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Population structure and genetic variation of the endangered species *Elaeagnus mollis* Diels (Elaeagnaceae)

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ABSTRACT. *Elaeagnus mollis* Diels is a group of shrubs and dwarf trees endemic to China and are endangered plants. However, the reason why these plants are endangered remains controversial. The current study aimed to explore the endangered status of *E. mollis* from a genetic perspective and to propose conservation strategies for this species. Using 16 polymorphic allozyme loci, the population genetic structure was investigated for three populations representing the taxa and variants. The variants exhibited relatively high levels of genetic variation compared to other woody shrubs with similar geographic distributions. The overall genetic diversity (H_E = 0.352) was elevated compared to long-lived woody angiosperms. The average number of alleles per locus (*A*), percentage polymorphic loci (*P*), and observed heterozygosity (H_O) were 2.0, 85.2, and 0.371, respectively. Furthermore, gene flow estimates within the population groups were also elevated. The life history and habitats of *E. mollis* play major roles in the levels of genetic diversity. The results of this study may help to device strategies for preserving the genetic diversity of *E. mollis* and

for promoting planting.

Key words: *Elaeagnus mollis*; Allozymes; Endangered species; Conservation; Population genetic structure

INTRODUCTION

Because rare species have a very uncertain fate, many studies have attempted to identify the features that characterize them (Young and Brown, 1996; Ge and Hong, 1999; Broadhurst and Coates, 2002).

Theoretically, species with limited ranges and few individuals will exhibit low levels of genetic polymorphism, and this phenomenon occurs because of selection under a narrow range of environmental conditions and genetic drift as well as inbreeding in small isolated populations (Ellstrand and Elam, 1993). Studies on genetic variation in plant species have revealed a strong association between the level of genetic diversity within a species and its geographic range (Hamrick and Godt, 1989). However, not all rare and endemic plant species are genetically depauperate, and some rare and endangered species maintain high levels of genetic variation (Young and Brown, 1996; Ge and Hong, 1999). Obviously, many other factors, such as life history traits, human activity, and evolutionary histories, may also influence the genetic variation and distribution of plant species (Karron et al., 1987).

Elaeagnus mollis Diels is a deciduous dwarf tree or shrub that is endemic to China. The species is a vestigial plant of the Quaternary Glaciation and is currently listed as endangered nationally (China State Environmental Protection Administration & Institute of Botany Chinese Academy of Sciences, 1991). *E. mollis* is disjunctly distributed primarily in the hills and lower mountains of southern Shanxi (three populations) and at the northern foot of the Qinling Range in Shaanxi (one population) (N34°05'-36°05', E110°37'-111°56') (Shangguan and Zhang, 2001). *E. mollis* is mainly pollinated by bees. The eco-environmental characteristics of *E. mollis* are typically efficient heat energy, little annual rainfall, and poor stands (Shangguan and Zhang, 2001; Zhang et al., 2001). Recent habitat fragmentation has affected all *E. mollis* populations by reducing their size and increasing their isolation (Qin et al., 2006, 2010).

To date, numerous researchers have investigated why *E. mollis* is endangered. The endangered status of *E. mollis* may not be related to low genetic diversity but instead to the specific breeding system of the species, as well as ecological factors (Qin et al., 2006, 2010; Xu et al., 2011). The endangered status of *E. mollis* may also be caused by strong artificial interference. A short seed life-span and low germination rate also endangers the species (Shangguan and Zhang, 2001). In addition, it is disadvantageous for seedlings and juvenile *E. mollis* to be in competition with other plants for various resources (Shangguan and Zhang, 2001). However, in these studies, the genetic diversity of *E. mollis* has been assessed using simple sequence repeat markers and randomly amplified polymorphic DNA, respectively. In conclusion, the definitive cause for the endangered status of the species remains controversial and further studies are required.

In this paper, for the first time, allozyme loci were used to examine *E. mollis* population genetics. Our objectives were to 1) determine the amount and distribution of genetic diversity within and between populations, 2) analyze the factors driving genetic diversity, and 3) provide recommendations for *E. mollis* conservation.

MATERIAL AND METHODS

Subjects

E. mollis Diels (Elaeagnaceae) is a long-lived deciduous tree or shrub that grows to 2-10 m in height. The leaves alternate and are papyraceous, rarely membranaceous, ovate to ovate-elliptic, 6-15 cm long and 3-11 cm wide; the petioles are 6-15 mm long and semi-orbiculate. The flowers are hermaphroditic, grayish green, fragrant, and densely covered in stellate hairs; the pedicels are 3-4 mm long. Four stamens are present along with ellipsoid anthers approximately 1.6 mm long. The fruits are subglobose or broadly ellipsoid and approximately 13 mm long, with eight conspicuous raphes, wings, cottony sarcocarp. The flowering period is between April and May, and fruiting occurs between August and September.

Areas investigated

Most of the plants studied grew on a shady or half-shady slope at altitudes of 780-1,520 m. The slopes were between 30° and 60°. For low mountain areas, they primarily grew on slopes of 15-25°. The relative positions of the three populations studied were as follows: Xiangning population-Yicheng population, 78 km; Yicheng population-Pinglu population, 108 km; and Xiangning population-Pinglu population, 132 km. The climate characteristics of the areas investigated are summarized in Table 1. The geographic locations of the areas studied are shown in Figure 1.

Figure 1. Locations of the study populations in Shanxi Province, China.

Table 1. Climate characteristics of the study areas.

	Xiangning	Yicheng	Pinglu
Average temperature (°C)	9.9	12.3	13.8
Maximal temperature (°C)	35	41.3	41.3
Minimal temperature (°C)	-19.8	-19.1	-13.2
Frost-free period (d)	212.6	227	238.4
Average annual rainfall (mm)	570.2	542.8	551.3
Altitude (m)	1,200-1,520	1,200-1,340	920-1,060

Sample collection

Fresh leaves (without wormholes or corruption) were collected, sealed in plastic bags with wet filter paper, and were stored in a pot on ice. After returning from the field, samples were stored at -20°C. To avoid selecting samples from the same clone, the collection sites were located 20 m apart.

Electrophoresis

A total of 268 leaf samples were collected from three Shanxi populations (Figure 1). The leaves were stored at -20°C for use in electrophoresis. Leaf tissues were ground in a chilled glass mortar with quartz powder. The fine powdered tissue was centrifuged for at 14,000 rpm for 30 min at 4°C, and the crude extract was mixed with an equal volume of 40% (w/v) sucrose and 3-5 drops of 0.2% (w/v) bromophenol blue. The solution extracted was divided into portions of 100 µl per EP tube and stored at -80°C until electrophoresis. This preparation and the isozyme methods were performed at the Institute of Loess Plateau, Shanxi University.

Allozyme diversity was determined via vertical slab polyacrylamide gel electrophoresis. The staining recipes for all enzymes have been previously described by Wang (1996) and were used with minor modifications. Individual gametophytes were smeared onto wicks that were pre-moistened with an extraction buffer-PVP solution. After the plant material was absorbed, the wicks were inserted into a vertical slit in the starch gel and subjected to horizontal electrophoresis at 4°C. After the first 10-20 min of electrophoresis, the wicks were removed from the gel for better band definition. A spacer was routinely placed at the cathode end of the gel to slightly compress the gel and to insure that the slit did not open.

Observation/measurement indices

Fourteen enzyme systems were assayed (Wang, 1996): aspartate aminotransferase (AAT, E.C. 2.6.1.1), acid phosphatase (ACP, E.C. 3.1.3.2), esterase (EST, E.C. 3.1.1.-), peroxidase (PER, E.C. 1.11.1.-), alcohol dehydrogenase (ADH, E.C. 1.1.1.1), superoxide dismutase (SOD, E.C. 1.15.1.1), phosphogluconate dehydrogenase (PGD, E.C. 1.1.1.44), phosphoglucomutase (PGM, E.C. 5.4.2.2), glucose-6-phosphate isomerase (PGI, E.C. 5.3.1.9), formate dehydrogenase (FDH, E.C. 1.2.1.2), glucose-6-phosphate dehydrogenase (G-6-PD, E.C. 1.1.1.49), lactate dehydrogenase (LDH, E.C. 1.1.1.27), isocitrate dehydrogenase (IDH, E.C. 1.1.1.41), and malate dehydrogenase (MDH, E.C. 1.1.1.37). Eighteen putative allozyme zones of activity were clearly resolved, and sixteen polymorphic loci were detected.

Standard measures of genetic diversity, including the average number of alleles per locus (A), percentage polymorphic loci (P), observed heterozygosity (H_O), and gene diversity (H_E), were calculated for each population and across the populations. Wright's fixation index (*F*), or the inbreeding coefficient measured deviation from Hardy-Weinberg (H-W) expectations.

Statistical analysis

The distribution of genetic variation within and between populations was calculated using Nei's gene diversity statistics. Interpopulation divergence was estimated using Nei's (1978) unbiased genetic distance (*D*), Nei's unbiased genetic identity (*I*), and the Modified Rogers distance (*DT*) (Wright, 1978). Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analyses of *D*, *I*, and *DT* examined the genetic associations between populations. The parameters above were determined using the BIOSYS-2 computer program (Swofford and Selander, 1997; Urbana, IL, USA).

An indirect estimate of gene flow (N_m) was calculated for all populations and within the two population groups based on the relationship $F_{ST} = 1/(4N_m + 1)$ (Wright 1978).

RESULTS

Among the fourteen assayed enzyme systems, two loci (*Acp-2* and *Ldh*) were monomorphic, and sixteen putative loci (*Per-1*, *Per-2*, *Est-2*, *Est-3*, *Aat-1*, *Pgi-1*, *Pgm*, *Pgd-2*, *Sod-1*, *Mdh-1*, *Mdh-3*, *G-6-pd-1*, *Adh-1*, *Adh-2*, *Fdh*, and *Idh*) were polymorphic across all populations.

The mean (Table 2) for all population estimates, including P = 85.2, *A* = 2.0, H_O = 0.371, and H_E = 0.352, revealed relatively high levels of genetic diversity for an animal-pollinated outcrossed species (Hamrick and Godt, 1989). *F* deviated from zero in all three populations, and there was generally more heterozygosity than expected based on geographical distribution and compared with other woody plant species with similar life histories. No significant departures from the H-W equilibrium were detected. Of the total genetic diversity observed in the Shanxi population, approximately 85.35% was between populations, and 14.65% was within populations (Table 3).

Table 2. Estimates of genetic variability in *Elaeagnus mollis* based on 16 variable loci in three populations.

Population	A	P*	H_O	H_E**	*F*
Xiangning	2.0 (0.1)	88.9	0.375 (0.042)	0.367 (0.037)	-0.033
Yicheng	2.1 (0.2)	88.9	0.370 (0.040)	0.363 (0.037)	-0.035
Pinglu	1.9 (0.1)	77.8	0.367 (0.063)	0.325 (0.048)	-0.094
Mean	2.0	85.2	0.371	0.352	-0.054

*A locus is considered polymorphic if more than one allele is detected. **Expected heterozygosities are unbiased estimates (Nei, 1978). A = mean number of alleles per locus; P = percentage of polymorphic loci; H_O = observed heterozygosity averaged over all loci; H_E = H-W expected heterozygosity (unbiased); and *F* = Wright's fixation index. Standard error is in parentheses.

Nei's unbiased genetic identity (*I*) values were generally elevated and ranged from 0.786 to 1.000, with a mean of 0.861. The values of Nei's unbiased genetic distance (*D*) were significantly reduced, and the mean was 0.156. The phenogram produced with UPGMA cluster analysis depicted a close genetic relationship between the Xiangning and Yicheng populations. The same result was observed when the phenogram was produced according to *I*, *D*, or *DT*.

Table 3. Summary of *F*-statistics at all loci in Shanxi *Elaeagnus mollis*.

Locus	F_{IS}	F_{ST}	F_{IT}
Per-1	0.0729	0.0743	0.1418
Per-2	0.0391	0.2000	0.2313
Est-2	-0.1387	0.0046	-0.1335
Est-3	0.0301	0.0055	0.0355
Aat-1	-0.1262	0.0001	-0.1261
Pgi-1	-0.2344	0.0885	-0.1252
Pgm	-0.2649	0.0055	-0.2579
Pgd-2	-0.0250	0.0382	0.0142
Sod-1	-0.1862	0.4674	0.3683
Mdh-1	-0.1055	0.0370	-0.0646
Mdh-3	-0.1766	0.1315	-0.0219
G-6-pd-1	-0.1226	0.4120	0.3399
Adh-1	-0.0700	0.3357	0.2891
Adh-2	-0.1514	0.3346	0.2338
Fdh	0.3085	0.1554	0.4159
Idh	0.2286	0.0016	0.2299
Mean	-0.0591	0.1465	0.0961

Gene flow estimates within population groups were elevated even though the minimum geographical distance between any population pair was 78 km in the southern Shanxi Province. The overall estimated gene flow level between the populations was also elevated ($N_m = 2.2$).

DISCUSSION

The level of genetic diversity observed in *E. mollis* ($H_E = 0.352$) was considerably higher than that expected of long-lived woody angiosperms ($H_E = 0.183$, se = 0.011) (Hamrick et al., 1992). The levels of diversity were more than five-fold the levels exhibited by other endemic taxa on a worldwide basis ($H_E = 0.078$, SE = 0.016). Compared with 26 long-lived woody endemics ($P = 0.263$, SE = 0.039; $A = 1.48$, SE = 0.09; and $H_E = 0.056$, SE = 0.010) and 61 such species with narrow geographic distributions ($P = 0.443$, SE = 0.028; $A = 1.61$, SE = 0.05; and $H_E = 0.143$, SE = 0.010) (Hamrick et al., 1992), *E. mollis* exhibited high genetic variability. These results suggest that *E. mollis* is not genetically depauperate.

Hamrick et al. (1992) found that species with narrow geographic distributions show low levels of genetic diversity ($H_E = 0.143$, SE = 0.010). However, *E. mollis* is not the only rare species with high genetic diversity. Young and Brown (1996) observed high genetic diversity among populations of the rare woodland shrub *Daviesia suaveolens*. Furthermore, Ge and Hong (1999) proposed that the high diversity of the rare and endangered species *Adenophora lobophylla* resulted from ecological factors and life history.

Although *E. mollis* is a rare and endangered species, high genetic diversity among *E. mollis* populations was observed. There are several possible explanations. 1) *E. mollis* populations are generally large and are not subject to a significant loss of variation through small-population effects, such as random genetic drift and inbreeding coupled with selection. 2) *E. mollis* is a long-lived species (Wang et al., 1992). This longevity supports the general findings of Hamrick and Godt (1989) that genetic variance will decay more slowly in long-lived species, and that populations will be less susceptible to drift. 3) *E. mollis* survived the Quaternary Glaciation. In addition, *E. mollis* has a high rate of root turion formation, which helps in maintaining the genetic diversity of their populations.

The overall estimated level of gene flow between all populations was elevated (N_m = 2.2). Generally, gene flow between populations of the same species is possible and can prevent local differentiation (Slatkin, 1987), and our study supports this assumption.

Compared with other endangered or rare species with high genetic diversity, conservative actions that suit local species were used, e.g., conservation measures for *Nouelia insignis* involved expanding the population range and enlarging the population size. Furthermore, introducing foreign germplasms to enhance the genetic diversity of this population may also be a viable option to protect this species (Luan et al., 2006). For *Eryngium alpinium*, conservation measures should preserve a maximum number of plants, with priority given to those individuals that are genetically most diverse and/or differentiated (Gaudeul et al., 2000). For *Olea europaea*, the vegetative propagation of existing individuals in different massifs or the production of novel plants stemming from seed germination has been proposed (Baali-Cherif and Besnard, 2005).

Although *E. mollis* is endangered and rare, it is not noticeably genetically depauperate. Based on these results, conservation strategies for *E. mollis* are recommended. Because of high gene flow, the possibility of local population adaptations is less likely compared with many other taxa. Reintroductions using seeds and seedlings from any convenient seed and seedling source may be feasible; however, a careful choice of seed source to match the reintroduction site according to proximity, soil type, or associated vegetation may not be necessary. Human activity, such as deforestation, has caused habitat destruction. Therefore, mixing seeds from different sources appears unlikely to jeopardize reintroduction efforts. Finally, a nature reserve should be established to protect *E. mollis* from human activity. We propose that future studies should focus on the evolutionary history of *E. mollis* to elucidate the causes of its endangered status.

Conflicts of interest

The authors declare no conflict of interest.

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