

# Genetic diversity and population structure of *Hibiscus aridicola*, an endangered ornamental species in dry-hot valleys of Jinsha River

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## ABSTRACT

*Hibiscus aridicola* is an endangered ornamental shrub of the family Malvaceae that is endemic to the dry-hot valleys of Jinsha River in southwestern China. This species is a typical plant species with extremely small populations (PSESP). To support and monitor future conservation, develop management measures, and genotype this species, we performed extensive field studies together with genetic analyses. Specifically, we screened eleven microsatellite loci of 69 individuals of *H. aridicola* from four accessions. The population genetics analyses indicated that *H. aridicola* possesses high genetic diversity at both the population (0.6962–0.7293) and species level (0.7837) compared to other endemic/endangered species in China. The low differentiation of populations ( $F_{st} = 0.0971$ ) and the high gene flow between populations of *H. aridicola* ( $N_m = 2.3236$ ) could be due to its distribution along rivers in the hot-valleys of the Jinsha River and the wind-mediated dispersal of its seeds. Furthermore, the genetic diversity of *H. aridicola* is slightly positively correlated with geographic distance. Two populations are undergoing a genetic bottleneck, and require more specific attention from conservationists. Additionally, our analyses of the population genetics of *H. aridicola* demonstrate that the declines in populations are not the result of the internal genetics of these populations but due to external human activities over the past decades.

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## 1. Introduction

The species *Hibiscus aridicola* is a shrub member of Malvaceae and is an endemic species in the dry-hot valleys of Jinsha River (upper reaches of the Yangtze River) in southwest China (Feng, 1979; Feng et al., 1984; Jin, 1999; Tang et al., 2007). This species, which has large flowers varying from white to whitish purple, is also cultivated for ornamental purposes (Li et al., 2003). The type material was collected near Shangri-La, Yunnan Province, China and, according to the *Flora of China* and *Flora of Yunnan*, in the past

*H. aridicola* was widely distributed along the valleys of Jinsha River at elevations between 1300 and 2100 m a.s.l. Currently, however, *H. aridicola* can be found at fewer than five locations and the area of occupancy continues to decline, which, in accordance with EN B2ab (ii) of the IUCN Red List Categories and Criteria, has led to its classification as endangered and its placement in 2004 on the China Species Red List (Wang and Xie, 2004). This species is a typical plant species with extremely small populations (PSESP) (Ma et al., 2013). Our preliminary investigations into *H. aridicola* indicate that populations of this species are severely threatened, possibly due to human activities such as cutting for firewood, reclaiming farmland, and constructing hydropower stations. As a consequence, the distribution *H. aridicola* is highly fragmented, and the survival of the species requires both in situ and ex situ conservation. To propose effective solutions for the long-term in situ and ex situ conservation of *H. aridicola*, it is urgent to elucidate the genetic background of this species.

The genetic diversity of endangered species has been elucidated previously through the use of molecular markers, particularly

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microsatellites (Chen et al., 2009; Tang et al., 2008; Wang et al., 2006; Yang et al., 2018; Zhao and Gong, 2015). These markers are abundant, uniformly distributed, and show a high degree of polymorphism and codominance. Furthermore, these markers are easily amplified by PCR, produce results that are relatively simple to interpret, and can be easily accessed by other laboratories via published primer sequences (Jarne and Lagoda, 1996; Maroof et al., 1994). In addition, they can give informative results even with a small sample size (Habel et al., 2010).

The aim of this study was to elucidate the genetic background of *H. aridicola*, a plant species with extremely small populations. For this purpose, we used 11 microsatellite markers to examine the population structure and genetic diversity of the four remaining populations of *H. aridicola*. Based on our findings, we propose specific strategies for conserving this species.

## 2. Materials and methods

### 2.1. Plant collection

*H. aridicola* samples were collected in the dry-hot valleys of Jinsha River in the provinces Yunnan and Sichuan, China between September 2007 and December 2011. In total, 69 individuals were collected from four populations: Labo Town (LB), Muxintu Village (MXT), Jinyang County (QJ), and Xiazhen Village (XZE) (for details, see Fig. 1 and Table 1). Firstly, around 20 individuals were collected from each population, with a distance of 10 m between each sample. The collected leaves were dried using silica gel. Two voucher specimens were collected for each population and deposited in the herbarium (KUN) of Kunming Institute of Botany, Chinese Academy of Sciences.

### 2.2. DNA extraction, primer selection, PCR procedure, and product detection

The genomic DNA was extracted from approximately 5 g of dried leaves of each collected sample using the modified CTAB method

(Doyle and Doyle, 1987). Zhang et al. (2011a) successfully isolated 15 suitable microsatellite loci for *H. aridicola* that showed polymorphisms of two to six alleles per locus. In the current study, we used 11 of these loci; relevant primer pairs were synthesized by Sangon Company (Shanghai). PCR was performed according to the method described by Zhang et al. (2011a) and the PCR products were separated on 8% denaturing polyacrylamide gels and visualized by silver staining with a 100 bp extended DNA ladder (Fermentas) as a size standard.

### 2.3. Data analysis

To estimate bias in the SSR markers, we tested Hardy–Weinberg equilibrium (HWE) and Hardy–Weinberg linkage disequilibrium (HWLD) by inputting data into GENEPOP 4.0 (Raymond and Rousset, 1995; Rousset, 2008). POPGENE V. 1.32 (Yeh et al., 1999) was used to calculate the following parameters at the population and species level: population averages of sample sizes ( $N$ ), observed number of alleles ( $N_a$ ), effective number of alleles ( $N_e$ ), Shannon's information index ( $I$ ), expected ( $H_e$ ) and observed ( $H_o$ ) frequency of heterozygotes, percentage of polymorphic loci ( $PPB$ ), and the Nei's (1973) expected heterozygosity ( $H$ ). Nei's genetic identities and distances were also calculated among populations.

To examine the similarity of the populations included and to assess the correlation between genetic distance and geographic distance, principal coordinates analysis (PCoA) and the Mantel test were conducted using GenAlEx V. 6.0 (Peakall and Smouse, 2006). The geographic distances were transferred from the longitude and latitude data of the populations using Geographic Distance Matrix Generator V. 1.2.3 (Ersts, 2018). Hierarchical analyses of molecular variance (AMOVAs) were performed to assess the genetic structure within and between population genetic groups identified using ARLEQUIN V. 3.5.1.3 (Excoffier et al., 2005), and significance tests were performed on basis of 10,000 permutations. Neighbor-Joining (NJ) analysis was conducted in PHYLIP V. 3.67 with the Nei's distances calculated in MICROSATELLITE ANALYSER 4.05. Tests for recent bottlenecks were performed using the software

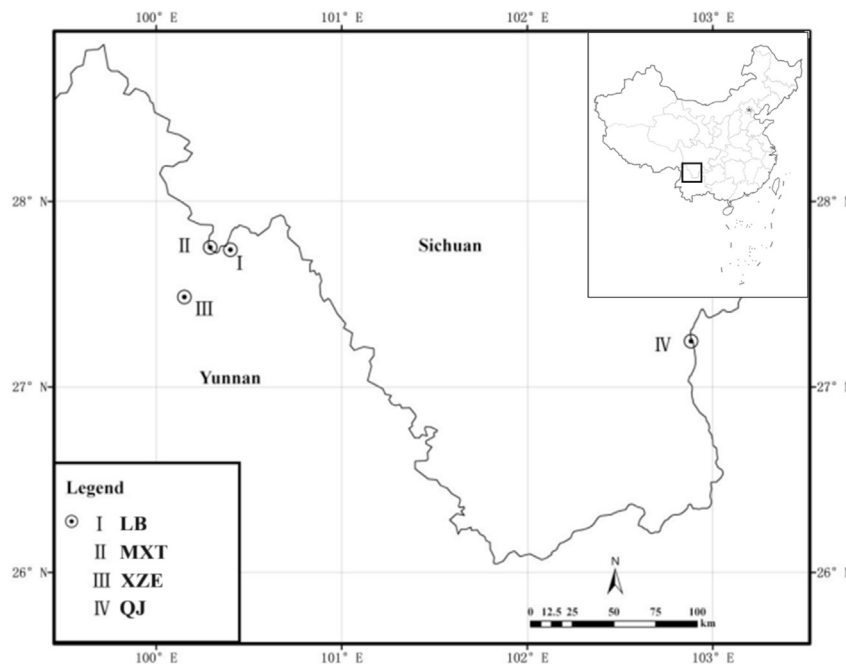


Fig. 1. Geographical distribution of *H. aridicola* along the Jinsha River in Southwest China.

**Table 1**  
Origins of the four studied populations of *H. aridicola*.

Population	Location	Altitude/longitude of location	Elevation (m)
LB	Tuodian Village, Labo Town, Ninglang County, Yunnan	N 27°44'20" E 100°24'01"	1645–1749
MXT	Muxintu Village, Luoji Town, Shangri-la County, Yunnan Province	N 27°45'06" E 100°17'32"	1420–2180
XZE	Xiazhen Village, Sanba Town, Shangri-la County, Yunnan Province	N 27°29'03" E 100°09'06"	1770–1920
QJ	Jinyang County, Sichuan Province	N 27°14'45" E 102°52'59"	710–835

BOTTLENECK V. 1.2.02. The stepwise mutation model (SMM) and two-phase model (TPM) were selected and both the *sign test* and the *Wilcoxon sign-rank test* were conducted for 1000 iterations. The Bayesian assignment test was conducted in STRUCTURE V. 2.2 to determine the number of genetic groups (Pritchard et al., 2000) using the admixture model and assumed correlated allele frequencies for 10,000 iterations, following a burn-in of 10,000 iterations. To quantify the variation of the likelihood for each K (number of groups assigned to the populations), ten runs each were performed for K varying from one to nine (number of populations plus five). The best value of K for the data set was determined based on the estimated posterior log probability of the data,  $L(K)$ , and the rate of change in probability ( $\Delta K$ ) between successive K values, following the work of Evanno et al. (2005). Graphics were drawn with DISTRUCT 1.1 (Rosenberg, 2004).

### 3. Results

#### 3.1. Hardy–Weinberg equilibrium and linkage disequilibrium tests

Among the 44 combinations for the four populations and eleven microsatellite loci, only five failed to deviate from Hardy–Weinberg equilibrium (11.36% of the total), seven deviated (15.91% of the total,  $P < 0.05$ ), six deviated significantly (13.64% of the total,  $P < 0.01$ ), 26 deviated highly significantly (59.09% of the total,  $P < 0.001$ ). Overall, 88.64% of the all combinations deviated from Hardy–Weinberg equilibrium (Table 2). Additionally, we found evidence of linkage disequilibrium between loci HA3 and HA4. The loci HA3 and HA13 showed highly significant P values ( $P < 0.001$ ) after Bonferroni correction, therefore all further analyses were conducted without locus HA3, due to linkage disequilibrium (Table 3).

#### 3.2. Genetic diversity

The related parameters of genetic diversity were calculated and summarized in Tables 4 and 5. Briefly, at both the population and

**Table 2**  
The P values for the Hardy–Weinberg test when H1 = heterozygote deficit, by population and loci.

	LB	MXT	QJ	XZE
HA-1	0	0	0	0
HA-3	0.0045	0.045	0	0.0122
HA-4	0.014	0.0009	0.0356	0.1295
HA-5	0	0	0	0
HA-6	0.0051	0	0	0.0287
HA-7	0.1609	0.0003	0	0.0004
HA-8	0	0.0004	0	0
HA-9	0.0352	0.0555	0.0082	0
HA-10	0.0002	0.022	0	0
HA-13	0.0907	0.8387	0	0.0041
HA-14	0.0003	0.0023	0.0061	0

species level, the percentage of polymorphic loci (PPB) was 100%. The number of different alleles ( $N_a$ ) and effective alleles ( $N_e$ ) varied from 5.8 (MXT) to 7.0 (QJ), 3.7325 (XZE) to 4.3000 (QJ) at the population level, 9.9 and 5.7401 at the species level, respectively. The average Shannon's Information Index ( $I$ ), Observed Heterozygosity ( $H_o$ ), Expected Heterozygosity ( $H_e$ ) and Nei's (1973) expected heterozygosity ( $H$ ) at the species level are 1.8381, 0.3580, 0.7894 and 0.7837, respectively and the LB population from Shangri-La, Yunnan Province showed the highest genetic diversity ( $I = 1.5198$  and  $H = 0.7293$ ), followed in descending order by the QJ, XZE and MXT populations. The value of the ratio of gene diversities of heterozygosities ( $F_{st}$ ) and the gene flow ( $N_m$ ) at the species level are 0.0971 and 2.3236, respectively.

The AMOVA results (Table 6) indicate that only 3.03% of the total variance occurs among groups and 7.61% among populations within groups, whereas the remaining 89.35% of variation occurs within populations.

In the two-phase model, two populations of *H. aridicola* showed heterozygosity excess: the LB population (sign test:  $P = 0.04417$ ; Wilcoxon test:  $P = 0.00684$ ) and the MXT population (Wilcoxon test:  $P = 0.01221$ ) (Table 7).

#### 3.3. Genetic structure

Genetic distance between the QJ and XZE populations was the longest (0.5491), followed by those between the LB and QJ, XZE and MXT, LB and MXT, LB and XZE populations (Table 8). When we quantified the number of clusters of individuals, we found that the maximum value of  $\Delta K$  was reached when K was 3, and the second maximum was reached when K was 2 (Fig. 2a). The assignment of sampled individuals improved when they were assigned to two groups rather than to three groups (Fig. 2b). This is because one of the three groups would consist of LB and MXT populations; however, due to high levels of gene introgression between these two populations, they cannot be separated distinctly from another possible group (XZE) (Fig. 2b). Principle coordinate analysis showed that the first three principle coordinates explained 65.57% genetic variation, and the 1st coordinate (27.05%) explained similar genetic variation with the 2nd coordinate (23.14%). Both on the 1st and 2nd coordinates, *H. aridicola* individuals can be subdivided into two major groups, and the two groups divided on 2nd coordinate are similar to the following NJ analysis with one group (Group A) consisting of the LB, MXT, XZE populations and the other group (Group B) including only the QJ population (Fig. 3).

Neighbor-Joining (NJ) analysis indicated that the populations LB and XZE are the most closely related, followed by MXT and QJ (Fig. 4), and population genetic diversity are positively correlated to geographic distance ( $R^2 = 0.3749$ , Fig. 5).

### 4. Discussion

#### 4.1. The potential reasons leading to the deviations of HWE and HWLD

In this study, we used 11 microsatellite loci to investigate the population structure and genetic diversity of four populations of *H. aridicola* along the Jinsha River in Yunnan and Sichuan Provinces in southwest China. We found that most combinations of loci and populations deviated from Hardy–Weinberg equilibrium. We also found that two of the four *H. aridicola* populations we examined have undergone genetic bottlenecks. Deviations from Hardy–Weinberg equilibrium may be the result of sampling small populations and heterozygote deficiencies in population bottlenecks. Hardy–Weinberg equilibrium tests are more reliable when the number of individuals in each population is above 50 (Peakall

**Table 3**  
Linkage disequilibrium of 11 polymorphic microsatellite loci.

	HA-1	HA-3	HA-4	HA-5	HA-6	HA-7	HA-8	HA-9	HA-10	HA-13	HA-14
HA-1											
HA-3	3.7854										
HA-4	3.2907	Infinity**									
HA-5	6.9134	7.8803	3.3162								
HA-6	1.9991	8.899	2.1217	2.0243							
HA-7	7.5805	3.4199	5.6183	13.0193	1.9646						
HA-8	12.1415	22.6385	15.8588	3.5358	2.3222	17.6733					
HA-9	7.4187	4.8007	3.9194	4.3736	3.9748	3.1536	3.1104				
HA-10	2.4377	11.1916	1.3975	7.1923	4.0474	11.4523	8.9064	1.9175			
HA-13	2.3706	Infinity**	9.408	0.6771	0.3201	7.769	15.0671	5.6453	8.3469		
HA-14	8.2961	0.9078	5.5292	1.7926	1.4751	3.0709	6.3767	0.8349	5.4327	5.1801	

\*\*p &lt; 0.001.

**Table 4**  
Statistics of genetic diversity for *H. aridicola*.

Populations	N	Na	Ne	I	Ho	He	H	PPB
LB	32	6.2	4.2207	1.5198	0.4375	0.7528	0.7293	100%
MXT	24	5.8	4.0034	1.4551	0.3583	0.7373	0.7066	100%
QJ	48	7	4.3	1.5177	0.35	0.7148	0.6999	100%
XZE	34	6.6	3.7325	1.4642	0.2941	0.7173	0.6962	100%
Total	138	9.9	5.7401	1.8381	0.358	0.7894	0.7837	100%

**Table 5**  
Summary of genetic statistics and Wright's F-statistics of each loci for *H. aridicola*.

Locus	Sample Size	Fis	Fit	Fst	Nm
HA1	138	0.842	0.8683	0.1668	1.249
HA4	138	0.1714	0.2463	0.0904	2.5168
HA5	138	0.6309	0.6668	0.0972	2.3224
HA6	138	0.3927	0.4353	0.0702	3.3129
HA7	138	0.5077	0.5755	0.1378	1.5646
HA8	138	1	1	0.0301	8.0661
HA9	138	0.3762	0.4254	0.0788	2.9228
HA10	138	0.6358	0.6923	0.1552	1.3612
HA13	138	0.1879	0.2552	0.0828	2.7676
HA14	138	0.4492	0.4804	0.0566	4.1644
Mean	138	0.4907	0.5402	0.0971	2.3236

and Smouse, 2006); however, *H. aridicola* is a plant species with an extremely small populations and all current populations are below 50 individuals. In addition, genetic bottlenecks further reduce heterozygosity (Wang et al., 1998; Crow and Kimura, 1970).

Hardy–Weinberg disequilibrium between populations of *H. aridicola* may indicate physical linkage among loci, recent species-wide reductions in effective population size, effects of geographical structure, or selection. Over the last decade, human activities have caused rapid reductions in population sizes of *H. aridicola*, which in previous years were widely distributed along the Jinsha River. In addition, the geographic complexity in this area (Hoang et al., 2009) resulted in the unique geographical structure of *H. aridicola*. Similar geographical structures in populations of

**Table 6**  
Hierarchical analyses of molecular variance (AMOVAs) based on SSR data for 4 populations of *H. aridicola*.

Source of variation	d.f.	Σ of Squares	Variance components	variation [%]
Among groups	2	39.059	0.12353	3.03
Among populations within groups	1	13.869	0.31023	7.61
within populations	134	487.811	3.64038	89.35
Total	137	540.739	4.07414	

*Terminalia franchetii* (Zhang and Sun, 2011; Zhang et al., 2011b), *Buddleja crispa* (Zhang et al., 2015; Yue et al., 2012), and *Osteomeles schwerinae* (Wang et al., 2015) have also led to changes in population genetic diversity and structure.

#### 4.2. Genetic diversity of *H. aridicola*

Understanding genetic diversity and differentiation within and among existing populations is a significant component of developing effective conservation strategies (Rivers et al., 2011). Our population genetic analyses indicate that genetic diversity for *H. aridicola* is high at both the population (0.6962–0.7293) and species level (0.7837) compared to those of endemic/endangered species in China, e.g., *Abies ziyuanensis* (He = 0.435) (Tang et al., 2008), *Cycas hongheensis* (He = 0.453) (Zhao and Gong, 2015), *Houpoa officinalis* (He = 0.600) (Yang et al., 2018), *Psathyrostachys huashanica* (He = 0.3504) (Wang et al., 2006), *Rheum tanguticum* (He = 0.515) (Chen et al., 2009). Furthermore, the genetic diversity of *H. aridicola* is also higher than that of the reported average value of genetic diversity for endemic species (He = 0.42) (Nybom, 2004). These findings suggest that *H. aridicola* is relatively well-adapted to a variety of habitats within the hot and dry valleys of the Jinsha River. Our results also show that the decline in the number of *H. aridicola* populations is not the result of a decrease in population genetic diversity. Taken together, these findings suggest that declines in population numbers may be caused by human activities. Specifically, frequent human activity may have decreased the number of *H. aridicola* populations severely over the last several decades, thus increasing the risk of extinction for this species.

Our population genetic analyses also show that gene flow is frequent among the current populations (Nm = 2.3236). This frequent gene flow between populations may be due to the special distribution pattern of *H. aridicola*, in which the individuals grow along a river and its seeds are dispersed by wind in hot-dry valleys. This is similar to *B. crispa* (Zhang et al., 2015), a plant species distributed in the same area.

#### 4.3. Genetic differentiation and structure of *H. aridicola*

We also found that variation among the *H. aridicola* populations is low, and the majority of variation exists within populations. This is also indicated by the low ratio of gene diversities of heterozygosities (Fst = 0.0971). Previous studies reported that high genetic variation within populations may be caused by environmental heterogeneity (Ewing, 1979; Gillespie and Turelli, 1989; Yeaman and Jarvis, 2006). Thus, variable habitats in the dry and hot valleys might promote accumulation and accommodation of new mutations in *H. aridicola*, enabling this species to adapt to different habitats. The current population genetic structure of *H. aridicola*

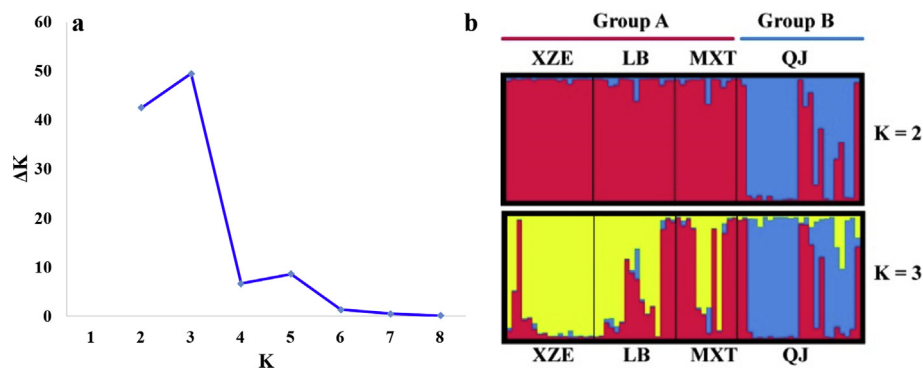
**Table 7**  
Results (P-values) of bottleneck testing under two models.

Population	N	Sign test				Wilcoxon test P-value	
		TPM		SMM		TPM	SMM
		No. of loci with heterozygosity excess	P-value	No. of loci with heterozygosity excess	P-value		
LB	16	5.96	0.04417	5.94	0.36629	0.00684	0.2158
MXT	12	5.82	0.13856	5.9	0.60859	0.01221	0.3125
QJ	24	5.77	0.32562	5.76	0.07447	0.1377	0.8125
XZE	17	5.86	0.59776	5.86	0.40323	0.34766	0.8623

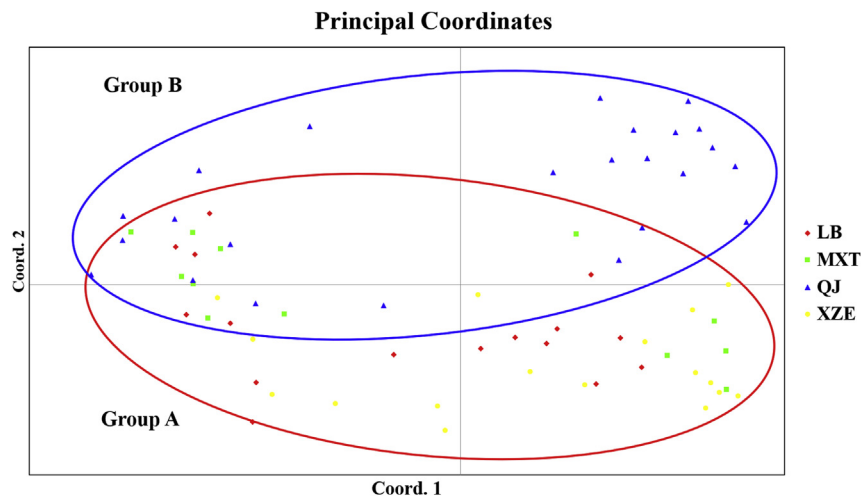
**Table 8**  
Nei's unbiased values of genetic identity (above diagonal) and genetic distance (below diagonal) between four populations of *H. aridicola*.

pop ID	LB	MXT	QJ	XZE
LB	****	0.7224	0.6657	0.7367
MXT	0.3252	****	0.7037	0.6698
QJ	0.407	0.3514	****	0.5775
XZE	0.3056	0.4008	0.5491	****

Asterisks means no values for the corresponding cells.



**Fig. 2.** Genetic structure of *H. aridicola* inferred by Bayesian clustering of SSR data. a: the best and the second-best grouping number was 3 and 2 ( $K = 3$  and 2) based on the  $\Delta K$  estimation. b: Assignment of 69 individuals into  $K = 2$  and  $K = 3$  genetically distinguishable groups. Each individual is represented by a vertical bar, colored according to the assigned group(s).



**Fig. 3.** Principal Coordinates Analysis of sampled individuals of *H. aridicola*. The first and second axes explained 27.05% and 23.14% of the genetic similarities among populations, respectively.

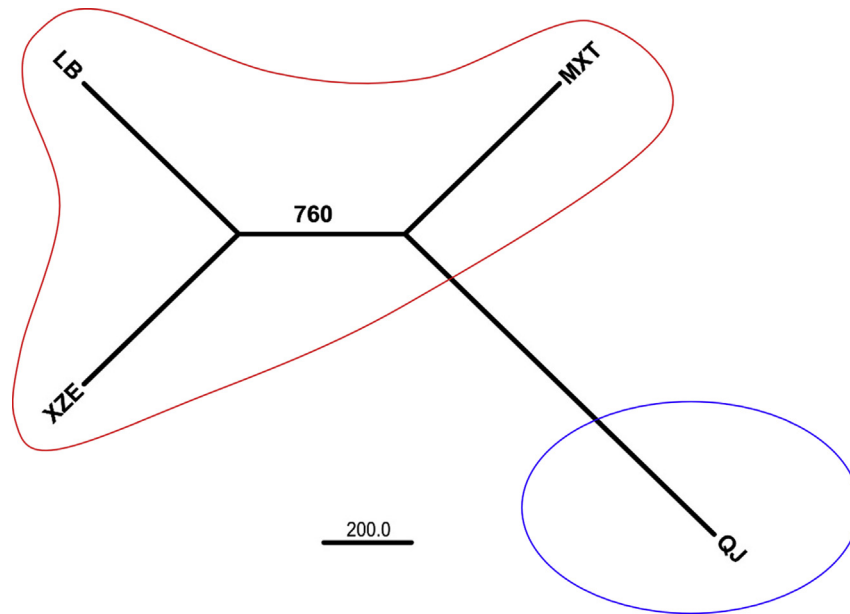


Fig. 4. Unrooted neighbor-joining dendrogram illustrating the genetic relationship among the four populations of *H. aridicola*.

(Figs. 2–4) could be caused by the geographic distances between the remaining populations.

#### 4.4. Conservation of *H. aridicola*

Taken together, our findings show that populations of *H. aridicola* have maintained high genetic diversity, low genetic differentiation and strong gene flow. However, we also found that two populations (LB and MXT) have undergone a genetic bottleneck, even though they have the highest genetic diversity. Therefore, in situ conservation efforts should focus on the LB and MXT populations; otherwise, the genetic bottleneck, which is known to lead to smaller population sizes, may decrease their ability to continue adapting to changes in the environment (Nei et al., 1975). Consequently, these populations may become extinct due to, among other factors, strong negative selective pressures such as human activities, inbreeding depression, and natural hazards.

*Ex situ* conservation efforts should focus on two populations, the isolated QJ population in Sichuan Province and the LB population in Yunnan Province. Because these populations have high genetic diversity, *ex situ* conservation would maintain gene flow among distinct populations and enrich the genetic pool of *H. aridicola*.

In conclusion, *H. aridicola* has high genetic diversity at both the population and species level, but low differentiation among

populations. The distribution of *H. aridicola* along the Jinsha River and wind-mediated dispersal of its seeds may contribute strongly to the current genetic structure of *H. aridicola* populations. Finally, two populations in Yunnan Province—the LB population in Ninglang County and the MXT population in Shangri-La County—have undergone a genetic bottleneck and require more attention from conservationists.

#### Conflicts of interest

The authors declare that there are no conflicts of interest.

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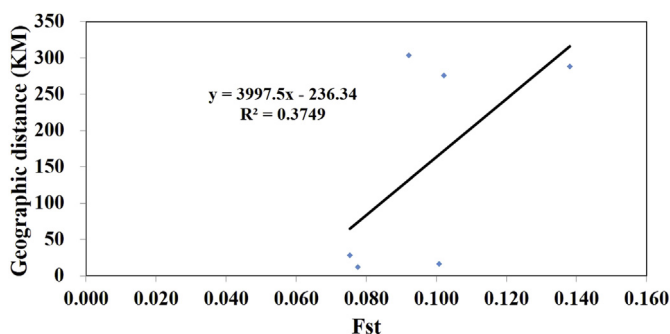


Fig. 5. Relationships inferred using the ratio of gene diversities of heterozygosities ( $F_{st}$ ) and the geographical distances (Mantel test).

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