

# Genetic Diversity of *Hippophae rhamnoides* Populations at Varying Altitudes in the Wolong Natural Reserve of China as Revealed by ISSR Markers

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## Abstract

*Hippophae rhamnoides* L., a dioecious and deciduous shrub species, occupies a wide range of habitats in the Wolong Nature Reserve, Southwest China. Our present study investigated the pattern of genetic variation and differentiation among five natural populations of *H. rhamnoides*, occurring along an altitudinal gradient that varied from 1,800 to 3,400 m above sea level in the Wolong Natural Reserve, by using ISSR markers. Based on fingerprinting patterns generated by fifteen primers, high levels of genetic variation were present within populations and subpopulations. Substantial genetic divergence was observed among populations, and also among female and male subpopulations, the  $G_{ST}$  values equaling 0.182, 0.222, and 0.238, respectively. Such considerable divergence present among populations and subpopulations may be caused by complex topography which effectively restricts gene flow, and by variable climatic conditions at different altitudes which may cause varying selective pressures. A significantly positive correlation between genetic and altitudinal distances was detected for the five populations and female subpopulations of *H. rhamnoides*. The cluster analysis including all populations or male subpopulations resulted in three altitude groups. Our results suggest that altitudinal gradients may be the prime cause affecting the genetic variation pattern of different populations and subpopulations in *H. rhamnoides* in the Wolong Nature Reserve, Southwest China.

**Key words:** Altitudinal gradients, Genetic variation, *Hippophae rhamnoides*, ISSR markers, Sex-specific genetic differentiation.

## 1. Introduction

*Hippophae rhamnoides* L. is a dioecious, wind-pollinated woody plant which mainly grows on sandy soils by sea shores or along river beds (BARTISH *et al.*, 1999). As one of the Frankia-non-leguminous nitrogen-fixing plant species, *H. rhamnoides* is not only a pioneer plant species in its natural ecosystems but also an exploitable species for several purposes, including medicine, wood, fuel wood, fodder and honey (LU, 1992). Nowadays, *H. rhamnoides* is widely cultivated as a berry crop in China, Russia, Central Asia and Europe (LU, 1992).

*H. rhamnoides* has a wide distribution in Southwest China. Its occurrence in a large range of habitats across different altitudes, although mainly restricted to sunny, south-facing slopes, implies wide adaptation to different environments (LU, 1992; WU and RAVEN, 1994). *H. rhamnoides* plays an important role in preventing soil erosion and soil water loss, and in regulating microclimate, as well as in retaining ecological stability in the region (LU, 1992).

With the advent of molecular markers, isozyme and RAPD (randomly amplified polymorphic DNA) markers have been extensively used in investigations aiming to reveal the level and apportionment of genetic diversity, genetic relationships among species or subspecies, and sex identification of *H. rhamnoides* (PERSSON and NYBOM, 1998; BARTISH, 2000; RUAN *et al.*, 2004). Among DNA-based molecular markers, compared to RAPDs, ISSRs (inter-simple sequence repeats) have an advantage of having longer primers which require more stringent annealing temperatures, further resulting in more reliable amplification patterns. Therefore, ISSRs have been widely used to detect polymorphisms, analyze phylogenetic relationships, evaluate variation within and among natural populations of many species, identify cultivars, and to distinguish wild and cultivated species (WOLFE and LISTON, 1998; CAMACHO and LISTON, 2001; NAN *et al.*, 2003).

Since elevation is a complex factor, especially in mountain regions, which produce heterogeneous environmental conditions, such as rapidly changing climate conditions, rugged topography and a complex pattern of vegetation. Furthermore, such variable conditions are likely to markedly affect the genetic variation pattern of a plant species (REHFELDT, 1994; SÁENZ-ROMERO *et al.*, 2003; SÁENZ-ROMERO *et al.*, 2006). Genetic differentiation between populations along altitudinal gradients has been observed in several plant species as a result of rapid, elevation-related changes in environmental conditions (NEVO *et al.*, 1983; FAHIMA *et al.*, 1999; Ettl and PETERSON, 2001; SEMAN *et al.*, 2003; BELLUSCI *et al.*, 2005; LIU *et al.*, 2006; ZHANG *et al.*, 2006). Yet, there are also studies which have reported only little or no differentiation with respect to altitude (ARADHYA *et al.*, 1993; OYAMA *et al.*, 1993; GEHRING and DELPH, 1999). According to our previous field investigations in the Wolong Nature Reserve of China, the growth, spatial pattern and population structure of *H. rhamnoides* are closely related to altitudinal gradients, although the changes in these characteristics occur non-linearly along increasing altitudes. Therefore, studies on the level of genetic diversity and its partitioning among *H. rhamnoides* pop-

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ulations at varying altitudes in this region are of a prime interest. In fact, there is relatively little previous work conducted on the level or partitioning of variation across altitudinal ranges, although a lot of such research has been done in relation to geography (YAO and TIGERSTEDT, 1993; BARTISH *et al.*, 1999; CHEN *et al.*, 2004; TIAN *et al.*, 2004a, b; WANG *et al.*, 2005; LU *et al.*, 2006).

In the present study, five natural populations of *H. rhamnoides* (each population consisting of a female and male subpopulation) occurring at altitudes ranging from 1,800 to 3,400 m above sea level in the Wolong Natural Reserve were chosen for the investigation to reveal the level and pattern of genetic variability. In addition, the possibility of genetic differentiation present between the female and male subpopulations of *H. rhamnoides* was also examined.

## 2. Materials and Methods

### 2.1 Plant material

During growing season 2004, 145 adult individuals of *H. rhamnoides* were collected from five different altitudes in the Wolong Natural Reserve of southwestern China (30°45' ~ 31°25' N, 102°25' ~ 103°24' E). Sampling locations were selected along a vertical transect that spanned approximately 1,600 m over a linear distance of about 50 km. Each sampling interval was about 400 m. The altitudinal populations 1–5, i.e. P1800, P2200, P2600, P3000 and P3400, correspond to the altitudes 1,800, 2,200, 2,600, 3,000 and 3,400 m, respectively. Each altitudinal population is comprised of a female subpopulation and a male subpopulation, i.e. 1800F, 1800M, 2200F, 2200M, 2600F, 2600M, 3000F, 3000M, 3400F and 3400M. In every population, twenty-five to thirty individuals were randomly sampled among adult individuals, each same-sex individual being separated by a distance of at least 50 m. Each population sample included 15 females and 15 males, except for P3400 which was composed of 10 female and 15 male individuals. For molecular analyses, fresh leaves from each plant were collected, then frozen quickly and stored at –80°C until DNA extraction.

### 2.2 DNA extraction

DNA was extracted from 0.5 g of fresh leaves following the protocol of CASTIGLIONE *et al.* (1993) with 1%  $\beta$ -mercaptoethanol (v/v) and 1% PVP 40000 (w/v) added to the CTAB extraction buffer. DNA concentrations were determined by comparison with a serial dilution of standard lambda DNA, and the quality of DNA was checked by a DNA-Protein instrument (Bio-RAD).

### 2.3 PCR amplification

The amplification of the ISSR markers was based on a modified protocol as described by YIN *et al.* (2002). All amplification reactions were performed in a GeneAmp® PCR System 9700 (Perkin Elmer Corp., Norwalk, CT, USA). Based on the clarity and reproducibility of the band patterns, fifteen primers (Table 1) out of the 45 primers (produced by the Biotechnology Laboratory,

University of British Columbia) first tested were selected for further use. The PCRs were performed in a volume of 25  $\mu$ l containing 2.5  $\mu$ l of the 10 x reaction buffer (TaKaRa, Dalian), 200  $\mu$ M dNTP (Promega), 0.25  $\mu$ M primer, 1.0 U Taq polymerase (TaKaRa, Dalian) and 40–50 ng of genomic DNA. For each primer, amplifications were carried out in 96-well plates using the following program: an initial step of 5 min at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 53–60°C, and 2 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).

### 2.4 Data analysis

Only bands that were unambiguously scored across all samples were taken into consideration in the further analysis. 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™ 100bp DNA Ladder Plus). The following parameters were generated using the program POPGENE 1.31 to describe genetic variation at intra- and inter-population or subpopulation level: Nei's gene diversity ( $h$ ), Shannon's information index ( $i$ ), the observed number of alleles ( $N_a$ ) and the effective number of alleles ( $N_e$ ) (LEWONTIN, 1972; NEI, 1973; YEH *et al.*, 1997). Genetic divergence between populations or subpopulations was investigated using Nei's unbiased genetic distances (GD) and genetic identities (GID) (NEI, 1978). Nei's unbiased genetic distances were used to construct dendrograms using UPGMA (unweighted pair-group arithmetic mean-method) for the mixed populations (including both sexes) (NEI, 1978). The genetic structure was further investigated using Nei's gene diversity statistics, including the total genetic diversity ( $H_T$ ), genetic diversity within populations ( $H_S$ ), and the relative magnitude of genetic differentiation among populations ( $G_{ST} = (H_T - H_S)/H_T$ ) (NEI, 1973). An estimate of gene flow among populations ( $Nm$ ) was computed using the formula of  $Nm = (1 - G_{ST})/2G_{ST}$  (McDERMOTT and McDONALD, 1993).

Using the program GenAlEx version 6 (PEAKALL and SMOUSE, 2006), Mantel tests (MANTEL, 1967) were conducted to investigate the possible relationship between the genetic and geographical distances. This analysis tested isolation by distance based on the linear regression of pairwise genetic distance matrix against the geographic distance matrix between populations and subpopulations. To obtain an additional representation of genetic relationships among populations, a principal coordinate analysis (PCO), using the program GenAlEx version 6, was conducted.

## 3. Results

### 3.1 ISSR profiles

A total of 326 markers generated by fifteen primers were scored, with sizes ranging between 400 and 1500

Table 1. – The nucleotide sequences and annealing temperatures of the fifteen selected ISSR primers, and the numbers of bands scored in *H. rhamnoides*.

Primer	Primer sequence (5'-3')	Annealing temp. (°C)	Number of bands recorded
IP-1	(GA) <sub>8</sub> C	53	16
IP-2	(AC) <sub>8</sub> CTG	53	19
IP-4	(GA) <sub>8</sub> CTT	54	24
807	(AG) <sub>8</sub> T	55	20
808	(AG) <sub>8</sub> C	53	20
809	(AG) <sub>8</sub> G	57	35
826	(AC) <sub>8</sub> C	53	25
834	(AG) <sub>8</sub> YT	51	22
836	(AG) <sub>8</sub> YA	49	20
ISSR-32	(AG) <sub>8</sub> AC	58	19
ISSR-44	(AC) <sub>8</sub> GA	53	21
ISSR-47	(AC) <sub>8</sub> GT	58	19
ISSR-62	(AG) <sub>8</sub> CA	54	22
ISSR-64	(AG) <sub>8</sub> CG	59	27
ISSR-65	(AG) <sub>8</sub> CC	60	17

Y = C or T.

Table 2. – Genetic variability parameters of *H. rhamnoides* populations based on ISSRs.

Population	$N_a$	$N_e$	$h$	$i$	$P$ (%)
P1800	1.752	1.384	0.233	0.358	75.2
P2200	1.856	1.428	0.265	0.409	85.6
P2600	1.810	1.432	0.259	0.395	81.0
P3000	1.776	1.402	0.242	0.370	77.6
P3400	1.755	1.408	0.247	0.375	75.5

$N_a$ , observed number of alleles;  $N_e$ , effective number of alleles;  $h$ , Nei's gene diversity;  $i$ , Shannon's indices;  $P$ , percentage of polymorphic loci.

bp. The number of scored markers per primer ranged from 16 (IP-1) to 35 (809) (Table 1), with an average of 21.7 markers per primer. At the population level, 324 markers were found polymorphic, with the percentage of polymorphic loci equaling 99.4%. At the subpopulation level, 318 markers (97.6%) were polymorphic among the five female subpopulations, while 322 markers (98.8%) were polymorphic across the five male subpopulations.

### 3.2 Intra-population and intra-subpopulation variation

In individual populations, the percentages of polymorphic loci ( $P$ ) ranged from 75.2% to 85.6%, with an average of 79.0% (Table 2). Nei's gene diversities ( $h$ ) varied from 0.233 to 0.265, with an average of 0.249, and Shannon's indices ( $i$ ) ranged from 0.358 to 0.409, with an average of 0.381. A similar trend was found in the values of the parameters  $h$  and  $i$ . The mean observed number of alleles ( $N_a$ ) ranged from 1.752 to 1.856, while the mean effective number of alleles ( $N_e$ ) varied from 1.384 to 1.432. When calculated across populations, the  $h$  and  $i$  values equaled 0.305 and 0.468, respectively, and the  $N_a$  and  $N_e$  values equaled 1.994 and 1.505, respectively. Among the five populations investigated, P2200 exhibited the highest level of variability while P1800 possessed the lowest value of variability (Figure 1).

In individual subpopulations, the percentages of polymorphic loci ( $P$ ) of each female and male subpopulation

ranged from 66.6% to 81.9%, and from 67.2% to 79.1%, with an average of 72.6% and 72.0%, respectively (Table 3). Nei's gene diversities ( $h$ ) of female and male subpopulations varied from 0.217 to 0.255, and from 0.212 to 0.253, with an average of 0.233 and 0.231, and Shannon's indices ( $i$ ) ranged from 0.331 to 0.392, and from 0.323 to 0.384, with an average of 0.355 and 0.351, respectively. The mean observed number of alleles ( $N_a$ ) ranged from 1.666 to 1.819, and from 1.672 to 1.791,

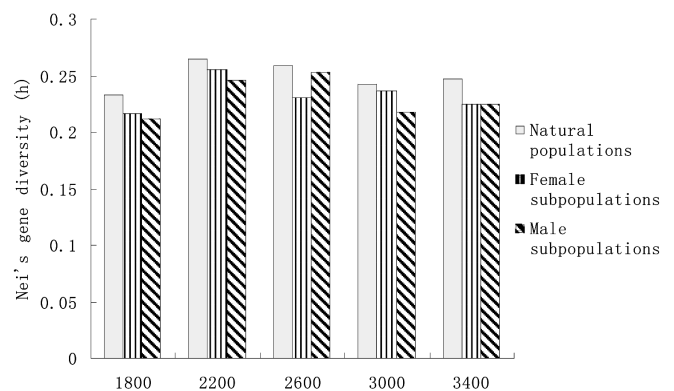


Figure 1. – Comparison of Nei's (1978) gene diversity among the natural populations, and among the male and female subpopulations of *H. rhamnoides* originating from the Wolong Natural Reserve.



Table 3. – Genetic variability parameters of *H. rhamnoides* in female and male subpopulations based on ISSRs.

Subpopulation	$N_a$	$N_e$	$h$	$i$	$P$ (%)
1800F	1.687	1.359	0.217	0.331	68.7
2200F	1.819	1.414	0.255	0.392	81.9
2600F	1.733	1.386	0.231	0.351	73.3
3000F	1.727	1.393	0.237	0.360	72.7
3400F	1.666	1.382	0.225	0.339	66.6
1800M	1.672	1.357	0.212	0.323	67.2
2200M	1.791	1.407	0.246	0.378	79.1
2600M	1.776	1.424	0.253	0.384	77.6
3000M	1.678	1.366	0.218	0.331	67.8
3400M	1.681	1.377	0.225	0.340	68.1

$N_a$ , observed number of alleles;  $N_e$ , effective number of alleles;  $h$ , Nei's gene diversity;  $i$ , Shannon's indices;  $P$ , percentage of polymorphic loci.

while the mean effective number of alleles ( $N_e$ ) varied from 1.359 to 1.414, and from 1.357 to 1.424 in female and male subpopulations, respectively. When calculated across female and male subpopulations, the  $h$  and  $i$  values equaled 0.300 and 0.459, and 0.303 and 0.465, respectively, while the  $N_a$  and  $N_e$  values equaled 1.976 and 1.499, and 1.988 and 1.499, respectively. Among the five female subpopulations investigated, 2200F exhibited the highest level of variability while 1800F possessed the lowest value of variability (Figure 1). However, among the five male subpopulations, 2600M exhibited the highest level of variability and 2200M almost as much variation, while 1800M possessed the lowest value of variability.

### 3.3 Inter-population and inter-subpopulation differentiation

The total gene diversity ( $H_T$ ) and gene diversity within populations ( $H_S$ ) revealed by ISSR analyses in the mixed populations of *H. rhamnoides* equaled 0.305 and 0.249, respectively. The coefficient of genetic differentiation ( $G_{ST}$ ), equaling 0.182, showed the presence of some degree of genetic differentiation among mixed populations. The level of gene flow ( $N_m$ , the number of migrating individuals among populations per generation) was estimated to equal 2.249.

When restricting the analysis to the five female and male subpopulations, the total gene diversity ( $H_T$ ) and gene diversity within populations ( $H_S$ ) were 0.299 and 0.233, and 0.303 and 0.231, respectively. The coefficient of genetic differentiation ( $G_{ST}$ ), equaling 0.222 and 0.238, respectively, showed considerable genetic differentiation among female and male subpopulations. The level of gene flow ( $N_m$ ) was estimated to be 1.758 and 1.605, respectively. The analysis of molecular variance (AMOVA) taking into account the female and male subpopulations showed that 22.2% of the total variation was maintained among populations, and only 3.8% of the variability resided between female and male subpopulations.

### 3.4 Genetic relationships among populations and subpopulations

Nei's unbiased genetic distances were calculated for populations and subpopulations to estimate their divergence (Tables 4). The mean distance among the five mixed populations equaled 0.0973 (pairwise range 0.0528–0.1600). The lowest genetic distance was found between P3000 and P3400, while the greatest distance was detected between P1800 and P3000. The mean distance among the five female subpopulations equaled 0.1150 (pairwise range 0.0590–0.1796) (Table 4). The

Table 4. – Nei's genetic distances and genetic identities between female and male subpopulations of *H. rhamnoides*.

Subp.	1800F	2200F	2600F	3000F	3400F	1800M	2200M	2600M	3000M	3400M
1800F	—	0.9079	0.8356	0.8390	0.8409	0.9684	0.8691	0.8397	0.8323	0.8359
2200F	0.0966	—	0.9096	0.9108	0.9052	0.8866	0.9632	0.9115	0.9063	0.8967
2600F	0.1796	0.0948	—	0.9169	0.9142	0.8286	0.9185	0.9647	0.9153	0.9038
3000F	0.1755	0.0935	0.0868	—	0.9428	0.8289	0.9221	0.9275	0.9715	0.9210
3400F	0.1733	0.0996	0.0897	0.0590	—	0.8309	0.9141	0.9147	0.9366	0.9596
1800M	0.0322	0.1203	0.1880	0.1876	0.1852	—	0.8468	0.8319	0.8177	0.8278
2200M	0.1403	0.0375	0.0851	0.0811	0.0898	0.1663	—	0.9268	0.9183	0.9143
2600M	0.1747	0.0927	0.0359	0.0752	0.0892	0.1840	0.0760	—	0.9222	0.9060
3000M	0.1835	0.0984	0.0885	0.0289	0.0655	0.2013	0.0853	0.0810	—	0.9242
3400M	0.1792	0.1090	0.1011	0.0823	0.0412	0.1890	0.0896	0.0987	0.0788	—

Above diagonal: genetic identities; below diagonal: genetic distances.

smallest genetic distance was found between subpopulations 3000F and 3400F, while the greatest distance occurred between subpopulations 1800F and 2600F. On the other hand, the genetic distances among the five male subpopulations varied from 0.0760 (subpopulations 2200M and 2600M) to 0.2013, (subpopulations 1800M and 3000M) with a mean value of 0.1250 (Table 4).

To investigate the presence of a possible association between the genetic and vertical distances among populations and subpopulations, we compared these two matrices with a Mantel test. A significantly positive correlation was found between the two types of distances for the five mixed populations ( $R = 0.636$ ,  $p = 0.037$ ) and for the five female subpopulations ( $R = 0.710$ ,  $p = 0.027$ ) (Figure 2), while the corresponding relationship among the male subpopulations was not significant ( $R = 0.505$ ,  $p = 0.062$ ) (Figure 2).

### 3.5 Cluster analyses

A UPGMA analysis was conducted on the basis of Nei's (1978) genetic distances for mixed populations,

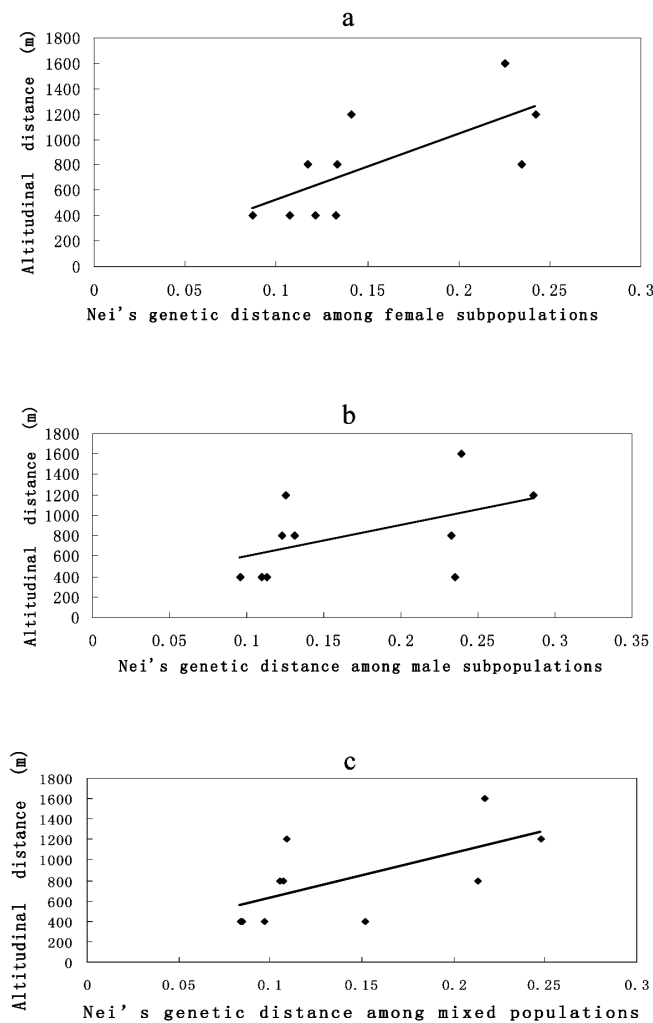


Figure 2. – Mantel tests for the relationship between genetic and altitudinal distances in the female ( $R = 0.710$ ,  $p = 0.027$ ) (a) and male ( $R = 0.505$ ,  $p = 0.062$ ) (b) subpopulations, and in the mixed populations ( $R = 0.636$ ,  $p = 0.037$ ) (c) of *H. rhamnoides* originating from the Wolong Natural Reserve.

female subpopulations and male subpopulations. Except for the dendrogram based on the female subpopulations, the dendrograms consisted of three clusters, the first one with low-elevation population or subpopulation P1800 or 1800M, the second one with mid-elevation populations or subpopulations P2200 and P2600, or 2200M and 2600M, and the third one with high-elevation populations or subpopulations P3000 and P3400, or 3000M and 3400M. The dendrogram based on female subpopulations displayed a slightly different trend. It showed that subpopulations 3000F and 3400F first formed a cluster, and then subpopulations 2600F, 2200F and 1800F were clustered with that one by one. When all subpopulations were included in the cluster analyses, the dendrogram showed a similar trend as detected in the dendrograms based on mixed populations or male subpopulations (Figure 3). Its specific character is that subpopulations of the same altitude, e.g. 1800F and 1800M, firstly formed a cluster.

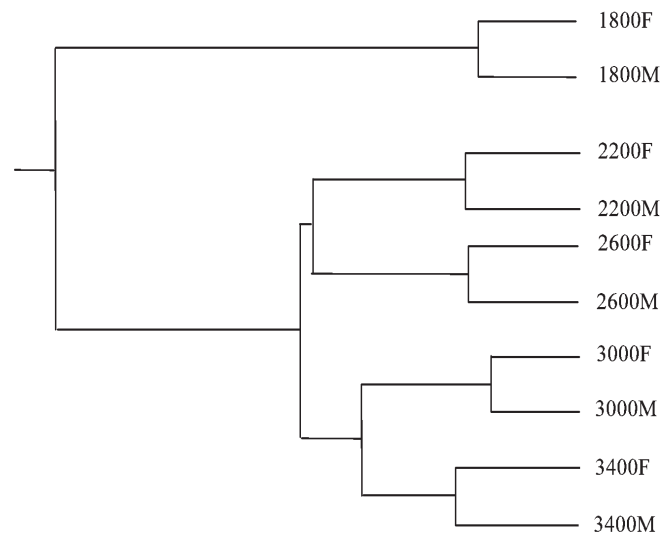


Figure 3. – Dendrogram for all subpopulations of *H. rhamnoides* originating from the Wolong Natural Reserve. Calculations were performed using the unweighted pair-group arithmetic mean-method for the genetic distance values given in Table 4.

### 3.6 Principal coordinate analysis

A PCO analysis was performed to provide spatial representation of the relative genetic distances among individuals and to determine the consistency of differentiation among populations defined by the cluster analysis (Figure 4). The first three principal coordinate axes obtained in the analysis of mixed populations accounted for 43.2%, 14.8% and 12.7% of the total variation, respectively (cumulative value = 70.7%). In an agreement with the cluster analysis, individuals from each population formed a separate plot and could be clearly distinguished from individuals originating from other populations. Population P1800 expressed the highest level of genetic differentiation in relation to other populations, while population P2200 formed two looser sub-clusters of individuals and less population-specific identity.

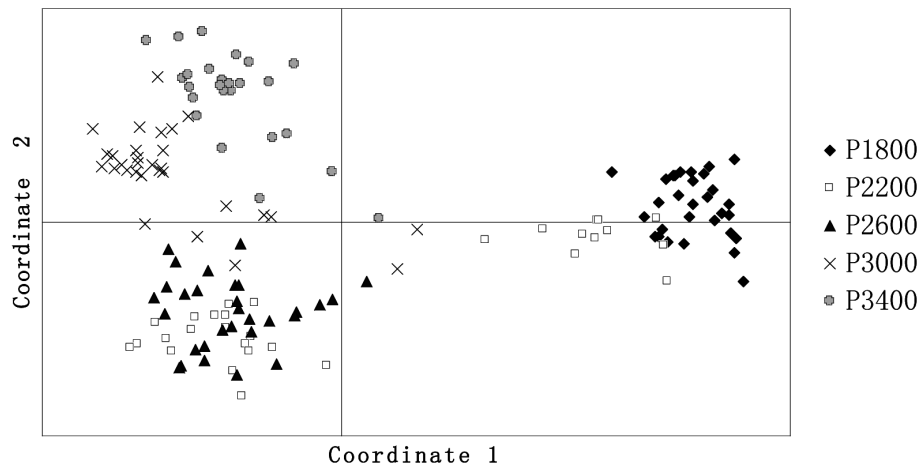


Figure 4. – PCO analysis for the populations of *H. rhamnoides* originating from different altitudes from the Wolong Natural Reserve.

#### 4. Discussion

The ISSR analysis conducted for the *H. rhamnoides* populations located in the Wolong Natural Reserve of China revealed the presence of high levels of genetic variation (as measured by mean  $h = 0.249$  and  $H_T = 0.305$ ). This is an expected result for a woody, moderately long-lived, outcrossing and wind-pollinated species (HAMRICK *et al.*, 1992). The degree of diversity was higher than the values previously reported for *H. rhamnoides* and other subspecies belonging to *H. rhamnoides* (BARTISH *et al.*, 1999, 2000; RUAN *et al.*, 2004; TIAN *et al.*, 2004a, b).

Furthermore, the genetic diversity of *H. rhamnoides* was found to vary significantly with changing elevation, showing a trend that mid-elevation populations (2,200 m and 2,600 m) were genetically more diverse than both low-elevation (1,800 m) and high-elevation populations (3,000 m and 3,400 m). *H. rhamnoides* is thought to be stressed by drought and high temperature at low elevations, and by low temperature at high elevations (LI *et al.*, 2007). Unfavourable environments at both low and high altitudinal zones may lead to an increase in vegetative reproduction and to a decrease in resource-demanding sexuality, which may result in a loss of genetic variation. In addition, from a geographical point of view, the low- and high-elevation populations can be considered as being marginal and isolated. Lower levels of genetic diversity in low-elevation populations may also be ascribed to the negative effects of anthropogenic pressures, such as road building and other human activity, which gradually disrupt the habitats of *H. rhamnoides* and result in reduced population density and size, and habitat fragmentation. The presence of lower genetic diversity in high-elevation populations is likely affected by decreased sexual reproduction imposed by pollination limitation and shortened flowering season (YOUNG *et al.*, 2002). Natural selection in harsh environments may also lead to a loss in genetic variability. On the other hand, the mid-elevation populations of *H. rhamnoides* are thought to be free from both drought and high- and low-temperature stresses. The evidence that there exists an optimum zone for *H. rhamnoides* in the Wolong Natural Reserve has been documented by LI *et al.*

(2007). The high genetic variability present in the mid-elevation populations of *H. rhamnoides* is assumed to be related to larger population sizes in the mid-altitudinal zone, where favorable ecological conditions permit a continuous distribution covering the zone from 2,200 m to 2,600 m above sea level. In general, genetic diversity is known to be positively correlated with population size (FRANKHAM, 1996).

The analysis restricted to female subpopulations and male subpopulations demonstrated the following: (1) In both sexes, the peak value of genetic variation consistently appears in mid-elevation subpopulations; (2) with the exception of the altitude 2,600 m, the genetic variability of female subpopulations is slightly higher than that of male subpopulations. Although only few previous reports are available with respect to sex-specific genetic differentiation in dioecious plant species, sex-related differences in morphological and physiological characteristics have been studied extensively (KRISCHIK and DENNO, 1990; GEHRING and MONSON, 1994; JONES *et al.*, 1999; WANG and GRIFFIN, 2003; LI *et al.*, 2004, 2005). Most of these studies confirm the finding that males are often impacted less than females when both sexes grow in the same stressful environment, or that males are more adaptive than females in a variety of ways when the sexes are compared in stressful environments. A higher frequency of vegetative reproduction in the males of *H. rhamnoides* may account for a partial loss of genetic diversity in males when compared to females at altitudes other than 2,600 m. The sex-specific diversity values peaking at 2,200 m in females and at 2,600 m in males may reflect the presence of sex-specific selection or differences in altitude optimums or in the prevalence of sexual reproduction.

The genetic structure of *H. rhamnoides* followed the general pattern detected in woody species with widespread distributions and outcrossing mating systems. Such plants possess more genetic diversity within populations and less variation among populations than do species with other combinations of traits (HAMRICK and GODT, 1989). In general, population differentiation is expected to be very restricted in long-lived woody, outcrossed, dioecious and wind-pollinated species (LOVELESS



and HAMRICK, 1984; HAMRICK and GODT, 1989, 1996; BARTISH *et al.*, 1999). Yet, the level of genetic differentiation, recorded in the present study for *H. rhamnoides* ( $G_{ST} = 0.182$ ) within a geographically restricted area using ISSR markers, is quite high. Lower levels of differentiation have been detected in previous studies on *H. rhamnoides* based on isozyme, RAPD and ISSR analyses (YAO and TIGERSTEDT, 1993; BARTISH *et al.*, 1999, 2000; BARTISH, 2000; RUAN *et al.*, 2004; TIAN *et al.*, 2004a, b). The relatively high genetic differentiation detected here can be mainly ascribed to the following two causes: 1) The sampling sites located in the Wolong Natural Reserve are separated by tall, zigzag positioned mountains, and, consequently, the gene flow among populations is limited. 2) A further reason can be the floral asynchrony at different altitudes (REISCH *et al.*, 2005). An increase in altitude causes a decrease in temperature and, further, it postpones the flower development. Generally, flowering is delayed by 2–3 days for every 200 m of elevation in the Wolong Natural Reserve. Therefore, the flowering periods do not overlap in populations or subpopulations with altitude differences greater than 500 m. It follows that the phenological gap also contributes to the observed differentiation.

In the present study, Mantel tests showed positive correlations between altitudinal distances and genetic distances among populations or subpopulations. The observed relationship between altitude and genetic distances, and the result of the cluster analysis including populations or male subpopulations that classified the groups into three altitude clusters suggest that altitude is a major factor that restricts gene flow between populations and subpopulations. A comparable situation has been reported in *Psathyrostachys huashanica* by WANG *et al.* (2006). In addition, a cluster analysis grouping the same altitude females and males into the same clusters shows that female and male subpopulations of the same elevations are genetically alike. The analysis of molecular variance showed that only 3.8% of the variability resided between female and male subpopulations. Such a very restricted proportion of the total molecular variance between females and males is due to common sexuality and the mixing of genetic material between the sexes.

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