp. *sinensis* or *H. neurocarpa* ssp. *neurocarpa*	*H. neurocarpa* ssp. *neurocarpa* or *H. rhamnoides* ssp. *sinensis*	RAPD	Bartish et al. (2000a)
	H. rhamnoides ssp. *sinensis* or *H. neurocarpa* ssp. *neurocarpa*	*H. neurocarpa* ssp. *neurocarpa* or *H. rhamnoides* ssp. *sinensis*	ITS	Sun et al. (2002, 2003)
	H. rhamnoides ssp. *sinensis*	*H. neurocarpa* ssp. *neurocarpa*	cpDNA RFLP	Bartish et al. (2002)
	H. rhamnoides ssp. *sinensis* and *H. neurocarpa* ssp. *neurocarpa*		*trn*L-F	Wang et al. (2008a)
H. litangensis	*H. rhamnoides* ssp. *yunnanensis* or *H. neurocarpa* ssp. *stellatopilosa*	*H. rhamnoides* ssp. *sinensis* or *H. neurocarpa* ssp. *stellatopilosa*	Morphology and isozymes	Lian et al. (1995)
	H. rhamnoides ssp. *yunnanensis* or *H. neurocarpa* ssp. *stellatopilosa*	*H. neurocarpa* ssp. *stellatopilosa* or *H. rhamnoides* ssp. *yunnanensis*	RAPD	Bartish et al. (2000a)
	H. rhamnoides ssp. *yunnanensis* or *H. neurocarpa* ssp. *stellatopilosa*	*H. neurocarpa* ssp. *stellatopilosa* or *H. rhamnoides* ssp. *yunnanensis*	ITS	Sun et al. (2002)
	H. rhamnoides ssp. *yunnanensis*	*H. neurocarpa* ssp. *stellatopilosa*	cpDNA RFLP	Bartish et al. (2002)
H. rhamnoides ssp. *mongolica*	*H. rhamnoides* ssp. *sinensis*	*H. rhamnoides* ssp. *turkestanica*	*trn*L-F, *trn*S-D, ITS	Jia et al. (2012)
Undescribed	Undescribed "*Grand Cannyon*"[a]	*H. rhamnoides* ssp. *yunnanensis*	*trn*L-F, *trn*S-D, ITS, *Chs*i	Jia et al. (2012); Xu et al. (personal communication, 2013)
Undescribed	Undescribed "*Grand Cannyon*"[a]	*H. gyantsensis*	*trn*L-F, *trn*S-D, ITS, *Chs*i	Jia et al. (2012); Xu et al. (personal communication, 2013)

(continued)

Table 14.4 (continued)

Taxon	Seed parents	Pollen parent	Source of data	References
Undescribed	*H. gyantsensis*	*H. rhamnoides* ssp. *yunnanensis*	*trn*L-F, *trn*S-D, ITS, *Chs*i	Jia et al. (2012); Xu et al. (personal communication, 2013)
Undescribed	*H. gyantsensis*	*H. neurocarpa* ssp. *stellatopilosa*	*trn*L-F, *Chs*i	Xu et al. (personal communication, 2013)

[a]"*Grand Cannyon*" is an informal name introduced by Dr. Kun Sun for currently undescribed cryptic taxon of *Hippophae,* revealed by recent molecular analyses of Jia et al. (2012) as a part of *H. rhamnoides* subsp. *yunnanensis sensu lato*

This study confirmed hybrid origin of these taxa, identified their putative parents, and suggested direction of crosses. *H. rhamnoides* subsp. *sinensis* and *yunnanensis* were identified as seed, while *H. neurocarpa* subsp. *neurocarpa* and *stellatopilosa* as pollen parents of subsp. *goniocarpa* and *litangensis*, respectively. Based on these results, Bartish et al. (2002) suggested raising the hybrid taxa to species level under new combinations: *H. goniocarpa* Y.S. Lian et al. ex Swenson & Bartish and *H. litangensis* Y.S. Lian & X.L. Chen ex Swenson & Bartish. These species were accepted in general by Swenson and Bartish (2002), but with a precautionary note that several morphological characters were not available for morphological studies of these hybrid species. Sun et al. (2003) confirmed hybrid origin of *H. goniocarapa* in analyses of ITS sequences, and suggested that hybridization between *H. rhamnoides* subsp. *sinensis* and *H. neurocarpa* was recent. Besides, their results indicated multiple hybridization events in origin of *H. goniocarpa*. In analysis of *trn*L-F sequences in 75 individuals of *H. goniocarpa*, *H. rhamnoides* subsp. *sinensis* and *H. neurocarpa* subsp. *neurocarpa* from two cites with sympatrically grown populations of these taxa, Wang et al. (2008a) revealed individuals of *H. goniocarpa* with cpDNA from both putative parental species. Their results thus confirmed the idea of multiple hybridization events in origin of this species. Thorough analyses of multiple morphological, cpDNA and nuclear markers in a representative sample of individuals of *H. goniocarpa* and *H. litangensis* from several localities are clearly necessary to better understand evolution of these species. Such analyses could allow settling the argument of earlier studies regarding the status of the taxa, which originally were described under these names (Lian et al. 1995; Bartish et al. 2002, Swenson and Bartish 2002). According to results of Sun et al. (2003) and Wang et al. (2008a), it is unlikely that *H. goniocarpa* is a genetically stable taxon, as suggested by Lian et al. (1995). More likely, it can be an evolutionary recent hybrid swarm of ephemeral populations. These populations should be of very different genetic backgrounds and originate from zones of secondary contact of numerous populations of the putative parental species. In the

latter case circumscription of all these hybrid populations and individual plants in two subspecies of the same species, as suggested by Lian et al. (2003b), will be problematic.

Molecular analyses revealed several additional putative hybrid taxa in *Hippophae*. All these taxa are cryptic hybrids, since no earlier taxonomic studies indicated their hybrid origin. Results reported by Jia et al. (2012) from analyses of sequences of two loci of cpDNA and ITS from a representative sample of populations of *H. rhamnoides* subsp. *mongolica*, *turkestanica*, and *sinensis* indicated possibility of hybrid origin of subsp. *mongolica.* A single chlorotype of this taxon was reconstructed as sister to a clade of multiple chlorotypes of subsp. *sinensis*. However, two of ITS haplotypes of *H. rhamnoides* subsp. *mongolica* created a clade sister to a clade of haplotypes of subsp. *turkestanica*, and another two ITS haplotypes were included within the clade of subsp. *turkestanica*. The incongruence in phylogenetic relationships between chloroplast and nuclear genomes of the three Asian subspecies of *H. rhamnoides* was further confirmed in analyses of a larger sample of five genes from each of the two genomes (cpDNA and nDNA) by Jia (2013). This study provides support to the hypothesis of an ancient and likely unique hybrid origin of *H. rhamnoides* subsp. *mongolica* and dismisses the hypothesis of incomplete lineage sorting as a possible explanation for the incongruence between phylogenetic reconstructions. Given the monophyly of both genomes of *H. rhamnoides* subsp. *mongolica* reconstructed in this study, currently wide but isolated range of the subspecies, and distinct climatic differentiation from each of the two putative parental taxa, this taxon should be considered as an ancient and stable hybrid with unique origin and ecological adaptations.

Several poorly characterized putative hybrid taxa were further indicated by analyses of Jia et al. (2012). One of them can be a cryptic taxon, currently identified as *Hippophae rhamnoides* subsp. *yunnanensis*. This taxon was represented by a group of populations from south QTP, where the flow of the Brahmaputra River turns from west–east to north–south direction. The provisional name of this taxon is "Grand Canyon," reflecting its currently identified range. The group of populations under this name was characterized by a clade of chlorotypes (clade C), which was sister to a clade of all subspecies of *H. rhamnoides* excluding subsp. *yunnanensis*. The clade of ITS haplotypes sampled from the same group of populations (clade II) was, however, sister to all subspecies of *H. rhamnoides* including subsp. *yunnanensis*. These results made taxonomic status of *H. rhamnoides* subsp. *yunnanensis* unclear and beg for thorough analyses of morphological characters in specimens from different populations, currently circumscribed within this taxon. Finally, results of Jia et al. (2012) revealed also putative hybridization between *H. gyantsensis* and *H. rhamnoides* subsp. *yunnanensis*. This hybridization was indicated by presence in individuals of population from Bomi, Xizang, of chlorotypes from clade A, which was later identified by Jia and Bartish (unpublished) as one of cpDNA clades of *H. gyantsensis*, and ITS haplotypes from clade III, representing *H. rhamnoides* subsp. *yunnanensis* sensu stricto. Interestingly, individuals with chlorotypes of clade A were also found in the population from Litang, which was defined as *H. rhamnoides* subsp. *yunnanensis* by Jia et al. (2012), although all ITS

haplotypes from this population belonged to a clade of haplotypes of *H. gyantsensis* (clade I). Adding to this confusion, the Litang population is located in the county referred to as locality of origin of the type of *H. litangensis* (Lian et al. 1995), a putative hybrid species, for which *H. rhamnoides* subsp. *yunnanensis* and *H. neurocarpa* subsp. *stellatopilosa* were suggested as parental taxa (see above). Hybridization between *H. gyantsensis* and *H. rhamnoides* has not been suggested in earlier studies in the genus. Populations from Bomi and Litang obviously need further investigation as localities of putative interspecific hybridizations, in which several species of *Hippophae* can be involved.

14.3 Biotechnological Advancements in *Hippophae Rhamnoides*

14.3.1 Assessment of Gene Bank Collections

Breeding programs of different crops are often focused on several agricultural traits most important for current economically efficient production, such as high yield and resistance to variable diseases and pests. Gene bank collections of crops represent an important part of breeding programs and are developed to conserve a wide range of naturally available traits together with achievements of past generations of breeders (Jeppsson et al. 1999; Nybom et al. 2003). Unfortunately, many traits are not easily determined on living plants, pedigree information on origin of cultivars is often lacking, and accessions are sometimes mislabeled. Different kinds of relatively cheap, easy-to-generate, and highly polymorphic molecular markers have been developed (Weising et al. 1994) and can help to improve effectiveness of using gene bank accessions (i.e., Hokanson et al. 1998). Analyses of genetic relatedness among accessions of a germplasm collection can provide valuable information on genetic background of different cultivars, helping to develop conservation strategies for preservation of important agricultural traits. Effectiveness of breeding programs can be improved using parental varieties with clearly defined taxonomic and geographic origins and highly resolved genetic relationships to other cultivars. Assessment of collections of cultivated varieties by molecular markers can also help to keep track of certified cultivars and prevent their illegal distribution (Congiu et al. 2000).

Modern breeding programs in sea buckthorn are relatively young and even the earliest of them from the ex-Soviet Union started only in the second half of the past century (Kalinina and Panteleyeva 1987). Nevertheless, a relatively large number of cultivars have been developed by now (Trajkovsky and Jeppsson 1999); for a list of cultivars from Lisavenko Institute of Horticulture for Siberia, Barnaul, the pioneer and one of the leaders of sea buckthorn breeding, see Zubarev et al. (2014). One of the most diverse gene banks of sea buckthorn varieties in the world was established at Balsgård, Department of Horticultural Plant Breeding of Swedish Agricultural

University (Bartish et al. 2000b). Assessment of genetic diversity in a subset of this collection (55 varieties) by RAPD analysis revealed three main groups of genotypes (Bartish et al. 2000b). The first of these groups represented Siberian cultivars with genetic background almost exclusively in *Hippophae rhamnoides* subsp. *mongolica* from Altai region. The second group included hybrids between cultivars from breeding programs at the Moscow Lomonosov University. In these programs breeders used selections of wild specimens of *H. rhamnoides* subsp. *rhamnoides* (mostly from the eastern Baltic coast) as one of the parents, and cultivars from Siberia as the second parent. Finally, the third group represented a mix of wild accessions and cultivars from crosses involving *H. rhamnoides* subsp. *carpatica*, *caucasica*, *fluviatilis*, and *rhamnoides* from Central and Eastern Europe and from Caucasus, and hybrids of these varieties. Although the three groups were significantly differentiated from each other, the major part of molecular variance (approximately 75 %) in the analyzed set of accessions was found within taxonomic or geographic groups. This study demonstrated the utility of molecular markers for clarification of geographic and taxonomic origin of accessions and cultivars of sea buckthorn.

Analyses of 14 cultivars involved into Chinese breeding programs using RAPD (Ruan et al. 2004), or the same cultivars plus one additional using AFLP molecular markers (Ruan and Li 2005, Ruan 2006) revealed strong presence of genetic component of *Hippophae rhamnoides* subsp. *mongolica* in these breeding programs. Nine of the analyzed cultivars were obtained from Russia and Mongolia (all represented *H. rhamnoides* subsp. *mongolica*). Among the remaining cultivars three represented hybrids between *H. rhamnoides* subsp. *mongolica* and *sinensis*, and the other three–*H. rhamnoides* subsp. *sinensis* (Ruan et al. 2004, Ruan and Li 2005). All three cultivars representing *H. rhamnoides* subsp. *sinensis* and all three hybrids were placed by cluster analysis of RAPD profiles either between two clusters of cultivars each representing *H. rhamnoides* subsp. *mongolica*, or among cultivars from this taxon. Besides, at least in the case of cultivars Hongguo (*H. rhamnoides* subsp. *sinensis*) and Hongyun (*H. rhamnoides* subsp. *mongolica*), representatives of two subspecies were difficult to distinguish from each other by phenotypic characters, and authors doubt that cultivar Zhongguoyou really represents subsp. *sinensis* (Ruan and Li 2005). These results thus indicated that all analyzed cultivars were hardly differentiated genetically from *H. rhamnoides* subsp. *mongolica*. Authors explained the unexpected placement of Chinese cultivars by geographic origin of the putative representatives of *H. rhamnoides* subsp. *sinensis* from Inner Mongolia, where natural hybridization between two subspecies might occur (Ruan and Li 2005). However, the gap between ranges of these subspecies is currently wide (Rousi 1971; Jia 2013). Neither RAPD (Bartish et al. 2000a), nor sequencing analyses (Jia et al. 2012) of representative samples of natural populations of two subspecies indicate recent hybridization between these taxa. Instead, these analyses revealed clear genetic differentiation between natural populations of *H. rhamnoides* subsp. *mongolica* and *sinensis*.

In a follow-up study from the same laboratory, 52 accessions from a wider taxonomic range of cultivars representing *Hippophae rhamnoides* subsp. *mongolica*,

rhamnoides, *sinensis*, and *H. salicifolia* were analyzed by ISSR markers (Ruan et al. 2009). These analyses demonstrated utility of ISSR markers for assessment of breeding collections of sea buckthorn and confirmed strong representation of *H. rhamnoides* subsp. *mongolica* in Chinese breeding programs. In addition, these markers support higher genetic polymorphism in Siberian cultivars relatively to Chinese selections (Liu et al. 2007).

Assessment of genetic relationships among selections from native stands in other subspecies of *Hippophae rhamnoides* has been so far scarce. Shah et al. (2009) used AFLP markers in analyses of 25 ecotypes from ten localities in northern Pakistan, representing *Hippophae rhamnoides* subsp. *turkestanica*. The ecotypes were clustered in three groups, which did not correspond to geographic or climatic distances between the localities. The authors explained this result by high levels of seed dispersal in the area by birds and river flows. In analyses of RAPD markers in ten selections of *Hippophae rhamnoides* subsp. *caucasica* from Erzurum province of Turkey Ercisli et al. (2008) reported high polymorphism in these accessions (92.2 %). All investigated genotypes were clearly differentiated by Jaccard's similarity indices. Authors of this study also reported lack of congruence between fatty acid composition and RAPD-based genetic relationships among the ten selections. Simon-Gruita et al. (2012) analyzed five accessions of natural populations of *H. rhamnoides* subsp. *carpatica* and five cultivars from the breeding programs in Romania. These authors used RAPD markers and found a relatively close relationship between natural populations grown at the same altitude. They also concluded that Romanian commercial varieties resulted mainly from recent selections from natural populations with little indication of coherent breeding programs in their genetic composition. Finally, a preliminary analysis of exotic naturalized populations of *H. rhamnoides* in Canada by Chowdhury et al. (2000) using RAPDs suggested relatively high levels of Shannon's phenotypic diversity in these populations. Estimates of the diversity index for this species were comparable with corresponding estimates of diversity in native populations of co-familial buffaloberry (*Shepherdia argentea*), and higher than diversity of another native representative of Elaegnaceae, silverberry (*Elaeagnus commutata*). Authors of this study do not specify, however, which subspecies of *H. rhamnoides* naturalized in the area of their collections (southwestern Saskatchewan).

In conclusion, assessment by molecular markers of numerous gene bank collections of cultivated sea buckthorn in different regions of the world reveals a relatively narrow taxonomic representation of *Hippophae rhamnoides* in many of these collections. The most productive so far Asian breeding programs in China (Huang 1995) and Russia (Zubarev 2014) have been focusing mostly on introgression into cultivars of genetic material from a relatively small part of the range of *H. rhamnoides* subsp. *mongolica* (it was mainly derived from Altai region). European cultivars were mostly represented by progenies of *H. rhamnoides* subsp. *rhamnoides* and by hybrids between these progenies and Siberian cultivars (Bartish et al. 2000b).

14.3.2 Molecular Breeding and Development of Genetic Markers of Important Traits

Marker-assisted selection (MAS) of superior varieties with combination of economically important traits can be an efficient way of introgression of useful genetic material from wild populations into breeding programs (Tanksley 1993; Xiao et al. 1998). Genetic maps of linkage groups of traits of interest with variable molecular markers can be a useful tool in successful molecular breeding. However, development of mapping populations for specific traits in trees and shrubs and in young crops such as sea buckthorn is time-consuming and laborious. Besides, success of MAS largely depends on the extent of genetic linkage between markers and relevant QTL loci (Virk et al. 1996). To increase the resolution of the approach and the likelihood of linkage between markers and traits of interest, saturation of linkage maps should be as high as possible. High levels of map resolution require large number of markers, which preferably should also be transferable between mapping populations for different traits. Co-dominant markers, such as SSRs and single nucleotide polymorphisms (SNPs) are relatively evenly distributed throughout genome (Akkaya et al. 1995), abundant, and highly polymorphic (Morgante and Olivieri 1993). Besides, these markers are also relatively stable and therefore express high levels of transferability between mapping progenies from the same species (Antanaviciute et al. 2012; Fernandez-Fernandez et al. 2012). Due to these characteristics SSRs and SNPs have been the main tools in many mapping projects. Unfortunately, development of these markers requires sophisticated personnel and expensive laboratory equipment, and has so far been both expensive and laborious (Gupta and Varshney 2000). This could be the main reason for the current lack of genetic maps in *Hippophae rhamnoides*. Fortunately, new technologies are being currently developed, which allow creation of highly saturated genetic maps even in tree species with relatively large genomes as in *Malus* from Rosaceae (Antanaviciute et al. 2012). These technologies hold great promise for considerable improvement in efficiency, and decreasing the costs of generation of linkage maps in young and minor crops without sequenced genomes. First primers for amplification of microsatellite markers have been already reported for *H. rhamnoides* subsp. *sinensis* (Wang et al. 2008b) and subsp. *turkestanica* (Jian et al. 2010; Jian et al. 2014), and development of thousands of SSRs using next-generation sequencing (NGS) is underway (Ghanghal et al. 2013). Until now, however, MAS in *Hippophae rhamnoides* has been focused on generation of molecular markers for a few important traits under simple genetic control, such as sex determination (Persson and Nybom 1998; Sharma et al. 2010; Korekar et al. 2012) and dried-shrink disease resistance (Ruan et al. 2009; Li et al. 2010).

Markers of genetic sex determination can be used for discrimination between male and female plants of *H. rhamnoides* at early stages of development. The first of attempts to obtain these markers used RAPD analyses and revealed a male-specific marker (OPD15-600) in one of two progenies of controlled crosses (Persson and Nybom 1998). Both parents of the first progeny were commercial

varieties from wild material selected in eastern Germany, within the range of *H. rhamnoides* subsp. *rhamnoides*. The marker was amplified in all 17 male plants and was absent in all 17 female plants of this progeny, used in the analyses. However, the marker was present in only one male plant of eight tested in the second progeny. The female parent of this progeny was derived from an open pollinated population of a Russian cultivar Dar Katuni, which was selected from plant material from the Altai, within the range of *H. rhamnoides* subsp. *mongolica*. The male parent was collected from a wild population of *H. rhamnoides* subsp. *rhamnoides* in Kilskiir, Uppland, Sweden. The different geographic and taxonomic origins of parents of these two crosses and high levels of mutations in RAPD loci (which decreases transferability of these markers between phylogenetically distant genomes) can be the main reason for the failure to amplify marker OPD15-600 in most males of the second progeny.

Sharma et al. (2010) obtained two gender-specific markers in isozyme and RAPD analyses of five female and five male plants of *Hippophae rhamnoides* subsp. *turkestanica* from Sunnam and Kinnaur districts of Himachal Pradesh, at the southeastern edge of the range of this taxon (I. V. Bartish, personal observation, 2013). One of these markers was a female-specific allele of peroxidase, and the other–a male-specific amplification of OPD20-911. However, the very low numbers of the tested in these analyses plants make utility of the developed markers uncertain.

In a more recent study, Korekar et al. (2012) reported development of sequence-characterized amplified region (SCAR) markers of genetic sex determination in *Hippophae rhamnoides* subsp. *turkestanica.* The analyzed plant material was collected from natural populations in Ladakh region. Authors of this study found consistent amplification of female-specific (FS) polymorphic fragments of 1,164, and 868 bp, named as OPA-04(FS) and OPT-06(FS), respectively. The fragments were cloned, sequenced, and specific to these fragments primers were developed. One of the fragments, OPA- 04(FS), was found to be 54 % similar with a hypothetical oxidoreductase and the other, OPT-06(FS), with BNR/Asp-box repeat domain containing protein of *Pyrenophora tritici-repentis* Pt-1C-BFP. These markers were tested on 120 female and 100 male plants (each from different locality) and showed consistent gender specificity. These results suggest that the markers developed by Korekar et al. (2012) can be a promising tool in detection of gender in plants of *H. rhamnoides* subsp. *turkestanica* at early stages of their development. However, it remains to be seen if these markers can be applied successfully across the whole taxonomic and geographic ranges of the species.

The second agronomical trait of sea buckthorn, which received considerable interest in molecular breeding studies, was dried-shrink disease (DSD) resistance. The disease is caused by several species of *Fusarium* and *Phomopsis* (Ruan et al. 2010), and work on development of molecular markers of resistance and susceptibility to this disease has started recently (Ruan et al. 2009; Li et al. 2010). Ruan et al. (2009) reported a significant correlation with resistance to DSD ($P < 0.001$) for four ISSR markers (809_{290}, and $811_{280,}$ 835_{700}, and 887_{190}) among analyzed accessions of cultivars and varieties from Chinese breeding programs. They

suggested using these markers in a selection of lineages resistant to DSD, when no other genetic information is available. Li et al. (2010) estimated variability of sequence-related amplified polymorphism (SRAP) markers in 77 accessions of 22 cultivars of *H. rhamnoides*, most of which were used in an earlier study by Ruan et al. (2009). They searched for association of these markers with DSD resistance using multiple regression analysis and revealed 11 SRAPs significantly ($P < 0.001$) correlated with this trait. These authors suggest application of the obtained markers for screening of resistant and susceptible to DSD genotypes at early stages of plant development. The proportion of known genes and co-dominant loci is relatively high in the total pool of SRAPs (Li and Quiros 2001). These markers should therefore express high levels of transferability between closely related taxa (such as subspecies of *H. rhamnoides*), and could be a more efficient alternative to taxon-specific AFLPs, ISSRs, and RAPDs.

14.3.3 Gene Cloning

Cloning of genes controlling expression of agronomical traits can considerably advance molecular breeding (Glick et al. 2010). For example, genes of traits under simple genetic control, as resistance to some diseases and pests (Gusberti et al. 2013), control of ripening (Barry et al. 2006), or tree architecture (Dardick et al. 2013) can be transferred to, or specifically blocked in selected genomes, triggering expression of the desired traits in superior cultivars. However, the task can be complicated, if genes controlling the traits of interest are not known or have not been sequenced. Identification of these genes usually requires availability of mapping populations with segregation of the trait of interest, and highly resolved genetic maps (Collard et al. 2005). As already mentioned, these maps are not available for sea buckthorn yet. Nevertheless, the first steps in direction for better understanding of expression of specific traits at the level of individual genes have been made also in *Hippophae*. Xu et al. (2009) studied adaptation to drought stress in seedlings of *H. rhamnoides* from Jiuzhai population. This locality is in the range of *H. rhamnoides* subsp. *sinensis*, according to the data from Jia et al. (2012). Xu et al. (2009) used two-dimensional electrophoresis and mass spectrometry for identification of drought-associated proteins in this population. They reported 13 drought stress-responsive proteins, four of which have not been found in higher plants. Functions of J-type co-chaperone (Hsc20), a putative ABC transporter ATP-binging protein, a probable nitrogen regulation protein (NtrX), and heat shock protein (HslU) were predicted either from their conserved domains or homologies to other organisms. Results of this study elucidate mechanisms of responses to drought stress in plants and could potentially open a way to cloning of genes involved in stress tolerance in sea buckthorn.

Ghangal et al. (2012) constructed a cDNA library of clones of *Hippophae rhamnoides*. Authors of this study do not indicate which subspecies was used in their analyses, but they state that leaf tissue for this work was sampled from plants

growing at Defense Institute of High Altitude Research, Leh, Ladakh. Because most populations from this region belong to *H. rhamnoides* subsp. *turkestanica* (Raina et al. 2012; I. V. Bartish, personal observations, 2013), the reported cDNA library was most likely created from genetic material of this taxon. The library comprises 3412 Expressed Sequence Tags (ESTs) and 1665 unigenes, of which 1278 were annotated by similarity search. Ghangal et al. (2012) reported identification of 43 unigenes responsive to biotic and abiotic stresses. The change in expression pattern under cold/freeze stresses was examined by real-time PCR in 13 of these genes, which earlier were shown to be associated with cold stress tolerance in *Arabidopsis*, and in three novel transcripts. These transcriptome analyses revealed dramatic changes in expression patterns in *H. rhamnoides* (3–12 fold increases) under freezing temperatures for *Pathogenesis Related Thaumatin*, *Chitinase*, *Rare Cold-Inducible* 2A, and *High Mobility Group* B2 genes. Authors suggest that further investigations might help in establishing some of the identified cold and freeze responsive genes as valuable resources for developing cold-resistant crop plants. In particular, breeding of better adapted to environmental stress cultivars of sea buckthorn might benefit from involvement of these and other associated with tolerance or resistance to cold and freeze genes into breeding programs. For example, Gupta et al. (2009) cloned partial cDNA (689 bp) of glycerol-3-phosphate acyltransferase (GPAT) and suggest that full-length cloning and overexpression of this gene could help cold-sussceptible plants to protect their photosynthetic machinery from photoinhibition under cold conditions and better resist freezing temperatures.

NGS techniques and methods have been used intensively in the past decade to generate a large amount of genetic data (Mardis 2008). Although these approaches are especially efficient in analyses of annotated genomes, they also hold great promise of considerable increase in efficiency of genetic analyses in non-model species. Fatima et al. (2012) applied high-throughput 454 sequencing methodology to uncover genes related to oil biosynthesis, other important metabolic pathways, and stress-response pathways in *Hippophae rhamnoides* subsp. *mongolica* (seeds of RC-4 cultivar from a Canadian breeding program were used in this study for RNA extractions). These authors reported identification of 89,141 putative unigenes in their dataset and most of the genes involved in fatty acid biosynthesis. In particular, Fatima et al. (2012) identified sequences for most enzymes involved in the biosynthesis and elongation of fatty acids (with the exception of ACP-S-malonyl transferase). They also identified genes involved in isoprenoid biosynthesis (113 sequences) and abiotic stress-related genes (gene ontology category "response to stress" resulted in 1525 sequences). The carotenoid biosynthesis genes were abundantly represented in the seed transcriptome, as would be expected. The largest number of sequences of stress-related genes fell within the heat, oxidative, osmotic, and cold stress categories, followed by wounding, DNA damage and water deprivation categories. Heat shock proteins were the most highly represented set in the mature seed transcriptome of the species. Fatima et al. (2012) concluded that their dataset forms a comprehensive genomic resource for sea buckthorn and establishes a basis for dissecting metabolic pathways related to the formation of oil and

bioactive components. They also suggested that by using knowledge of the metabolic pathways, new strategies can be developed to enhance the agricultural production of variable desirable compounds. These compounds could potentially be utilized in pharmacology, cosmetic, and nutritional industries.

The promising perspectives of NGS technologies in analyses of transcriptome composition of sea buckthorn were further confirmed by a recent study of Ghanghal et al. (2013). Authors of this work used for their sequencing analyses RNA extracts from leaf and root tissues of plants from the same region as in the study of Ghangal et al. (2012). Applying Illumina HiSeq 2000 platform, six frequently used short read assemblers, and following two different strategies to select the best transcriptome assembly, Ghanghal et al. (2013) reported de novo short read assembly of 88,297 transcripts (>100 bp), representing about 53 Mb of sea buckthorn transcriptome and their functional annotation of this transcriptome revealed conservation of genes involved in various biological processes.

The studies by Fatima et al. (2012) and Ghangal et al. (2013) demonstrated the power of NGS for gene function discovery in *Hippophae*. The transcriptome data generated in these studies could provide a valuable resource for gene discovery and development of functional molecular markers in sea buckthorn breeding programs. By widening the taxonomic range of transcriptomes analyzed by NGS methods, further studies should advance our understanding of how genetic variability within *Hippophae* is translated into the incredibly high adaptability of its different species to abiotic stress and biotic pressure. This knowledge can be especially useful for efficient engineering of highly productive cultivars with wide range of adaptations to different environments of cultivation.

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