

Genetic structure in fragmented populations of *Hippophae rhamnoides* ssp. *sinensis* in China investigated by ISSR and cpSSR markers

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Abstract *Hippophae rhamnoides* ssp. *sinensis* is an ecologically and economically important species that has been widely used as a pioneer plant in China. In this study, we employed both nuclear ISSR and maternal cpSSR markers to survey the genetic diversity and structure of populations of ssp. *sinensis* representing three different landscapes, the northwestern desert and grassland region, the alpine vegetation region of the Qinghai-Tibetan Plateau, and the northeastern humid forest region. In all, 12 natural populations with a scattered distribution in the area were studied. The genetic diversities of populations were found to be uneven, and the total genetic diversity was low on the basis of both types of marker. Mantel tests based on both individual Euclidean distance matrices and population genetic distance (measured by Φ_{pt}) matrices showed that the two marker systems detected similar trends with respect to genetic distances between populations. The analysis of molecular variance (AMOVA) revealed significant differentiation among populations and among regions for both types of marker. Although the detected pattern of isolation-by-distance among all sampled populations confirmed the earlier colonization pathway, the low level of gene flow and the lack of isolation by distance within each region suggested the presence of an additional dispersal barrier. UPGMA dendrograms and PCA plots also revealed clear clustering and significant regional differentiation. Our results indicate that the genetic structure of ssp. *sinensis*

has been affected by habitat fragmentation and restricted population sizes. We propose that the biology of reproduction and ecology have played determinant roles in the development of the regional structure of populations. The genetic information obtained will help to establish conservation strategies and programs for sustainable management of *H. rhamnoides* ssp. *sinensis*.

Keywords *Hippophae rhamnoides* ssp. *sinensis* · Genetic variation · Habitat fragmentation · ISSR · cpSSR

Introduction

Hippophae L. is a small genus of Elaeagnaceae. Several scholars have recognized only one species in *Hippophae* (Servettaz 1909; Avdeyev 1983). The “one species” classification was denied by some authors based on the highly variable morphology. However, the systematic treatment and the relationships between taxa in this genus remain controversial, the number of species and subspecies being the focus of this dispute. Rousi (1971) divided the genus *Hippophae* into three species with nine subspecies in the species *H. rhamnoides*. After the revision of Rousi (1971), Liu and He (1978) described a new species, *H. neurocarpa* S. W. Liu and T. N. He, from the Qinghai Plateau. Lian (1988) established two sections and increased the number of taxa to seven species and nine subspecies on the basis of extensive investigations and systemic studies (Lian and Chen 1996; Lian et al. 1997, 1998). However, the taxonomical treatment that divided the genus into two sections was not supported by Sun et al. (2002) on the basis of ITS sequences. According to the latest systematic treatments of the genus *Hippophae* L. (Bartish et al. 2002; Swenson and Bartish 2003), the genus comprises seven species, and the

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species *H. rhamnoides* circumscribes eight subspecies. All species are diploid ($2n = 24$), wind-pollinated, and dioecious, and are restricted to the Qinghai-Tibetan Plateau and adjacent areas, with the exception of the species *H. rhamnoides* L. that occurs widely but sporadically in Asia and Europe (Rousi 1965, 1971; Lu 1997; Lian et al. 2000; Bartish et al. 2000, 2002). Ssp. *sinensis* and ssp. *yunnanensis* from *H. rhamnoides* are endemic to China. Ssp. *sinensis* is found on the eastern edges of the distribution of *H. rhamnoides* and is most widely distributed at China at altitudes ranging from 400 to 3,900 m. Some plants belonging to ssp. *sinensis* also occur at altitudes below 400 m.

Because of its strong ecological adaptability and high tolerance of environmental stresses, especially to extreme conditions, including temperature, drought, high altitude, salinity, alkalinity, and inundation (Lu 1992; Ruan and Li 2002; Sheng et al. 2006), sea buckthorn (*Hippophae*) is a major pioneer species in arid and semiarid zones and can provide a protective windbreak. Sea buckthorn also has enormous nutritional, medical and ornamental value. It is a multi-purpose plant which has been a target of plant breeding programs, mainly in China, Russia, Central Asia and Europe (Yao and Tigerstedt 1993; Aitzetmuller and Xin 1999; Tang and Tigerstedt 2001). However, the accelerated and uncontrolled use of this important resource has led to deforestation in some parts of its natural distribution, with consequent adverse impact on the ecosystem (Tian et al. 2004a). Knowledge of its genetics, especially of the genetic variation and structure of natural populations, is urgently needed to guide the exploitation of this valuable germplasm and breeding programs.

H. rhamnoides ssp. *sinensis* is an ecologically and economically important species that has been widely used as a pioneer plant in China. The major regions of northern China within the Loess Plateau areas, where ssp. *sinensis* occurs, are characterized by orographically diverse landscapes and an array of ridges and ravines. It mainly occurs on sandy soils by river banks or dry river beds, mountain slopes, and valleys in the transition zone of the northwestern desert and grassland region. Plants belonging to ssp. *sinensis* also grow in the marginal areas of the alpine vegetation of the Qinghai-Tibet Plateau and the northeastern humid forest region, where they mix with other trees and form patchy forests. The discontinuous but extensive distribution in diverse environmental conditions, together with the dioecious breeding system and competition with other species, may have contributed to the genetic structure of this subspecies during its long-term evolution. Substantial morphological variations in the fruits, leaves, and chemical components have been detected (Rousi 1971; Zhao et al. 1991; Lian et al. 2000). Several studies based on molecular markers have been

conducted on the genetic diversity and population genetic structure of ssp. *sinensis* (Yao and Tigerstedt 1993; Sun et al. 2006). However, previous studies were performed on the basis of a single type of molecular marker representing either the nuclear or cytoplasmic genome. Comparisons of population diversity and structure represented in different genomes, and patterns of gene flow by seed and pollen in different environments would certainly be an advance compared with previous genetic studies in ssp. *sinensis*.

In this study, we used nuclear ISSR and maternal cpSSR markers to investigate the genetic diversity and structure of populations of ssp. *sinensis* representing three different landscapes. Our primary purpose was to detect the level and pattern of genetic variation of the subspecies, to compare population structures represented in different genomes, and to estimate gene flow in natural populations via different types of propagules. Knowledge of regional differentiation in two marker systems may provide an important insight into the population genetics of ssp. *sinensis* and the effect of eco-environmental and climatic factors on the genetic variation. This knowledge could be useful for conservation and breeding programs.

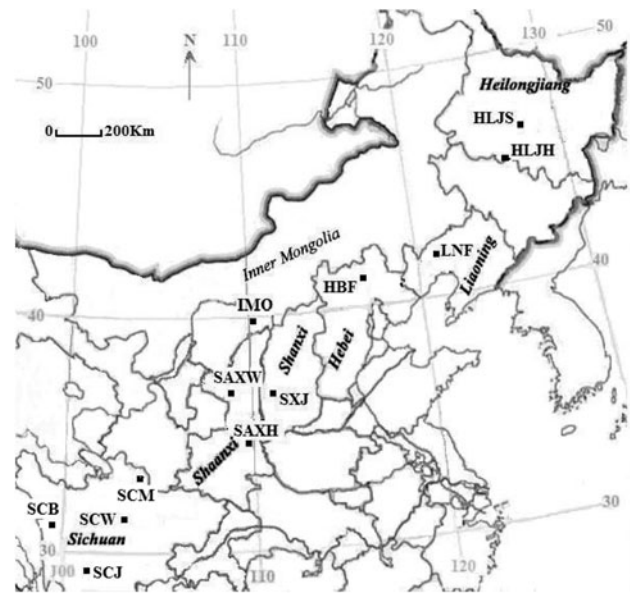
Materials and methods

Plant material

Ssp. *sinensis* has a zonal distribution pattern controlled by rainfall and heat. Its geographical range is roughly located in the transitional zone of three vegetation regions in China, i.e. the northwestern desert and grassland region, the alpine vegetation region of the Qinghai-Tibetan Plateau, and the eastern humid forest region (Lian and Chen 1992). Twelve populations of ssp. *sinensis* which occur in various habitats with different climates and topographies within the major distribution area were selected for the study (Table 1; Fig. 1). The 12 populations were divided into three groups according to their geographical regions and the zonal distribution pattern controlled by heat and rainfall. Region 1 consisted of five populations (IMO, HBF, SAXH, SAXW, and SXJ), collected from the northwestern desert and grassland region. Region 2 comprised four populations (SCJ, SCB, SCM, and SCW) from the alpine vegetation region of the Qinghai-Tibetan Plateau. Three populations (LNF, HLJS, and HLJH) from the northeastern humid forest region were included in region 3. All populations were sampled from natural forests, and sampled stands were 150–3,000 m away from the closest neighboring populations. Population sizes varied widely, from about 200 m² to hundreds hm², as estimated by the eye.

Table 1 Locations, landform feature, geographical data, and ecological variables of the populations of *Hippophae rhamnoides* ssp. *sinensis* surveyed

Region	Location	Population code	Landform feature	Longitude (E)	Latitude (N)	Altitude (m)	Annual Rainfall (ml)	Annual Average temperature (°C)
Northwestern desert and grassland region	Ordos, Inner Mongolia	IMO	Mongolia Plateau	110°01'	39°55'	1,300	400	6.7
	Fengning, Hebei	HBF	Highland of Mongolia Plateau	116°35'	41°20'	1,500	480	6.1
	Huanglong, Shaanxi	SAXH	Hilly and gully region of Loess Plateau	109°53'	35°35'	1,500	602	8.6
	Jiaokou, Shanxi	SXJ	Hilly and gully region of Loess Plateau	111°10'	36°57'	1,600	450	7.0
Alpine vegetation region of the Qinghai-Tibetan Plateau	Wuqi, Shaanxi	SAXW	Hilly and canyon region of Loess Plateau	108°11'	36°57'	1,700	473	7.8
	Baiyu, Sichuan	SCB	Alpine and canyon	99°35'	31°15'	3,860	599	7.7
	Jiulong, Sichuan	SCJ	Alpine and canyon	101°45'	29°05'	3,120	892	8.8
	Wolong, Sichuan	SCW	Alpine and canyon	103°25'	31°25'	2,400	890	5.5
Northeastern humid forest region	Jiuzhaigou, Sichuan	SCM	Alpine and canyon	103°50'	33°38'	2,550	552	7.8
	Suileng, Heilongjiang	HLJS	Transitional zones of mountain plain	127°12'	47°24'	300	551	1.4
	Ha'erbin, Heilongjiang	HLJH	Plain of eastern humid forest region	126°46'	45°45'	150	570	3.6
	Fuxin, Liaoning	LNF	Highland of Plateau	121°35'	42°03'	450	481	7.6

**Fig. 1** The locations of the 12 populations of *Hippophae rhamnoides* ssp. *sinensis* in China. Different gray lines stand for the territorial boundaries of local provinces of selected populations

All plant material was raised from seeds collected in natural populations with two exceptions:

- 1 In population SCM, sampled material was collected from a population with approx. 120 individuals grown in the Maoxian Mountain Ecosystem Research Station of the Chinese Academy of Science, introduced from their original location in the Jiuzhaigou Nature Reserve, China;
- 2 In populations SAXW, SAXH, and IMO, sampled material was collected from a ssp. *sinensis* nursery representing populations with their original location in the Wuqi county and Huanglong county in the Shaanxi province, and in Ordos in the Inner Mongolia Autonomous Region, respectively.

Seed was generally collected from 16 to 20 widely separated mother plants in each population. The sampled mother plants were separated by a minimum distance of 10 m to prevent collection of ramets from a single individual. Thus, each analyzed individual had a different mother plant.

DNA extraction

DNA was isolated from the fresh leaves of seedlings by the CTAB-based method (Chen et al. 2008). Leaf materials were ground to fine powder in liquid nitrogen and then transferred to 2.0 ml Eppendorf tubes and mixed with 900 μ l preheated 2 \times CTAB extraction buffer containing 1% (v/v) β -mercaptoethanol and 1% (w/v) PVP 40000. DNA quality and quantity were checked by a DNA-protein instrument (Bio-Rad).

ISSR analysis

Based on the clarity and reproducibility of the band patterns, nine primers out of 45 ISSR primers, i.e. 807, 808, 809, 811, 826, 840, 848, 881, and 887, were selected from UBC set #9 of ISSR primers (<http://www.biotech.ubc.ca/>). The PCRs were performed in a volume of 12.5 μ l containing 1.3 μ l 10 \times reaction buffer (TaKaRa, Dalian), 300 μ M dNTP (Promega), 0.25 μ M primer, 0.5 U Taq polymerase (TaKaRa, Dalian), and 20 ng genomic DNA. For each primer, amplifications were carried out in 96-well plates using the program: initial denaturation step 3 min at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 53–57°C, and 1.5 min at 72°C, and a final extension step of 10 min at 72°C. The PCR products were separated on 1.8% agarose gels and stained with 0.1% ethidium bromide. The molecular weights were estimated using the GeneRuler™ 100 bp DNA Ladder Plus (Fermentas). The gel images were recorded and the band sizes were quantified using a Gel Doc 2000 system (Bio-Rad). All of the plant-primer combinations were run more than once to ensure reproducibility. The fragments amplified by ISSR primers were scored for each individual as present (1) or absent (0) on the basis of size comparison with external standards (GeneRuler™ 100 bp DNA Ladder Plus).

cpSSR analysis

To date, no chloroplast microsatellite sequences have been reported for *Hippophae rhamnoides*. In this study, ten conserved primer pairs developed for dicotyledonous angiosperms (Weising and Gardner 1999), six universal primer pairs developed from *Arabidopsis thaliana* (Cheng et al. 2006), and three primer pairs developed on the basis of chloroplast genomes of Solanaceous plants (Bryan et al. 1999) were used to detect cpDNA microsatellite loci. In addition, seven primer pairs denoted CCMP2, CCMP3, CCMP4, CCMP6, CCMP7, ARCP4, and NTCP9, which produced fragments unambiguously scored across all samples, were taken into consideration for further analysis. The amplification reactions were performed in a volume of 10 μ l containing 1.0 μ l 10 \times reaction buffer (TaKaRa, Dalian), 300 mM dNTPs (Promega), 0.2 mM of both primers specified for each microsatellite locus, 0.5 U Taq polymerase (TaKaRa), and 10–20 ng DNA. PCR amplifications were performed using the profile: 3 min of denaturation at 94°C; 30 cycles of denaturation (45 s at 94°C), annealing (30 s at 48–55°C), and extension (45 s at 72°C), followed by a final extension step for 7 min at 72°C. The amplified PCR products were separated on a LI-COR 4200 DNA Analyzer Sequencer using 6% denaturing polyacrylamide gels (KB-plus solution, LI-COR). The electrophoresed gels were silver-stained, and the fingerprints were

visualized and scored using Genescan™ Analysis 3.1 software (Applied Biosystems). Fragment sizes were estimated using a standard molecular weight marker pBR322 DNA/*Hae*III. In all cases, PCR reactions were performed at least twice in order to ensure the reproducibility of the reactions. Because the chloroplast genome is haploid and does not undergo recombination, it can be viewed as a single locus, and the size scores for the fourteen fragments included in the analysis were combined in order to derive the chloroplast haplotype of each individual. Nevertheless, we use the term locus to refer to a cpSSR site, and allele to refer to a size variant at a given cpSSR site.

Data analysis

The genetic diversity statistics based on ISSR markers, including the percentage of polymorphic loci per population (PLP), Nei's gene diversity (H_{Enc}), the effective number of alleles (N_e), the total genetic diversity (H_{Tnc}), and genetic diversity within populations (H_{Snc}) (Nei 1978), were generated by use of the software Popgen 1.31 (Yeh et al. 1997) to describe genetic variation at intra and inter-population levels. The Arlequin ver 3.11 software package (Excoffier et al. 2007) was used to compute variation data for the chloroplast haplotype within and among populations, including the number of haplotypes (n_h), the effective number of haplotypes (n_E), the unbiased haplotypic diversity (H_{Ecp}), the average diversity over populations (H_{Scp}), and the total gene diversity (H_{Tcp}) (Nei 1987).

Pairwise genetic distances were calculated between both individuals and populations. Calculation of pairwise Euclidean distances between individuals for binary and haploid data followed the method of Huff et al. (1993). The degree of relatedness between the genetic distance matrices generated by the two types of marker was measured using the Mantel matrix-correspondence test (Mantel 1967). To facilitate comparisons between patterns of molecular variance of cpSSR and binary ISSR markers, the degree of genetic differentiation based on both marker types was estimated using the fixation indices at three hierarchical levels: among populations (Φ_{pt}), among populations within regions (Φ_{pr}), and among regions (Φ_{rt}) by analysis of molecular variance (AMOVA) in GenAlEx software ver 6 (Peakall and Smouse 2006). Significance values were computed by a permutation test from 1,000 permutations. The effective number of migrants (N_m) was indirectly estimated using Wright's (1969) formula $N_m = 0.25 (1 - F_{st})/F_{st}$, where F_{st} was substituted by Φ_{pt} , because Φ_{pt} is analogous to F_{st} when the data are haploid or binary (Peakall et al. 1995). To visualize similarities between populations, modified Roger's distances (Wright 1978) were calculated to construct dendrograms using UPGMA (unweighted pair-group arithmetic mean-method) for the

12 populations. The bootstrap values were obtained by use of TFGPA software (Miller 2000), with 1,000 replications to evaluate the internal support for the tree. Furthermore, in order to combine the information provided by different loci, a multivariate approach based on principal-components analysis (PCA) was performed with the informative ISSR loci allele frequencies and the transformed haplotype frequencies from the cpSSR data set. The possible relationship between genetic and geographical distances was assessed by a Mantel test in GenAlEx software ver 6, which tested isolation by distance on the basis of the modified Roger's distances between populations against the natural logarithm of geographical distances between populations.

Results

Genetic diversity

The ISSR patterns obtained using the nine selected primers out of 45 tested ISSR primers were found to be highly reproducible and polymorphic in all populations. A total of 252 reproducible bands were scored, of which 237 bands were polymorphic among the 12 populations. Of the total of 252 bands scored, four bands were population-specific, present in single populations (two in population IMO and two in population SCW). In individual populations, the percentage of polymorphic loci (PLP) ranged from 38.5 to 61.1%. Nei's gene diversities (H_{Enc}) and effective numbers of alleles per locus (N_e) varied from 0.106 to 0.187 and from 1.21 to 1.30, respectively (Table 2). Among the 12 populations investigated, population IMO had the highest variability and population HLJH had the lowest. The average Nei's gene diversity (H_{Snc}) calculated across populations equaled 0.144. For the entire sample set, the total genetic diversity (H_{Tnc}) and N_e value were 0.276 and 1.45, respectively. At the region level, genetic diversity was lowest in region 2, with H_{Enc} and N_e values of 0.201 and 1.31.

The analyses proved the high reproducibility of cpSSR fingerprints. Six loci (CCMP2, CCMP3, CCMP4, CCMP6, ARCP4, and NTCP9) out of the nineteen chloroplast microsatellites screened were polymorphic, with a total of 14 alleles. Among these, two alleles (ARCP4-1 and CCMP2-2) were found only in population SCW. The 14 alleles produced 15 different haplotypes, of which 13 haplotypes were common to all populations but two haplotypes were found only in population SCW (Table 2). Nei's unbiased haplotypic diversity (H_{Ecp}) and the number of haplotypes (n_h) were highest in populations HLJH and SXJ, and lowest in populations HLJS and SCM. The level of polymorphism obtained with the cpSSR was very low for all the populations, with the total gene diversity (H_{Tcp})

Table 2 Diversity statistics of the ISSR and chloroplast simple-sequence repeat (cpSSR) for the 12 populations of *Hippophae rhamnoides* ssp. *sinensis*

Population	n	H_{Enc}	N_e	H_{Ecp}	n_E	n_h
IMO	17	0.187	1.297	0.112	1.157	5
HBF	17	0.177	1.272	0.077	1.093	4
SAXH	19	0.141	1.221	0.130	1.172	6
SXJ	19	0.128	1.210	0.133	1.170	7
SAXW	16	0.134	1.211	0.092	1.238	3
SCB	17	0.130	1.220	0.061	1.055	3
SCJ	19	0.126	1.216	0.044	1.048	3
SCW	16	0.162	1.255	0.156	1.230	3
SCM	19	0.123	1.193	0.015	1.020	2
HLJS	19	0.157	1.250	0.028	1.030	2
HLJH	20	0.106	1.160	0.155	1.320	7
LNF	20	0.158	1.250	0.087	1.130	4
H_S		0.144		0.091		
H_T		0.276	1.447	0.207	1.310	
Region 1	88	0.261	1.429	0.117	1.159	10
Region 2	71	0.201	1.307	0.128	1.156	6
Region 3	59	0.263	1.441	0.214	1.366	9

n , sample size; H_{Enc} , Nei's gene diversity; N_e , effective number of alleles; H_{Ecp} , unbiased haplotypic diversity; n_E , effective number of haplotypes; n_h , number of haplotypes; H_S , genetic diversity within populations; H_T , total genetic diversity

and the average diversity (H_{Scp}) equalling 0.207 and 0.091 (Table 2). At the region level, region 3 had the highest level of haplotypic diversity, with H_{Ecp} and n_E values of 0.214 and 1.37, respectively, and genetic variability was lowest in region 1.

Genetic differentiation

Pairwise Euclidean distances between individuals were computed on the basis of ISSR and cpSSR data. A standardized Mantel statistic of $r = 0.177$ ($P = 0.001$) was obtained for the relationship between pairwise individual genetic distances computed with the two types of marker. Mantel's test showed that two individual genetic distance matrices based on ISSR and cpSSR markers were weakly but significantly correlated. The two marker systems therefore detected similar trends with regard to genetic distances between populations.

Pairwise genetic distances between populations were estimated on the basis of ISSR and cpSSR data using the Φ_{pt} estimator. Based on ISSR data, the values ranged from 0.019 to 0.822, populations HLJS and LNF being the most differentiated populations (Table 3). On the basis of cpSSR data, the Φ_{pt} values ranged from 0.000 to 0.867 (Table 3). Large genetic distance estimated by Φ_{pt} and significant genetic structure were found for most pairwise population

Table 3 Matrix of pairwise population Φ_{pt} values based on ISSR (below) and cpSSR data (above)

	IMO	HBF	SAXH	SXJ	SAXW	SCB	SCJ	SCW	SCM	HLJS	HLJH	LNF
IMO	–	0.000 ^{NS}	0.000 ^{NS}	0.022 ^{NS}	0.070 ^{NS}	0.592***	0.628***	0.674***	0.685***	0.767***	0.027 ^{NS}	0.000 ^{NS}
HBF	0.161***	–	0.000 ^{NS}	0.000 ^{NS}	0.133*	0.664***	0.699***	0.716***	0.760***	0.828***	0.074 ^{NS}	0.000 ^{NS}
SAXH	0.416***	0.448***	–	0.000 ^{NS}	0.075 ^{NS}	0.559***	0.595***	0.662**	0.644***	0.741***	0.023 ^{NS}	0.440***
SXJ	0.454***	0.452***	0.236***	–	0.139**	0.528***	0.561***	0.652***	0.606***	0.721***	0.072*	0.432***
SAXW	0.468***	0.509***	0.447***	0.475***	–	0.665***	0.704***	0.706***	0.764***	0.841***	0.000 ^{NS}	0.520***
SCB	0.436***	0.472***	0.436***	0.462***	0.535***	–	0.103*	0.587***	0.078 ^{NS}	0.761***	0.575***	0.647***
SCJ	0.451***	0.489***	0.462***	0.476***	0.547***	0.137**	–	0.601***	0.095 ^{NS}	0.794***	0.611***	0.680***
SCW	0.383***	0.427***	0.447***	0.473***	0.500***	0.479***	0.485***	–	0.665***	0.775***	0.660***	0.377***
SCM	0.444***	0.485***	0.454***	0.479***	0.545***	0.097*	0.104*	0.488***	–	0.867***	0.654***	0.489***
HLJS	0.409***	0.444***	0.385***	0.416***	0.494***	0.341***	0.348***	0.391***	0.348***	–	0.737***	0.465***
HLJH	0.569***	0.606***	0.532***	0.563***	0.314***	0.622***	0.618***	0.575***	0.622***	0.580***	–	0.597***
LNF	0.352***	0.359***	0.000 ^{NS}	0.019 ^{NS}	0.025 ^{NS}	0.482***	0.486***	0.712***	0.734***	0.822***	0.019 ^{NS}	–

Negative pairwise Φ_{pt} converted to zero

NS, not significant

*, significant at $P < 0.05$; **, significant at $P < 0.01$; ***, significant at $P < 0.001$

comparisons within regions 2 and 3. Low Φ_{pt} values based on cpSSR data were found between populations within region 1. The Mantel's test showed that there was a weak but significant correlation between the ISSR and cpSSR divergence matrices based on the Φ_{pt} estimator ($r = 0.185$ and $P = 0.05$).

At the region level, the Φ_{pt} values based on ISSR markers were 0.425, 0.309, and 0.476 for regions 1, 2, and 3, respectively (Table 4). The overall gene flow among the 12 populations was 0.282, and the gene flow among populations within the three regions was 0.338, 0.559 and 0.276, respectively. For the chloroplast genome, region 1 was least differentiated with the Φ_{pt} value equal to 0.072, and the Φ_{pt} values of regions 2 and 3 equal to 0.489 and 0.586, respectively, showing significant differentiation (Table 4). Consequently, based on cpSSR data, the gene flow among populations within the three regions was 3.222, 0.261, and 0.176, respectively, and the overall gene flow among the 12 populations was 0.165.

Analysis of molecular variance

The apportionment of genetic variation among populations and among regions was measured by fixation indices. For ISSR markers, almost a half of the genetic variation was found among populations and the other half within populations. Among hierarchical levels, differentiation among populations and among populations within regions was higher ($\Phi_{pt} = 0.470$, $P = 0.001$; $\Phi_{pr} = 0.431$, $P = 0.001$) than that among the three regions ($\Phi_{rt} = 0.067$, $P = 0.001$) (Table 5).

For cpSSR markers, analysis of molecular variance showed that approximately 40.1% genetic variation was found within populations, and 30.9 and 29% among the

three regions and among populations within regions, respectively (Table 5). Among hierarchical levels, differentiation among populations, among populations within regions, and among regions equaled 0.602 ($P = 0.001$), 0.423 ($P = 0.001$), and 0.309 ($P = 0.001$), respectively (Table 5).

Relationships among populations

The UPGMA dendrograms based on modified Roger's distances between populations revealed clear clustering with above 60% bootstrap value support, except for a few clusters with very low bootstrap values (<50%) (Fig. 2). The two phenetic trees based on the two types of marker showed relatively similar clustering patterns, except for a few populations. For instance, populations IMO, HBF, and LNF, SAXW and HLJH, and SCJ, SCJ, and SCM congruently formed three sub-clusters according to the phenetic trees (Fig. 2). The main difference between the two trees concerned population SCW, which clustered together with other populations for ISSR data but differed substantially from the others according to cpSSR data. The second difference between the two trees concerned populations SAXW and HLJH, which differed substantially from the others according to ISSR markers but clustered together with other populations according to cpSSR data.

The relationships among populations were further illustrated by the results from principal-components analysis (PCA) based on the ISSR and cpSSR matrices. All populations could be clearly distinguished by both principal-components analyses (Fig. 3), and the percentages of the total variation explained by the first two coordinates were 57.7 and 88.9%, respectively. The plots showed different types of

Table 4 Coefficient of genetic differentiation and gene flow based on ISSR and cpSSR data in the three regions of *Hippophae rhamnoides* ssp. *sinensis*

	ISSR			cpSSR		
	Gene flow	Fixation indices	<i>P</i>	Gene flow	Fixation indices	<i>P</i>
Subspecies	0.282	0.470	0.001	0.165	0.602	0.001
Region 1	0.338	0.425	0.001	3.222	0.072	0.045
Region 2	0.559	0.309	0.001	0.261	0.489	0.001
Region 3	0.276	0.476	0.001	0.176	0.586	0.001

Table 5 Analysis of molecular variance (AMOVA) based on ISSR and cpSSR loci in the 12 populations of *Hippophae rhamnoides* ssp. *sinensis*

Source of variation	ISSR							cpSSR						
	<i>df</i>	SSD	MSD	% Variation	Fixation indices	Value	<i>P</i>	<i>df</i>	SSD	MSD	% Variation	Fixation indices	Value	<i>P</i>
Among regions	2	983.0	491.5	7.00	Φ_{rt}	0.067	0.001	2	45.7	22.7	30.9	Φ_{rt}	0.309	0.001
Among populations within regions	9	2701.8	300.2	40.00	Φ_{pr}	0.431	0.001	9	41.8	4.65	29.0	Φ_{pr}	0.423	0.001
Within populations	206	4,199.0	20.4	53.00	Φ_{pt}	0.470	0.001	206	67.1	0.33	40.1	Φ_{pt}	0.602	0.001

distribution among populations, the ISSR points being more evenly dispersed than those of cpSSR data. For example, in the case of cpSSRs, populations from regions 1 and 2 demonstrated regional differentiation between populations sampled from these two areas. Populations SCW and HLJS were far from each other, which was probably responsible for most among-population divergence in cpSSR.

To explore how much genetic differentiation could be explained by geographical distances between pairs of populations, modified Roger's distance values were plotted against the natural logarithm of geographical distances between populations. Although the overall Mantel test revealed a significant correlation between genetic and geographical distances based on both ISSR ($r = 0.269$, $P = 0.043$) and cpSSR ($r = 0.386$, $P = 0.006$) markers, the genetic distances between pairs of populations within regions 1 and 2, on the basis of ISSR and cpSSR markers, were not positively correlated with geographical distances ($P > 0.28$ and $P > 0.15$, respectively).

Discussion

Habitat fragmentation is expected to have a detrimental impact on the genetic diversity of plant species (O'Connell et al. 2006; Isagi et al. 2007; Lu et al. 2009), but several studies have revealed that population responses to fragmentation are variable (Jump and Penuelas 2006; Kettle et al. 2007). In this study, the genetic diversity ($H_{Sne} = 0.144$) based on ISSR markers was within the general range previously detected for ssp. *sinensis* and other subspecies belonging to *H. rhamnoides* using RAPD and ISSR markers

($H_e = 0.117$ – 0.204) (Bartish et al. 1999, 2000; Tian et al. 2004a, b; Sun et al. 2006; Chen et al. 2010), but lower than the observed diversity ($H_e = 0.249$) for *H. rhamnoides* based on the same type of marker (ISSR) in the Wolong Nature Reserve of China (Chen et al. 2008). The observed genetic diversity in ssp. *sinensis* on the basis of cpSSRs was lower than cpSSR diversities found in natural populations of other angiosperms, for example *Dipteronia* Oliv (Yang et al. 2008). Unfortunately, as far as we are aware, there is lack of available diversity data for the chloroplast genome of *Hippophae*, and no comparison of cpSSR diversity can be performed here.

H. rhamnoides ssp. *sinensis* forests, which have endured habitat fragmentation for several centuries, occur in the hilly-gully region of the Loess Plateau and the desert-grassland region in northwestern China, the transition zones of the mountain-plain of the northeastern humid forest region, and the alpine vegetation region of the Qinghai-Tibetan Plateau. The different landscapes and rugged topography have divided ssp. *sinensis* forests into fragmented and small populations. Decreasing migration between and within populations and the effects of genetic drift may be responsible for the low level of genetic diversity present in ssp. *sinensis* and reduced ability to withstand variable climatic conditions during early successional stages. In addition, the high degree of vegetative reproduction may be a reason for the relatively low genetic diversity within populations of ssp. *sinensis*.

The amount of genetic diversity varied among populations of ssp. *sinensis* and between landscapes. Fluctuations in population size, and different landscape and variable climatic conditions in fragmented habitats may account for

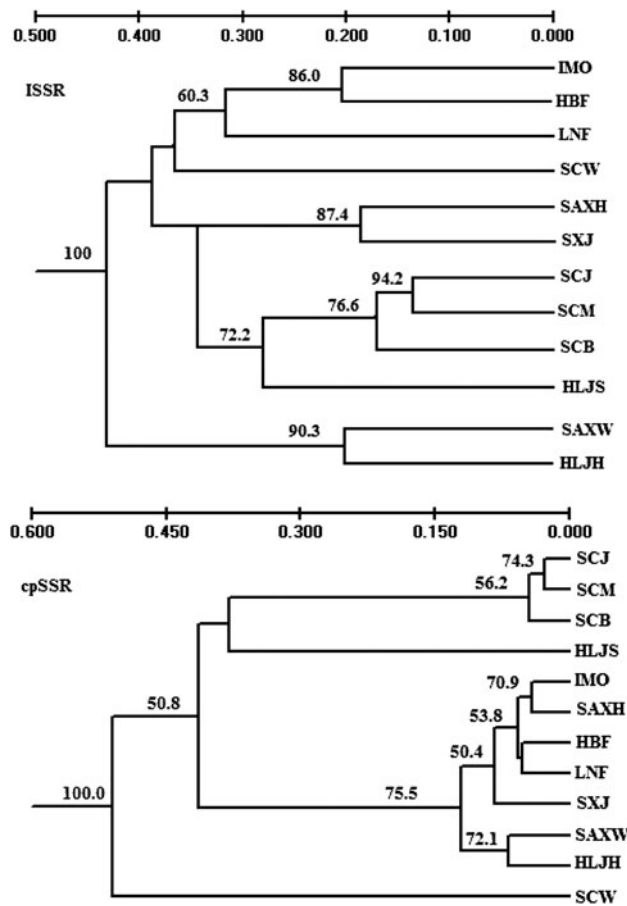


Fig. 2 UPGMA dendrogram obtained for the 12 populations of *Hippophae rhamnoides* ssp. *sinensis* by nuclear ISSR and cpSSR analysis using the modified Roger index (Wright 1978). Bootstrap values above 50% obtained from 1,000 replicate analyses are shown

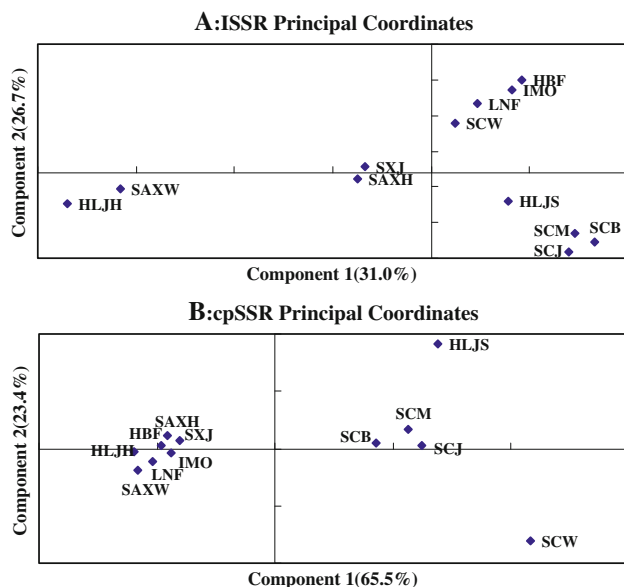


Fig. 3 The PCA plot of coordinates via a covariance matrix with data standardization based on pairwise Nei's genetic distances. PCA plot of coordinates: **a** based on ISSR markers; **b** based on cpSSR markers

the uneven distribution of genetic diversity. For example, population SCW had a high level of genetic diversity when compared with populations from the same region, which may be related to a larger population size, high density, and ecological optima. Population SCW is located in the middle altitudinal zone in the Wolong Nature Reserve of China, where favorable ecological conditions (e.g. abundant rainfall and optimum temperature; Table 1) enable its continuous distribution covering the zone from 2,000 to 2,600 m above sea level. Evidence for the optimum zone for *H. rhamnoides* occurring in the Wolong Natural Reserve has also been documented by Li et al. (2007). In general, genetic diversity is known to be positively correlated with population size (Frankham 1996). Limited gene exchange and competition with other species may contribute to the observed lower genetic diversity of populations SCM, SCJ, and SCB, originally located on the fringe of the forest, where plants belonging to ssp. *sinensis* mix with other trees and form patchy forests. Several mountains, for example the Hengduan Mountains and Saluli Mountain, and Jinsha River cross this area. Mountains, river, and tall trees form barriers and are important in preventing gene exchange, which consequently leads to isolation, genetic drift effects, and low genetic diversity in populations of ssp. *sinensis* in this region.

In populations from the northeastern humid forest region, the different landform features between populations and variable hydrothermal conditions may account for the difference in the level of genetic variation. Population HLJH is located in the plain region, which is a landform that enables effective gene exchange by seed dispersal. Populations HLJS and LNF occur in the transition zone of the mountain-plain area, where the rugged topography of the transition zone isolates populations and hampers gene exchange. In populations from the northwestern desert and grassland region, different population sizes and levels of gene flow may contribute to the observed varying levels of genetic variability among populations. There is an array of ridges and ravines in most areas of the Loess Plateau with the altitude range between 1,000 and 2,000. Steep slopes and ravines divide the ssp. *sinensis* forest into discrete fragmented populations with different sizes and block gene exchange. Hence variable levels of genetic diversity occur.

Genetic analyses revealed a high level of genetic differentiation among populations of ssp. *sinensis* based on ISSR ($\Phi_{pt} = 0.470$). The estimate is close to the values reported in earlier RAPD and ISSR analyses in ssp. *sinensis* ($F_{st} = 0.418$, Tian et al. 2004b) and other subspecies belonging to *H. rhamnoides* ($G_{st} = 0.459$, Chen et al. 2010). Comparably, the cpSSR markers revealed high genetic differentiation for regions ($\Phi_{rt} = 0.309$) and for populations ($\Phi_{pt} = 0.602$). The existence of disjunct regions with distinct nuclear DNA band patterns and

cpDNA haplotypes (e.g. IMO and SCW) suggests that large areas of unsuitable habitat (>100 km) impose significant barriers to pollen and seed dispersal. It is therefore likely that the genetic structure observed has been affected by habitat fragmentation. According to Heuertz et al. (2003), gene flow mediated by pollen and seed play determinant roles in the establishment of genetic structure. Previous research shows that the distance of pollen dispersal in *H. rhamnoides* is less than 20 m with 60–90% falling within 12 m (Tian et al. 1993; Wang et al. 1989). Long-distance dispersal by birds between isolated populations is expected to be rare, in view of the high mountains between populations of ssp. *sinensis*. For neutral genes, N_m values below unity indicate that genetic drift is a major factor affecting population structure (Hartl and Clark 1989). The indirect estimates of gene flow based on both types of marker in ssp. *sinensis* suggest that levels of gene flow are not high enough to counteract differentiation due to genetic drift.

Isolation-by-distance was observed in the investigated populations of ssp. *sinensis* but there was no extreme barrier to dispersal throughout the area of our study. Given the low dispersal ability and gene flow observed, some breakdown in dispersal has occurred. The lack of isolation by distance within regions 1 and 2 further suggests some additional breakdown in dispersal within each region. At present, we are unable to explain this puzzle and offer two possible explanations, which are not mutually exclusive. First, our results confirmed the pathway of the earlier colonization events in ssp. *sinensis*. According to Lian and Chen (1992), this subspecies originated in the eastern Himalayas and subsequently dispersed to the southwestern region of the Greater Khingan Mountains along the eastern and northeastern marginal zone of the Qinghai-Tibetan Plateau and Loess plateau.

Again, chance events during expansion after earlier colonizations of ssp. *sinensis* and habitat fragmentation may explain the lack of isolation by distance within regions. The lack of correlation between geographical and genetic distances within the alpine vegetation region of the Qinghai-Tibetan Plateau could be because of multiple independent colonization events or sub-differentiation during expansion. The gene flow within the northwestern desert and grassland region is mainly because of seed dispersal, which may in part explain the lack of isolation by distance. During cold winters, when snow covers most other plants, fruits of ssp. *sinensis* become the main food source for many birds in the northwestern desert and grassland region. Birds feeding on ssp. *sinensis* are likely to move irregularly among the isolated populations in the landscape, as seed resources on any one site become quickly exhausted, and consequent gene flow effects on the genetic structure are expected. Data collected from fruit

foraging patterns of frugivorous birds also indicate that seed dispersal via birds' digestion processes plays a vital role in renewal and maintenance of the community of ssp. *sinensis* (Lu et al. 2006).

Regional differentiation between the northwestern desert and grassland region and the alpine vegetation region of the Qinghai-Tibetan Plateau was observed. This result may be explained largely by the ecology and biology of reproduction of ssp. *sinensis*. The northwestern region of China belongs to the Loess plateau, where topography and climate conditions are more uniform than those in the northeastern humid forest region and the alpine vegetation region of the Qinghai-Tibetan Plateau. Uniform landscape and climate conditions tend to reduce genetic differentiation among populations. The northeastern humid forest region and the alpine vegetation region of the Qinghai-Tibetan Plateau have extremely complex topography and variable climatic conditions, which consequently, leads to genetic difference among populations. Additionally, morphological traits provide some support to the clustering pattern. As a result of adaptation to dry and barren habitats, plants occurring in the northwestern region are usually shrublike and have small, prickly fruits. Given the more suitable conditions (heat and rainfall), plants of ssp. *sinensis* in the alpine vegetation region and the northeastern humid forest region are trees and/or hemi-arbor and have big, succulent fruits. Therefore, the adaptation of populations of ssp. *sinensis* to the local environment in the three regions, as Lian et al. (2000) suggested, may account for the observed population structures and relationships, given that the habitats of ssp. *sinensis* vary greatly across its distribution.

The overall conclusions of this study are that populations of ssp. *sinensis* have a relatively low level of genetic diversity and significant genetic differentiation exists among populations and regions. Complex topography and heterogeneous climatic conditions throughout the range of ssp. *sinensis* could explain habitat fragmentation and restricted size of populations. Reduced gene flow between populations and increased genetic drift within populations were probably the main factors responsible for the genetic structure revealed in our samples. Our results have a number of implications for the management and conservation of ssp. *sinensis*. Conservation measurements should be focused on the conservation of regional populations in or ex situ. In a situation of limited management capacity of germplasm conservation in situ, the advised approach is to establish seed orchards of ssp. *sinensis* based on seed harvesting and collection of vegetative tissue. Using populations with high genetic variation and uniqueness, on the basis of both nuclear and chloroplast markers, as seed reservoirs is of great benefit to maintaining a wide genetic base. This would guarantee the maintenance of most of the

subspecies' genetic variation, including uncommon and unique alleles. Further advanced research on ssp. *sinensis*, especially investigations on eco-physiological, demographic, and genetic characteristics, will facilitate future programs of sustainable management and conservation of *H. rhamnoides* ssp. *sinensis*.

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