How much genetic variation is stored in endangered and fragmented shrub *Tetraena mongolica* Maxim?

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Tetraena mongolica Maxim (Zygophyllaceae) is an endangered species endemic to western Inner Mongolia and China, and is currently threatened by habitat loss and human over-exploitation. We explored the genetic background (genetic diversity, population structure and demographic history) of *T. mongolica* based on 12 polymorphic nuclear microsatellite loci. Our results indicated high genetic diversity in extant populations, but no distinguishable gene cluster corresponding with a specific biogeography. Population demography analysis using a Markov-Switching Vector Autoregression indicated a strong, recent population decline approximately 5,455 years ago. These results suggest that the Yellow River and Zhuozi Mountain range may not prevent pollination between populations. Finally, we surmised that the population demography of *T. mongolica* was likely to have been affected by human activities.

How Much Genetic Variation is Stored in Endangered and Fragmented Shrub *Tetraena mongolica* Maxim?

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ABSTRACT

Tetraena mongolica Maxim (Zygophyllaceae) is an endangered species endemic to western Inner Mongolia and China, and is currently threatened by habitat loss and human over-exploitation. We explored the genetic background (genetic diversity, population structure and demographic history) of *T. mongolica* based on 12 polymorphic nuclear microsatellite loci. Our results indicated high genetic diversity in extant populations, but no distinguishable gene cluster corresponding with a specific biogeography. Population demography analysis using a MSVAR indicated a strong, recent population decline approximately 5,455 years ago. These results suggest that the Yellow River and Zhuozi Mountain range may not prevent pollination between populations. Finally, we surmised that the population demography of *T. mongolica* was likely to have been affected by early mankind activities.

Keywords:

Tetraena mongolica, Genetic diversity, Population structure, Population decline, Yellow River

INTRODUCTION

Understanding population history and genetic structure is a key aspect of ecological research (Rockwood, 2006). Endemic species with restricted geographic distributions have become a central concern of biologists faced with the problem of preserving rare species endangered by habitat destruction and fragmentation (Ge et al., 2003). For endemics with narrow ranges and declining populations, information about historical patterns of demography, genetic structure, and genetic variation within and among natural populations helps to clarify population structure, and the organism's evolutionary history, supporting conservation and management efforts (Moritz, 1994; Ge et al., 2011). Intraspecific genetic variation is the most fundamental level of biodiversity, providing the basis for evolutionary change and the ability of species to adapt to new environmental conditions (Frankham, Ballou & Briscoe, 2002). In contrast, in most plants and invertebrates with high fecundity and human-mediated dispersal, populations can be successfully establishment and experience range expansion despite of its genetic polymorphism (Song et al., 2013).

Previous studies revealed that natural landscape features such as mountains and rivers can act as genetic boundaries and shape the population structure of the wildlife (Funk et al., 2001; Whiteley et al., 2004). Besides, anthropogenic landscape features also impact upon genetic structure (Gaines et al., 1997) and population dynamics (Nupp & Swhart, 1998). Anthropogenic disturbance, such as roads, has dramatically increased the physical isolation of populations and it has been assumed that such isolation will lead to reduced gene flow and consequently reduced genetic diversity in populations (Byrne et al., 2007). Besides, habitat destruction and land-use

change may also influence gene flow at the landscape scale (Manel et al., 2003; Eckstein et al., 2006). These anthropogenic effects may also occur in the center of a species' range and may thus be superimposed on natural geographic patterns.

Tetraena mongolica Maxim is a narrowly monotypic genus of the family Zygophyllaceae endemic to western Inner Mongolia around the Yellow River basin, and is nationally endangered in China (Fu, 1992; Xu et al., 1998; Zhang & Yang, 2000). Its distribution is restricted to the western Gobi, the largest desert in Asia, and one characterized by extremely low annual rainfall (Xu et al., 1998; Zhang & Yang, 2000), where *T. mongolica* is able to survive because of its extensive root system, and acts as a windbreak and soil stabilizer (Dong & Zhang, 2001; Zhang et al., 2003). Its stems contain high levels of triacylglycerol (Wang, Ma & Zheng, 2000), and are combustible, even when green. For this reason, *T. mongolica* is a popular firewood species, and its range has declined alarmingly through overexploitation (Zhang & Yang, 2000; Ge et al., 2011). Presently, neither the impacts of natural barriers to dispersal, nor human influences on the genetic structure and demographic history of *T. mongolica* have been ascertained.

Evolutionary, demographic and genetic analyses all contribute to conservation and management of species (Beaumont & Bruford, 1999; O'Brien, 1994). We generated a comprehensive genetic characterization for *T. mongolica* with the aim of supporting a conservation strategy. We used twelve microsatellites genotyped onto an extensive dataset to evaluate the current genetic diversity in *T. mongolica* populations, and to assess the effect of natural landscape barriers (Yellow River and Zhuozi Mountains) in shaping population structure. Lastly, we modeled the demographic history of *T. mongolica* to assess the effects of historic

events on current genetic variation. Our findings may be useful for the conservation and management of *T. mongolica* and other species endemic to the Yellow River basin.

MATERIAL AND METHODS

Ethical statement

The collection of samples was performed within a investigation project on plants of *T. mongolica*. This investigation project and the sample collection were approved by the West Ordos National Nature Reserve, Inner Mongolia Province, China. Field experiments were also approved by the West Ordos National Nature Reserve, Inner Mongolia Province, China.

Sample collection

Between 2010 and 2014, 339 leaf samples of *T. mongolica* were collected from eight populations along the G6 Road: Shizuishan (SZS, N = 32); Dishan (DS, N = 64); Hainan (HN, N = 51); Dongalashan (DALS, N = 62); Wuda (WD, N = 32); Qianlishan (QLS, N = 32); Wujiamiao (WJM, N = 36) and Taositu (TST, N = 30) (Fig. 1). Leaves sample were powdered in liquid nitrogen and stored in a freezer at -80 °C.

DNA extraction, PCR amplification and microsatellite genotyping

Total genomic DNA was extracted from the powdered tissue following a modified CTAB procedure (Doyle & Doyle, 1987), and purified by EasyPure PCR Purification Kit (TransGene). In present study, we used twelve high polymorphic loci for *T. mongolica* (Zhi et al., 2014) as genetic marker. PCR reaction mixtures (25 μL) consisted of 1 μL genomic DNA (concentration 10-50 ng/μL), 2 μL 10× buffer, 1 μL of 2.5 mM $MgSO_4$, 2 μL of 2 mM dNTPs, 1 U *Taq* polymerase, 0.3 mM of each primer (forward primer fluorescently labeled with FAM, HEX or

101 TAMRA) and sufficient water. The amplification program was conducted with following
102 conditions: 5 min at 95°C; followed by 35 cycles of 30s at 95°C, 20 s at the annealing
103 temperature (55 / 60 °C), 30 s at 72°C; and 5 min at 72°C. PCR products were genotyped on an
104 ABI 3730 semi-automated sequencer (PE Applied Biosystems) with GS500 marker, and then
105 analyzed under GeneMarker 1.85 (SoftGenetics LLC) (Holland and Parson, 2011).

106 **Data analysis**

107 The presence of null alleles and genotyping errors in microsatellite genotyping was detected
108 by Micro-Checker v2.2.3 as previous described in Van Oosterhout et al., (2004), while the
109 linkage disequilibrium was tested with GENEPOP 4.2.1 as described in Rousset (2008). In
110 addition, several population genetic summary statistics to describe genetic variation were
111 estimated by GENETIX v.4.02 as described in Belkhir, Borsa, Chikhi, Raufaste, & Bonhomme
112 (2001), including mean number of alleles per locus (MNA), observed heterozygosities (H_O),
113 expected heterozygosities (H_E) and inbreeding coefficients (F_{IS}). Besides, allelic richness (AR)
114 was also calculated to estimate the allelic diversity that compensates for unequal sample size by
115 FSTAT and averaged across loci (Goudet, 2001). Genetic differentiation (F_{ST}) between
116 populations was estimated using ARLEQUIN 3.0 (Excoffier, Laval, & Schneider, 2005), and
117 statistical significance of F_{ST} values was tested with 10,000 permutations. In addition, the
118 association between the estimates of $F_{ST} / 1 - F_{ST}$ (Rousset & Raymond, 1997) and land-based
119 Manhattan distance were assessed using the Mantel test, implemented in the Isolation by
120 Distance Web Service (IBDWS) software (Jensen, Bohonak, & Kelley, 2005); the statistical
121 significance of the values was obtained by 10,000 randomization steps.

A Bayesian analysis of population structure as previously described in Pritchard, Stephens, & Donnelly (2000) was carried out to estimate the number of potential clusters present in the microsatellite data and to assign individuals to inferred clusters by STRUCTURE. Specifically, five independent runs were carried for different values of K between 1 and 8, using no prior information about individual location, and assuming admixture and correlated allele frequencies. The Markov Chain Monte Carlo (MCMC) was run for a total of 1 million generations discarding the first 100,000 as burn-in. The most likely K explaining the variation in the data was selected estimating the maximal value of the log likelihood [Ln Pr(X/K)] of the posterior probability of the data for a given K (Pritchard, Stephens, & Donnelly, 2000), and the ΔK statistic (Evanno, Regnaut, & Goudet, 2005). The population structure results were graphically displayed by the software DISTRUCT (Rosenberg, 2004). Furthermore, we visualized the genetic differentiation among all samples with a factorial correspondence analysis (FCA) in GENETIX version 4.0.

Demographic history was performed in BOTTLENECK 1.2.02 (Piry, Luikart, & Cornuet, 1999) and assessed using Wilcoxon's sign rank test and mode-shift test as previously described in Cornuet & Luikart (1996) and Luikart & Cornuet (1998), respectively. Besides, the software MSVAR v.1.3 was used to characterize the recent demographic history of the whole *T. mongolica* population based on the microsatellite data as described in Storz, Beaumont, & Alberts (2002). Specifically, this method assumes that a current population (of size N_0) passed through a demographic change (a bottleneck or an expansion) at time T in the past, which changed its size from N_1 to N_0 following an exponential model. Five independent simulations were run to estimate the distributions of these three parameters. For *T. mongolica*, the average

143 generation time is four years (Xu et al., 1998), and this period was adopted for the simulation.
144 Each MSVAR run consisted of 2×10^9 iterations of the MCMC algorithm discarding the first 10%
145 of the coalescent simulations as burn-in. The median (50%) of the posterior distributions were
146 calculated from five runs data. Finally, we plotted the marginal posterior distributions of the
147 three parameters by the LOCFIT package (Loader, 2007) implemented in R based on five runs.

148 **RESULTS**

149 **Genetic diversity**

150 In this study, a total of 339 individuals were genotyped at 12 loci. Micro-Checker did not
151 indicate null alleles or genotyping errors such as large allele dropout or stuttering. There was no
152 linkage disequilibrium at any locus in any population. The MNA for the eight populations varied
153 between 13.17 and 17.67 with an overall value of 15.25. The overall observed heterozygosity
154 (H_O) was 0.840 (0.810 – 0.873), while the overall expected heterozygosity (H_E) was 0.868 (0.832
155 – 0.882) (Table 1). Allelic richness (AR) ranged from 6.860 – 10.529, with an overall allelic
156 richness across loci of 9.382 (Table 1). Inbreeding coefficient analysis generated negative values
157 in SZS and QLS populations (Table 1). For the population as a whole, genetic diversity as
158 characterized by microsatellite markers was higher than that reported for other shrub species
159 (Table 2).

160 **Population structure and gene flow**

161 Based on STRUCTURE analysis, the Dealt *K* statistics output showed a clear maximum at
162 $K = 2$, but no obvious maximum log likelihood of posterior probability was found (Fig. 2A and
163 2B). The ΔK value was notable at $K = 6$ (Fig. 2B), with an obvious maximum log likelihood of

posterior probability. These data suggest that six potential genetic clusters may exist among them. Notably, factors such as recent admixture, admixture with unsampled / unobservable "ghost" populations, and recent bottlenecks may lead to misinterpretation of STRUCTURE results (Gilbert et al., 2012; Lawson et al., 2012; Falush, Van Dorp & Lawson, 2016). According to this framework, K = 6 may be a psendophase. The highly mixed color bars in the DISTRUCT diagram (for K = 2 – 6, Fig. 2C) indicated strong admixture among the eight populations. Furthermore, F_{ST} values among these populations ranged from 0.00034 to 0.04284, indicating extremely weak genetic differentiation among them (Table 3). Besides, IBD tests detected no significant correlation between geographical distances and genetic distance for the whole samples. Furthermore, no separate groups were identified in the FCA analysis (Fig. 3). Specifically, all populations were highly clumped and overlapped (Fig. 3).

Population demography

In present study, there is no significant signal of recent bottlenecks in eight populations under both TPM and SMM model, meanwhile, the mode-shift test also showed a normal L-shaped distribution of allele frequencies. However, the MSVAR results showed the posterior distribution of N_0 and N_1 did not overlap under exponential models, which disclosed that the whole population passed through significant reduction in effective population size (Fig. 4A). Statistically, the average medians of the posterior distributions were approximately 2.9652 for log N_0, and approximately 4.7938 for log N_1 (Fig. 4A). Therefore, for *T. mongolica* population, the current effective population size (N_0) was approximately 923, while the ancestral effective population size (N_1) was approximately 62,214, showing an approximate 67 times population

185 decrease. Furthermore, the medians of the posterior distribution log T = 3.7368 (Fig. 4B),
186 indicating a recent population decline took place in 5,455 years ago.

187 **DISCUSSION**

188 **Genetic diversity**

189 Because of human overexploitation, *T. mongolica* has undergone dramatic population
190 decline in past decades (Ge et al., 2011). However, our assessment of genetic variation based on
191 microsatellite data revealed high levels of genetic diversity in this species. In the population as a
192 whole, high microsatellite diversity was detected, with MNA, H_O and H_E values of 15.45, 0.84
193 and 0.868, respectively (Table 1). Compared with Zhang and Yang's study, we determined high
194 genetic diversity for nuclear DNA in this species (Zhang & Yang, 2000). Using microsatellite
195 markers, we also detected high genetic diversity in *T. mongolica* compared with other shrub
196 species such as *Zygophyllum xanthoxylon*, *Ziziphus celata*, *Adiantum capillus-veneris*, *Grevillea*
197 *macleayana*, *Arabidopsis lyrata*, *Calothamnus quadrifidus*, *Myrtus communis* and *Schiedea*
198 *adamantis* (Table 2). Generally, species with high genetic diversity are members of large
199 populations that were geographically widespread in recent history (González et al., 1998).
200 Therefore, in this study, the high genetic diversity of *T. mongolica* may reveal the large effective
201 sizes of ancestral populations, as supported by the demographic analysis using MSVAR.
202 Furthermore, from a conservation perspective, it also implies that the recent sharp population
203 decline event did not have a significant effect on the genetic diversity of *T. mongolica*.

204 **Population genetic structure**

205 Landscape features such as rivers and mountains can function as geographical barriers to

dispersal and gene flow, shaping population structure (Funk et al., 2005; Whiteley et al., 2004). STRUCTURE analysis did not clearly identify genetic clusters corresponding to specific populations (Fig. 2). Clustering results indicated unobstructed admixture and thus weak genetic differentiation among *T. mongolica* populations. This result was corroborated by the pairwise F_{ST} estimates and FCA analysis (Table 2, Fig. 3). This genetic structure pattern suggests that the Yellow River and Zhuozi Mountain do not act as significant barriers to pollination between populations.

The Yellow River, the second longest river in China, is well-known for its frequent flooding and heavy silt load (Sinclair, 1987). In the last 3,000 years, the river's levees have breached more than 1,500 times and its course has changed approximately 26 times (Leung, 1996). As a result, *T. mongolica* populations on the flood plain have been exposed to periodic habitat destruction and fragmentation (Ge et al., 2011). It is established that species with narrow distributions and small population sizes face a high risk of extinction, especially when gene flow between sub-populations is restricted (Frankham, Ballou & Briscoe DA, 2002; Hanski & Gilpin 1997). In seed plants, such gene flow occurs via the movement of pollen or seeds. Fortunately, *T. mongolica* is primarily pollinated by insects (Zhen & Liu, 2008), negating the potential barrier effect of the Yellow River and, to some degree, the Zhuozi Mountain (Fig. 1). Hence, neither distinguishable genetic clusters nor population differentiation were detected in populations separated by these barriers.

Population demography

In present study, neither heterozygosity excess nor mode-shift tests suggested a recent

population bottleneck for *T. mongolica*. However, MSVAR simulation indicated a severe recent population decline in all populations (Fig. 4). Under the exponential model, the posterior distribution of N_0 and N_1 (50% quantile) indicates a 67 fold population decline, starting approximately 5,455 years ago, and mirrored in similar declines for animals. For example, in Northeastern Malaysia, human-induced deforestation and habitat fragmentation resulted in a recent population collapse in orangutans, *Pongo pygmaeus*, approximately 210 years ago (Goossens et al., 2006). Humans in southwestern China, over the course of thousands of years, have caused the dramatic decline of the giant panda, *Ailuropoda melanoleuca* (Zhang et al., 2007) and the tufted deer, *Elaphodus cephalophus* in the Yangtze River area (Sun et al., 2016). These events suggest the possibility of an anthropogenically induced decline for *T. mongolica*. In addition, it is worth noting that the high-density human activities along the G6 road would also siginificant impact on *T. mongolica* populations in the next few decades.

Implications for conservation

Population genetics studies can help to identify management units (MUs) and evolutionarily significant units (ESUs) for conservation (Moritz, 1994). In this study, all of the analysis results indicating weak genetic differentiation among extant populations. Our work suggests that the eight *T. mongolica* populations sampled may be deemed a single MU for conservation purposes. With rapidly increasing human disturbance, *T. mongolica* populations are suffering from overexploitation, habitat loss and fragmentation, most noticeably along the G6 road, where priority should be given to the conservation and restoration of its habitat. To better maintain the population size of *T. mongolica*, we propose that the Chinese government should plant more

artificial populations in the core area of its current range along the G6 Road.

CONCLUSION

In this study, 339 individuals from eight populations were genotyped at 12 nuclear loci, successfully. Based on microsatellite data, high levels of genetic diversity was revealed in this endangered species. It implies that the wild *T. mongolica* populations still harbor a surprisingly rich gene pool. Furthermore, neither distinguishable genetic clusters nor population differentiation were detected among extant *T. mongolica* populations. Finally, a strong and recent population decline event was discovered, which was likely to be affected by early mankind activities.

ACKNOWLEDGEMENTS

The authors would like to thank Qiang Wang for his help in sample collecting. We would also like to thank Tao Pan, Chaochao Hu and Guiyou Wu for their laboratory assistance and advice.

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Figure 1

Figure 1 Map showing the population location of *T. mongolica* sampled in this study.

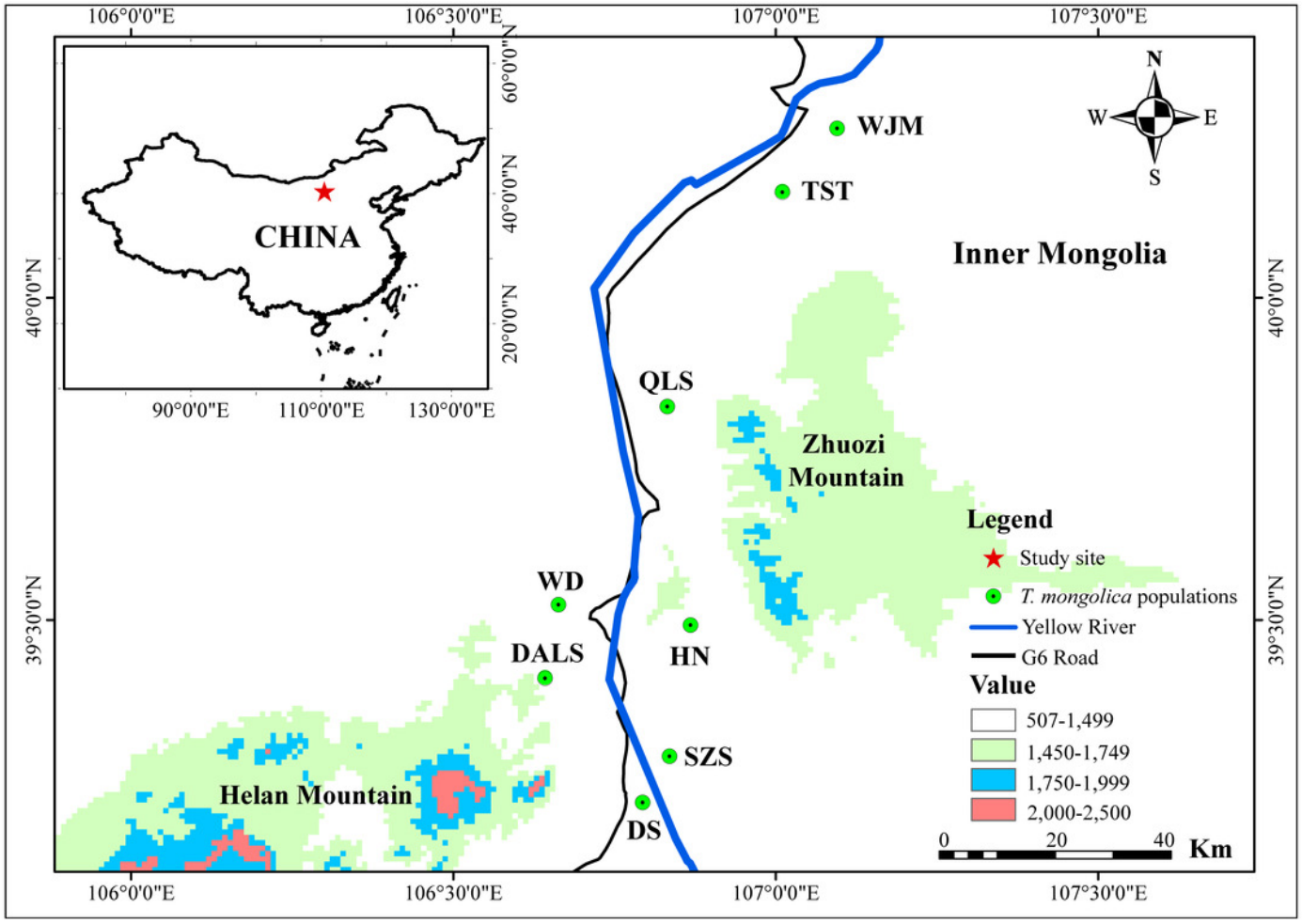

Figure 2

Figure 2 Bayesian STRUCTURE clustering results of microsatellite variation among *T. mongolica* populations.

(A) The linear relationship between LnP(D) and *K*, (B) Dealt *K* values as a function of *K* based on 5 runs and (C) STRUCTURE output from *K* = 2 to 6.

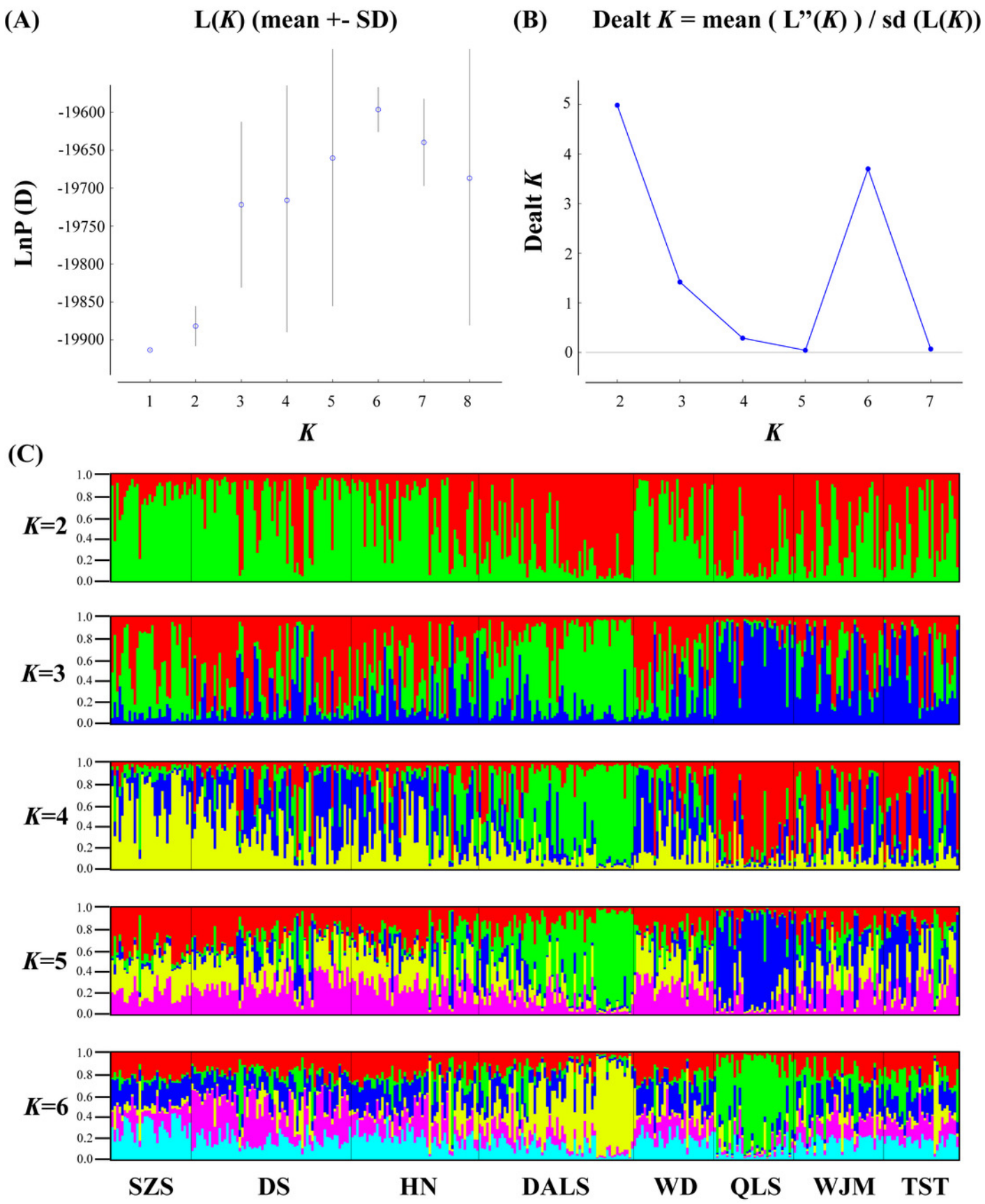
(A)
L(K) (mean +- SD)
LnP (D)
-19600
-19650
-19700
-19750
-19800
-19850
-19900
1
2
3
4
5
6
7
8
K
(B)
Dealt K = mean (L"(K)) / sd (L(K))
Dealt K
5
4
3
2
1
0
2
3
4
5
6
7
K
(C)
K=2
K=3
K=4
K=5
K=6
1.0
0.8
0.6
0.4
0.2
0.0
SZS
DS
HN
DALS
WD
QLS
WJM
TST

Figure 3

Figure 3 Factorial correspondence analysis performed for *T. mongolica* based on nuclear microsatellite loci.

Symbols and colors represent individuals from different populations.

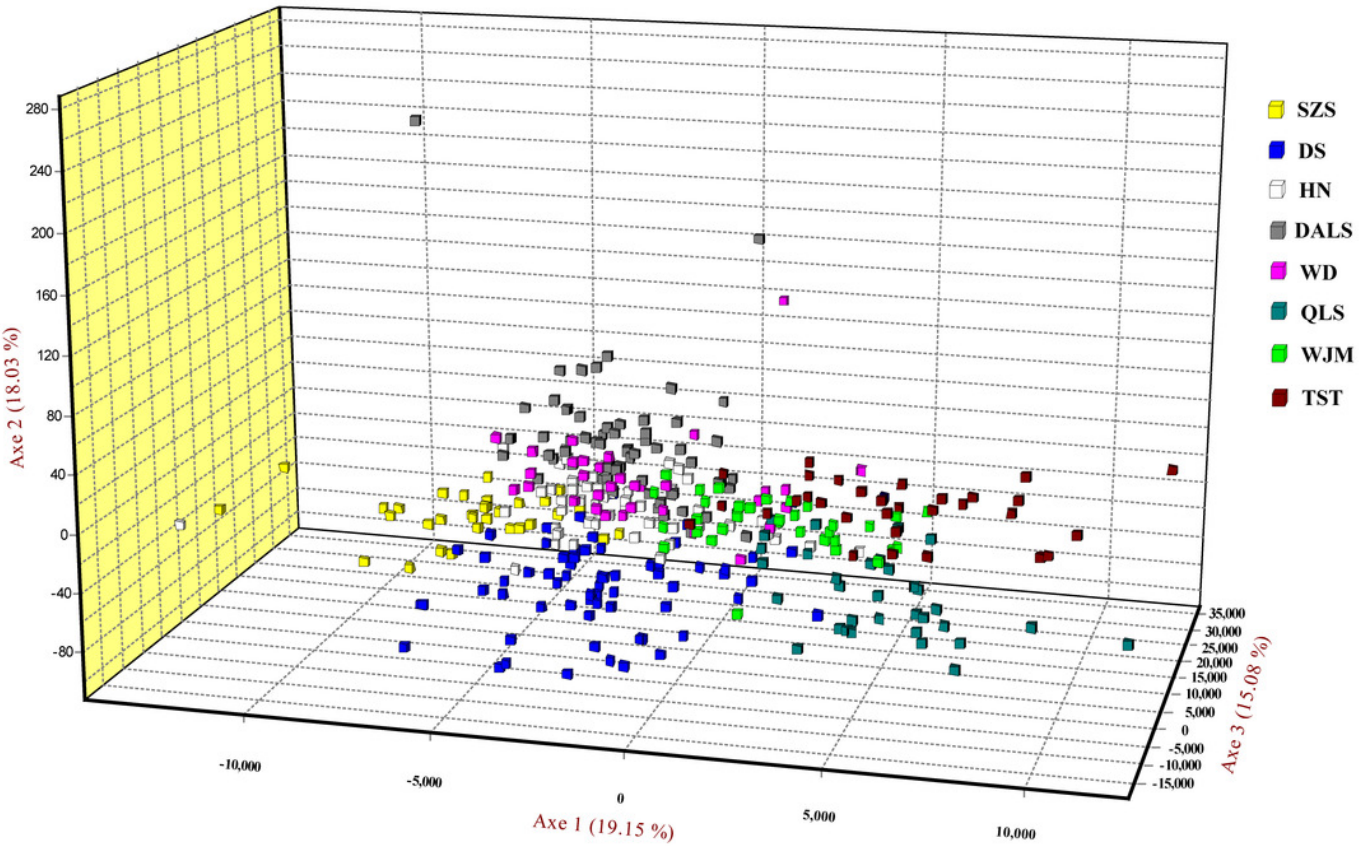

Figure 4

Figure 5 Estimated posterior distributions of N_0, N_1 and T using MSVAR.

N_0, current effective population sizes (blue curve); N_1, ancestral effective population sizes (red curve); T, time since population change (black curve). All densities are represented in a log10 scale.

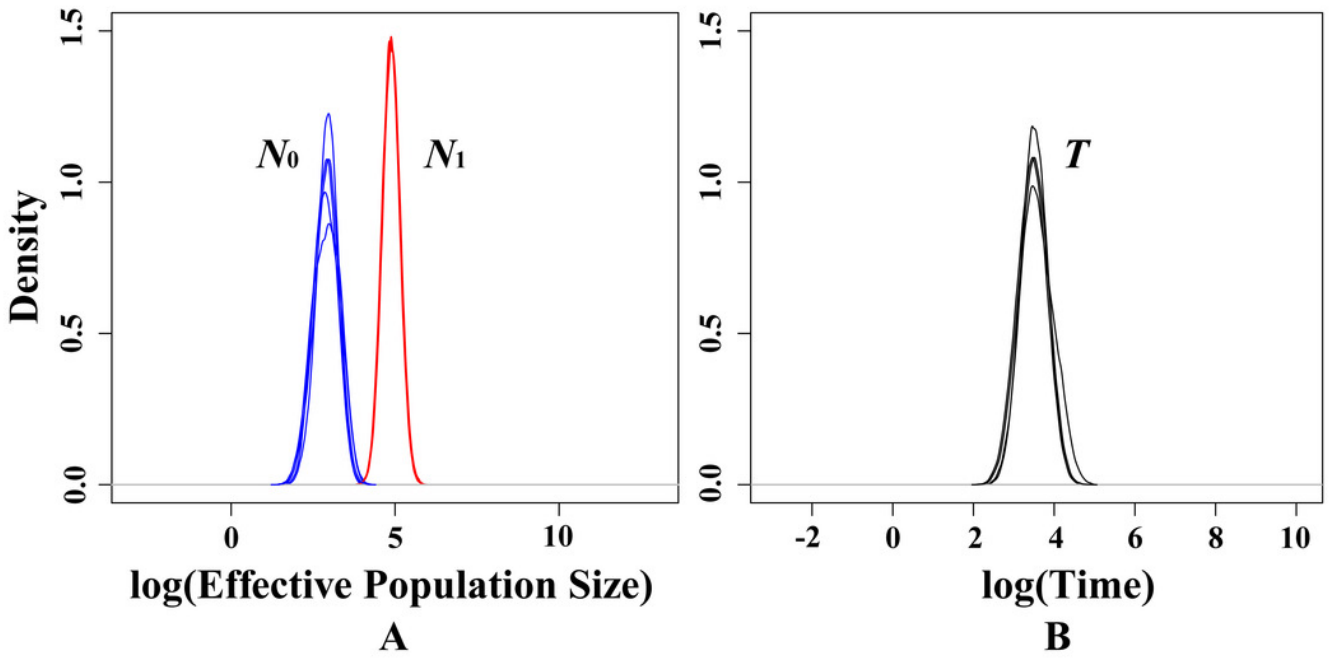

Table 1(on next page)

Table 1 Genetic variability observed within populations using microsatellite loci.

Note: N, number of individuals; MNA, mean number of allele per locus; AR, allelic richness; H_o and H_E, observed and expected heterozygosity; F_{IS}, inbreeding coefficient.

Table 1. Genetic variability observed within populations using smicrosatellite loci.

Population	N	MNA	AR	H_O	H_E	F_{IS} (IC 95%)
SZS	32	13.17	6.860	0.850	0.832	-0.00163 (-0.03378 - 0.00409)
DS	64	17.67	8.284	0.836	0.881	0.06051 (0.02421 - 0.07440)
NH	51	17.67	10.499	0.812	0.880	0.08889 (0.04226 - 0.09998)
DALS	62	16.75	8.974	0.810	0.867	0.07559 (0.04021 - 0.09279)
WD	32	14.67	8.829	0.851	0.867	0.03657 (-0.02664 - 0.07773)
QLS	32	13.92	8.532	0.873	0.854	-0.00596 (-0.05695 - 0.00596)
WJM	36	15.67	10.529	0.852	0.882	0.04891 (-0.01434 - 0.05576)
TST	30	14.08	9.397	0.840	0.877	0.06018 (0.02117 - 0.06018)
Total	339	15.45	9.382	0.840	0.868	0.06760 (0.05360 - 0.06888)

Note: N, number of individuals; MNA, mean number of allele per locus; AR, allelic richness; H_o and H_E, observed and expected heterozygosity; F_{IS}, inbreeding coefficient.

Table 2(on next page)

Table 2 Genetic diversity of *Tetraena mongolica* and other shrub based on microsatellite loci.

1 **Table 2. Genetic diversity of *Tetraena mongolica* and other shrub based on nuclear microsatellite loci.**

Species	N	MNA	H_O	H_E	Reference
Tetraena mongolica	339	15.45	0.84	0.868	In this study
T. mongolica	338	1.6	0.199	0.345	Zhang and Yang 2000
Zygophyllum xanthoxylon	61	2.2	0.43	0.392	Zhang and Yang 2000
Ziziphus celata	595	2.23	0.69	0.39	Gitzendanner et al., 2001
Adiantum capillus-veneris	151	-	0.13-0.37	0.2-0.63	Pryor et al., 2001
Grevillea macleayana	321	-	0.248-0.523	0.420-0.523	England et al., 2002
Arabidopsis lyrata	344	9.3	0.48	0.52	Clauss and Mitchell-Olds 2006
Calothamnus quadrifidus	114	19.67	0.584	0.867	Byrne et al., 2007
Myrtus communis	48	-	0.258-0.802	0.125-0.875	Albaladejo et al., 2010
Schiedea adamantis	49	-	0.125-0.755	0.041-0.787	Culley et al., 2008

2

Table 3(on next page)

Table 3 Pairwise F_{ST} estimates based on microsatellite loci.

Note: The asterisks (*) mean $P<0.05$.

Table 3. Pairwise F_{ST} estimates based on nuclear microsatellite loci.

Populations	1	2	3	4	5	6	7	8
1. SZS								
2. DS	0.01599*							
3. HN	0.00607*	0.00620*						
4. DALS	0.01648*	0.01623*	0.0076*					
5. WD	0.01795*	0.00834*	0.00793*	0.01522*				
6. QLS	0.04284*	0.02743*	0.01740*	0.02972*	0.03673*			
7. WJIM	0.01580*	0.00944*	0.00034	0.00769*	0.01095*	0.01839*		
8. TST	0.02699*	0.01837*	0.01373*	0.01829*	0.02349*	0.02138*	0.00912*	

Note: The double asterisks (*) mean $P<0.05$.