



# Analysis of the genetic diversity of the coastal and island endangered plant species *Elaeagnus macrophylla* via conserved DNA-derived polymorphism marker

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## ABSTRACT

The genetic diversity and genetic structure of five natural populations of the island and coastal endangered plant species *Elaeagnus macrophylla* were analyzed via conserved DNA-derived polymorphism molecular markers. A total of 289 discernible loci were obtained from 102 individuals via fifteen primers, and 100% of the loci were polymorphic. The observed number of alleles was 1.9654, and the effective number of alleles was 1.2604. Nei's genetic diversity index was 0.1724 on average, and Shannon's information index was 0.2869, indicating that *Elaeagnus macrophylla* had lower levels of genetic diversity than those reported for its continental relatives and other continental species. The average percentage of polymorphic loci was 42.1%, and the maximum and minimum were 80.97% and 14.88%, respectively, which were associated with the Nanji Island and Liugong Island populations, respectively. The populations of *Elaeagnus macrophylla* were highly differentiated. Cluster analysis revealed that the similarity between the tested samples was related to their geographical location, that the samples from the same island tended to cluster together, and that there was no cross-clustering between samples. The Nanji Island and Da Rushan populations differentiated into two subpopulations. Last, we detected no correlation between genetic distance and geographic distance between populations (Pearson's correlation coefficient  $r = 0.256579$ ,  $p$ -value = 0.8309).

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## INTRODUCTION

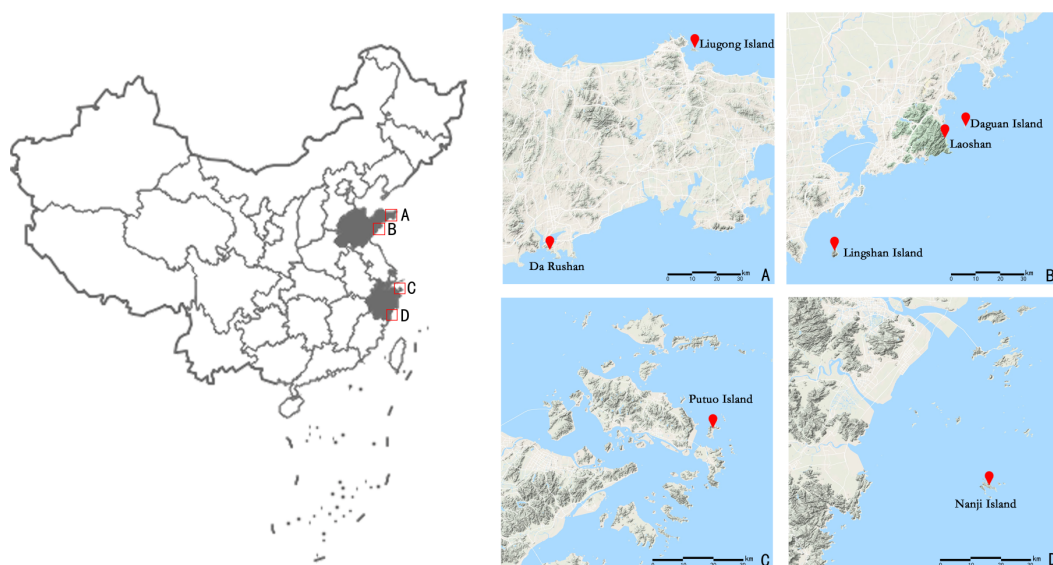
*Elaeagnus macrophylla* is an endangered evergreen shrub species of East Asian coastal areas and islands. It is distributed in the Shandong, Zhejiang, and Jiangsu Provinces of China, mainly on offshore islands and in coastal lowlands (*Chinese Flora Editorial Board of the Chinese Academy of Sciences, 1983*). Owing to its unique geographical distribution pattern, *E. macrophylla* is highly valuable for studying coastal flora and can be widely used in coastal greening because of its tolerance to sea breeze, salinity, drought, and poor

soil (Zang, 2016). This species also has potential economic value; for example, it can be used for the production of fruit juice and wine (Zang, 2016). In recent years, with the rapid development of the economy and coastline, intensification of human interference, and continuous reduction in suitable environments, the number, and size of natural populations have decreased sharply, causing this species to become endangered.

The genetic diversity of island species is generally lower than that of continental species, the risk of extinction is greater for the former (Raven, 1998). From the 17th century to the 20th century, 384 species of vascular plants went extinct worldwide, 139 of which were island plant species. Moreover, forty percent of vulnerable or endangered vascular plant species are island species (Reid & Miller, 1989). Human disturbances, such as habitat destruction and invasion by alien species, are considered to be the main factors threatening island species (Wolf & Harrison, 2001). Studies of *Ilex integra* based on inter simple sequence repeat (ISSR) molecular markers (Leng et al., 2005) and *Neolitsea sericea* based on random amplified polymorphic DNA (RAPD) molecular markers (Wang et al., 2004) showed that the geographical isolation of islands had a significant effect on the genetic differentiation of island populations and that the genetic diversity of island relatives was lower than that of close continental relatives. However, no studies of the genetic diversity of the island plant species *E. macrophylla* have been conducted.

The conserved DNA-derived polymorphism (CDDP) method is based on a single primer amplification reaction, with primers designed to target conserved sequences of plant functional genes, mostly transcription factors such as WRKYs, MYBs, MADs, ERFs, KNOXs, and ABP1. Because of the strong conservation of some sequences of plant DNA, CDDP molecular marker technology can be used across different species. Studies of rice (*Oryza sativa*) have shown that CDDP markers have many advantages, including convenience, low cost, and rich polymorphism, and can effectively mark sequences of target traits (Collard & Mackill, 2009). Compared with traditional DNA molecular methods, the CDDP method is practical because the primers used in CDDP are specific for conserved gene sequences. By amplifying these conserved sequences, which tend to be linked to phenotypic traits, CDDP markers can provide advantages for plant genetic diversity assessment (Andersen & Lübberstedt, 2003). Since Poczai first successfully used CDDP markers and within-target markers to investigate the genetic diversity and group structure of *Solanum dulcamara* (Poczai et al., 2011), CDDP markers have proven useful in the analysis of several other plant species, such as *Chrysanthemum* (Li et al., 2014), *Paeonia suffruticosa* (Li, 2013), *Vaccinium vitis-idaea* (Fang et al., 2016), and *Rosa rugosa* (Jiang & Zang, 2018). However, CDDP markers have not yet been used to study *E. macrophylla*.

In this study, CDDP markers were used to analyze the genetic diversity and genetic relationships between major natural populations of *E. macrophylla* in China, with the aim of revealing the level of genetic diversity and degree of genetic differentiation, assessing the relationships between populations, examining the influence of geographical isolation and human factors on the genetic structure, and providing a scientific basis for the protection and rational utilization of *E. macrophylla*.



**Figure 1** Geographical location of the seven sampling points (including Putuo Island and Lingshan Island where only one sample was collected) of *E. macrophylla* in China. Show the geographical location of seven sampling points by zooming in on the four areas A, B, C, and D.

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**Table 1** Sampling information for *E. macrophylla*. The table shows information such as population name, abbreviation, geographic location, latitude and longitude, and altitude.

Population	Population abbreviation	Number	Locality	Geographical location	Altitude (meters)
Liugong Island	LGD	8	Weihai Bay, Weihai city, Shandong Province	37°30'N, 122°10'E	22
Da Rushan	DRS	20	Rushan city, Weihai city, Shandong Province	36°45'N, 121°30'E	5.2
Lingshan Island	LSD	1	Huangdao District, Qingdao city, Shandong Province	36°27'N, 121°58'E	20
Daguang Island	DGD	26	Laoshan District, Qingdao city, Shandong Province	36°13'N, 120°46'E	11
Laoshan	LS	13	Laoshan District, Qingdao city, Shandong Province	36°7'N, 120°39'E	20
Putuo Island	PTD	1	Zhoushan Islands, Zhoushan city, Zhejiang Province	30°0'N, 122°24'E	96
Nanji Island	NJD	33	Pingyang County, Wenzhou city, Zhejiang Province	27°28'N, 121°3'E	42

## MATERIALS AND METHODS

### Plant materials

A total of 102 individual leaf samples were collected from 7 islands and offshore sites (Fig. 1 and Table 1) from April to July 2018; sampling was conducted within the natural distribution area of *E. macrophylla*. Interval sampling was applied except within small populations (such as the Liugong Island population, where samples from all individual plants found were collected). Only one individual each was found on Lingshan Island and Putuo Island. After the samples were collected, silica gel was used to quickly dry the specimens, after which they were stored at 20 °C.

**Table 2** Site information for 15 CDDP markers and genetic diversity parameters at each locus of *E. macrophylla*. Table contains information such as primer code, primer names, sequence, annealing temperature, number of bands recorded, number of polymorphic bands and the percentage of polymorphism.

Primer code	Primer name	Sequence (5'–3')	Annealing temperature	Number of bands recorded	Number of polymorphic bands	The percentage of polymorphism/%
Pr1	WRKY-F1	TGGCGSAAGTACGGCCAG	50	21	21	100
Pr2	WRKY-R1	GTGGTTGTGCTTGCC	52	30	30	100
Pr3	WRKY-R3	CCGCTCGTGTGSACG	50	21	25	100
Pr4	MYB1	GGCAAGGGCTGCCGC	50	19	19	100
Pr5	MYB2	GGCAAGGGCTGCCGG	50	13	13	100
Pr6	ERF1	CACTACCGCGGSGTSCG	50	30	30	100
Pr7	ERF2	GCSGAGATCCGSGACCC	50	11	11	100
Pr8	ERF3	TGGCTSGGCACSTTCGA	50	11	11	100
Pr9	KNOX-1	AAGGSAAGCTSCCSAAG	50	21	21	100
Pr10	KNOX-2	CACTGGTGGGAGCTSCAC	50	19	19	100
Pr11	KNOX-3	AAGCGSCACTGGAAGCC	50	15	15	100
Pr12	MADS-1	ATGGGCCGSGGCAAGGTGC	50	14	14	100
Pr13	MADS-4	CTSTGCGACCGSGAGGTG	50	28	28	100
Pr14	ABP1-1	ACSCCSATCCACCGC	50	14	14	100
Pr15	ABP1-3	CACGAGGACCTSCAGG	50	18	18	100

### DNA extraction and PCR amplification

Total DNA was extracted from *E. macrophylla* via the modified cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1987). The quality and purity of DNA were determined by 2% agarose gel electrophoresis, and a spectrophotometer (Thermo Fisher Scientific Inc., USA) was used to ensure DNA quantification. All DNA samples were stored at 20 °C for later use.

The DNA from one sample per population was selected to screen 21 CDDP primers (Collard & Mackill, 2009) (synthesized by Sangon Biotech, China). The results revealed 15 primers with clear and reproducible amplification bands were screened out (Table 2). PCR was conducted in a total reaction volume of 20 µl consisting of 10 µl of 2× Ex Taq MasterMix (dye), 7.5 µl of double-distilled H<sub>2</sub>O, 1 µl of 30 ng/µl DNA template, and 1.0 µl of 10 pmol/µl primers (Sangon Biotech, China). A standard PCR thermocycler (RT-PCR 7500, Thermo Fisher Scientific Inc., USA) was used and the PCR program was as follows: an initial denaturation step at 94 °C for 3 min; 35 annealing cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min; and a final extension of 72 °C for 5 min. The PCR products were subsequently stored at 4 °C. The products were then electrophoresed on a 2% agarose gel (110 V and 110 mA) for 1.5–2 h; a DL2000 marker was used as a size marker. The electrophoresis results were imaged and recorded by a gel imaging system. All amplification procedures were repeated at least twice to ensure the repeatability of the experiment.

## Statistics and data analysis

We used POPGEN v.1.32 (Yeh, Yang & Boyle, 1999) to compute the following parameters: observed allele number (Na), effective allele number (Ne), Nei's genetic diversity index (H), Shannon's information index (I), polymorphic loci, percentage of polymorphic loci (PPL, %), total genetic diversity (Ht), genetic diversity within populations (Hs), the genetic differentiation coefficient (Gst) and gene flow (Nm) between populations. Estimates were also calculated within sampling localities when significant differences among specimens were detected.

A dendrogram was generated by the unweighted pair group method with arithmetic mean (UPGMA) clustering procedure in NTSYS-pc 2.10e software (Rohlf, 1994). The relationship between geographic distance and genetic distance was analyzed with Pearson's correlation coefficients in R.

The genetic structure of populations was further assessed via the Bayesian clustering approach implemented in STRUCTURE v.2.3.4 (Pritchard, Stephens & Donnelly, 2000). The number of potential genetic clusters ( $K$  values) was set from 1 to 10, with 10 independent runs for each  $K$ . The contribution of the accessions to the genotypes was calculated on the basis of a  $10^5$  iteration burn-in period and  $10^5$  iteration sampling period. The optimal number of  $K$  clusters was then identified according to the methods of Evanno, Regnaut & Goudet (2005).

## RESULTS AND ANALYSIS

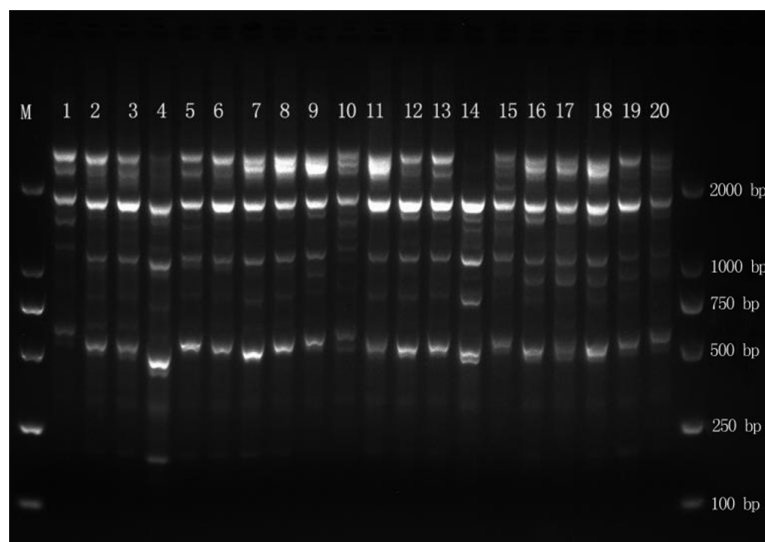
### Population- and species-level diversity of *E. macrophylla*

The DNA of 102 samples was amplified with 15 primers, yielding 289 bands, and the fragment length was between 500 and 2,000 bp (Fig. 2). The number of amplified bands ranged from 11 to 30, and the average number of amplified bands was 19.3. The number of Pr2 and Pr6 amplification bands was the highest, at 30, and the number of Pr7 and Pr8 amplification bands was the lowest, at 11. The percentage of polymorphisms reached 100% (Table 2), which indicated that the genomic DNA polymorphism of *E. macrophylla* was high.

At the population level, the PPL ranged from 14.88% to 80.28%, with an average of 48.928%, whereas it was 96.54% at the species level. The Na ranged from 1.1488 to 1.8028, while the Ne varied from 1.0739 to 1.2410. H varied from 0.0446 to 0.1580, with an average of 0.1149, and I ranged from 0.0690 to 0.2613, with an average of 0.1848. At the species level, H and I were 0.1724 and 0.2869, respectively (Table 3). The Na, Ne, H, I, and PPL were consistent among populations, with the NJD population presenting the largest values and the LGD population presenting the lowest values, all of which were lower than those at the species level.

### Genetic differentiation of the populations of *E. macrophylla*

The Ht and Hs were 0.1706 and 0.1149, respectively, as calculated by POPGEN v.1.32 software. The Gst was 0.3263, indicating that 67.37% of the variation was within the populations and that 32.63% of the variation occurred between the populations. A certain



**Figure 2** Amplification results for MYB1 in the Liugong Island population and the Da Rushan population. Amplification results for MYB1 in the Liugong Island population (1–8) and the Da Rushan population (9–20), Marker=DL2000.

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**Table 3** Genetic diversity in five populations of *E. macrophylla*. The table contains information such as population name, number of samples,  $N_a$ ,  $N_e$ ,  $H$ ,  $I$ , PPL, and standard deviation in parentheses.

Population name	Number of samples	$N_a$	$N_e$	$H$	$I$	PPL (%)
LGD	8	1.1488 (0.3565)	1.0739 (0.2178)	0.0446 (0.1210)	0.0690 (0.1785)	14.88
DRS	20	1.5398 (0.4993)	1.2002 (0.2850)	0.1290 (0.1622)	0.2070 (0.2381)	53.98
NJD	33	1.8028 (0.3986)	1.2410 (0.2931)	0.1580 (0.1566)	0.2613 (0.2198)	80.28
LS	13	1.4998 (0.4983)	1.1941 (0.3020)	0.1208 (0.1680)	0.1898 (0.2458)	50.52
DGD	26	1.5502 (0.4983)	1.1912 (0.2878)	0.1222 (0.1615)	0.1968 (0.2355)	44.98
Mean	20	1.5083	1.1801	0.1149	0.1848	48.928
Species level	100	1.9654 (0.1831)	1.2601 (0.2845)	0.1724 (0.1532)	0.2869 (0.2098)	96.54

**Notes.**

The PTD and LSD populations were not included because there was only one sample for each.

degree of genetic differentiation was observed between the populations. The  $N_m$  was 1.0325, indicating that there was some (albeit limited) genetic exchange between populations.

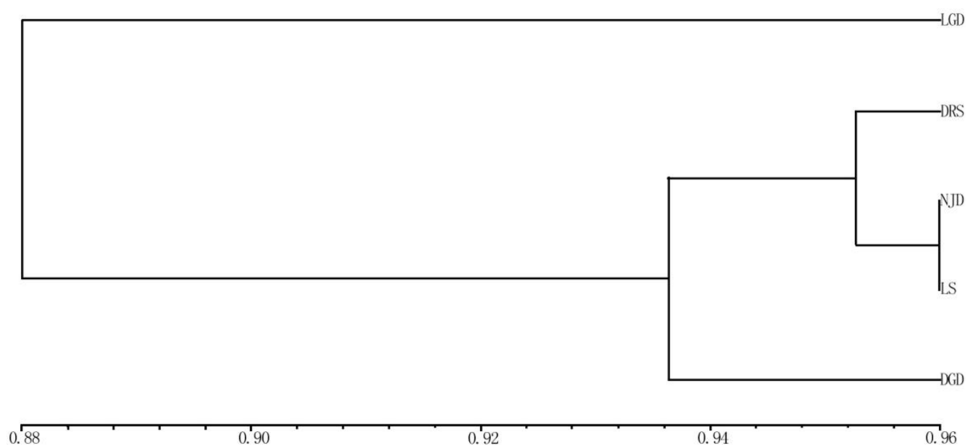
Genetic differentiation between populations can be further analyzed on the basis of Nei's genetic distance and genetic identity. For the five populations of *E. macrophylla*, the genetic distance was between 0.0490 and 0.1443 (Table 4), with a mean of 0.08127, and Nei's genetic identity was between 0.8656 and 0.9588, with a mean of 0.9226.

### UPGMA cluster analysis

The applied measure of genetic similarity was used to construct UPGMA dendrograms (Fig. 3). The clustering map showed that the five populations could be divided into three groups. One group represented the LGD population. The DRS, NJD, and LS populations

**Table 4** Nei's genetic identity (above diagonal) and genetic distance (below diagonal) for five populations. Liugong Island (LGD), Da Rushan (DRS), Nanji Island (NJD), Laoshan (LS), Daguean Island (DGD).

Population	LGD	DRS	NJD	LS	DGD
LGD	****	0.9253	0.8656	0.8730	0.8697
DRS	0.0776	****	0.9588	0.9569	0.9431
NJD	0.1443	0.0421	****	0.9522	0.9391
LS	0.1358	0.0440	0.0490	****	0.9427
DGD	0.1396	0.0585	0.0628	0.0590	****

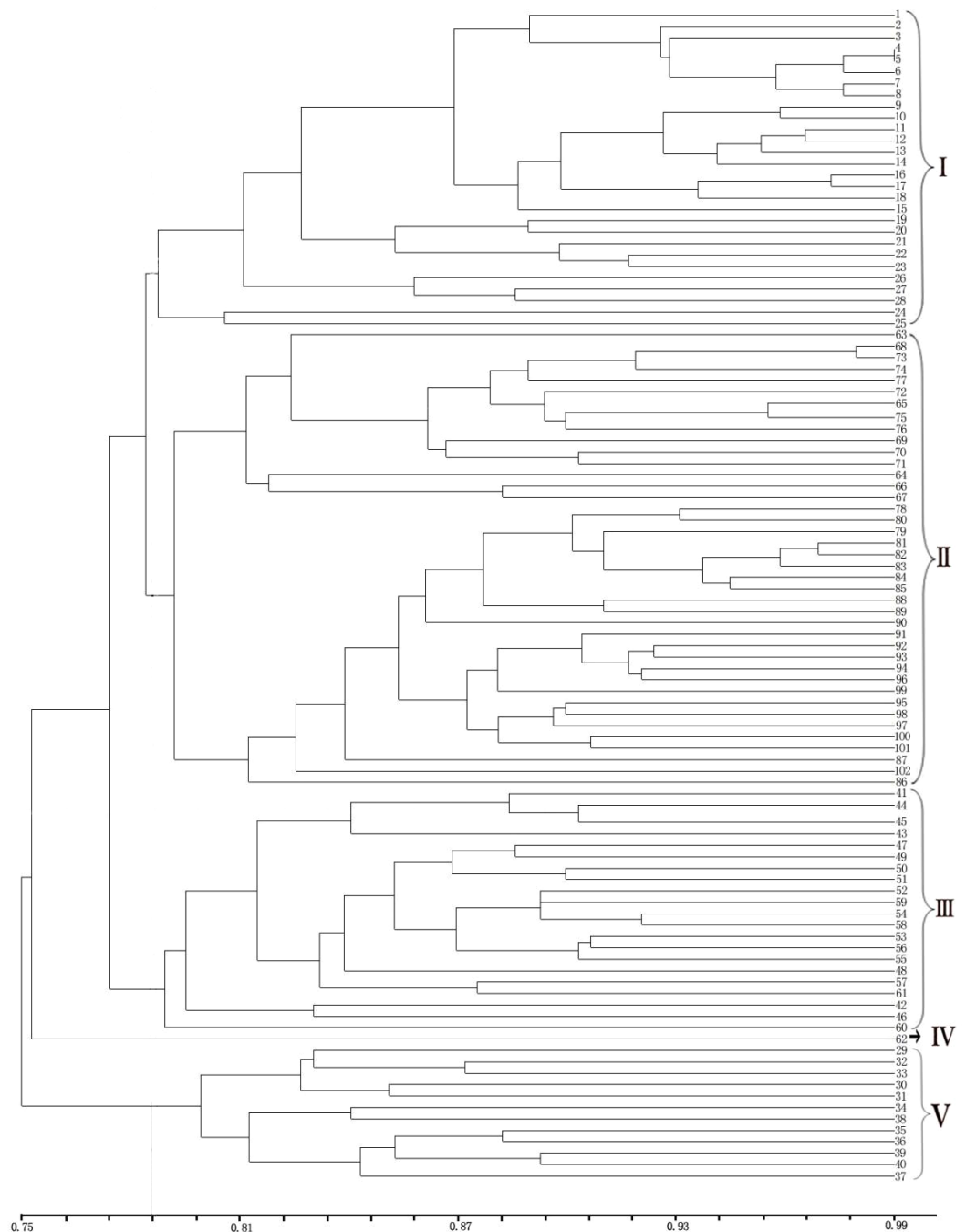


**Figure 3** UPGMA cluster analysis of genetic similarity of 5 populations. UPGMA cluster analysis of genetic similarity of five populations: LGD, DRS, NJD, LS, DGD.

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formed a second group, indicating that these three populations are closely related, the third group represented the DGD population. Populations with similar geographical distances were not clustered into the same group, indicating that the genetic distance between the populations of *E. macrophylla* was not related to geographical distance. Consistent with these results, the Pearson correlation coefficient test revealed no significant correlation between geographic and genetic distance (Pearson's correlation coefficient  $r = 0.256579$ ,  $p$ -value = 0.8309).

The UPGMA clustering map provided a clear division of the 102 samples (Fig. 4). Notably, cross-clustering occurred between samples from different populations, and samples from the same island tended to cluster together. The groups in the clustering results were generally consistent with the regional sources of the samples. As clearly shown by the clustering map, all the samples from the LGD population formed a small branch and then formed another branch with all the samples from the DRS population. Among the samples, Nos. 9–18 from the DRS population were more closely related to the samples from the LGD population than to Nos. 19–28 from the DRS population; thus, differentiation within the DRS population was observed between Nos. 9–18 and Nos. 19–28. All samples from the LS population formed a group, all samples from the DGD population formed



**Figure 4** UPGMA cluster analysis of 102 samples of *E. macrophylla*. LGD (1-8), DRS (9-28), NJD (29-61), PTD (62), LSD (63), LS (64-76), DGD (77-102).

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another group, and the two groups formed a large branch. One sample from PTD formed a separate group. Samples from the NJD population were composed of two subpopulations. On the basis of the sampling location and latitude and longitude, samples 29–40 were collected in the northwestern part of NJD ( $121^{\circ}3'24''$ – $121^{\circ}3'8''$ ,  $27^{\circ}27'53''$ – $27^{\circ}28'21''$ ), and



samples 41–61 were collected in the southeastern part of NJD ( $121^{\circ}5'52''$ – $121^{\circ}6'11''$ ,  $27^{\circ}26'54''$ – $27^{\circ}27'12''$ ). Twenty-one samples (Nos. 41–61) formed a group, 12 samples (Nos. 29–40) formed another group, and these two groups formed different branches. Therefore, the samples could be easily divided into five groups on the basis of the clustering results.

### Population structure analysis

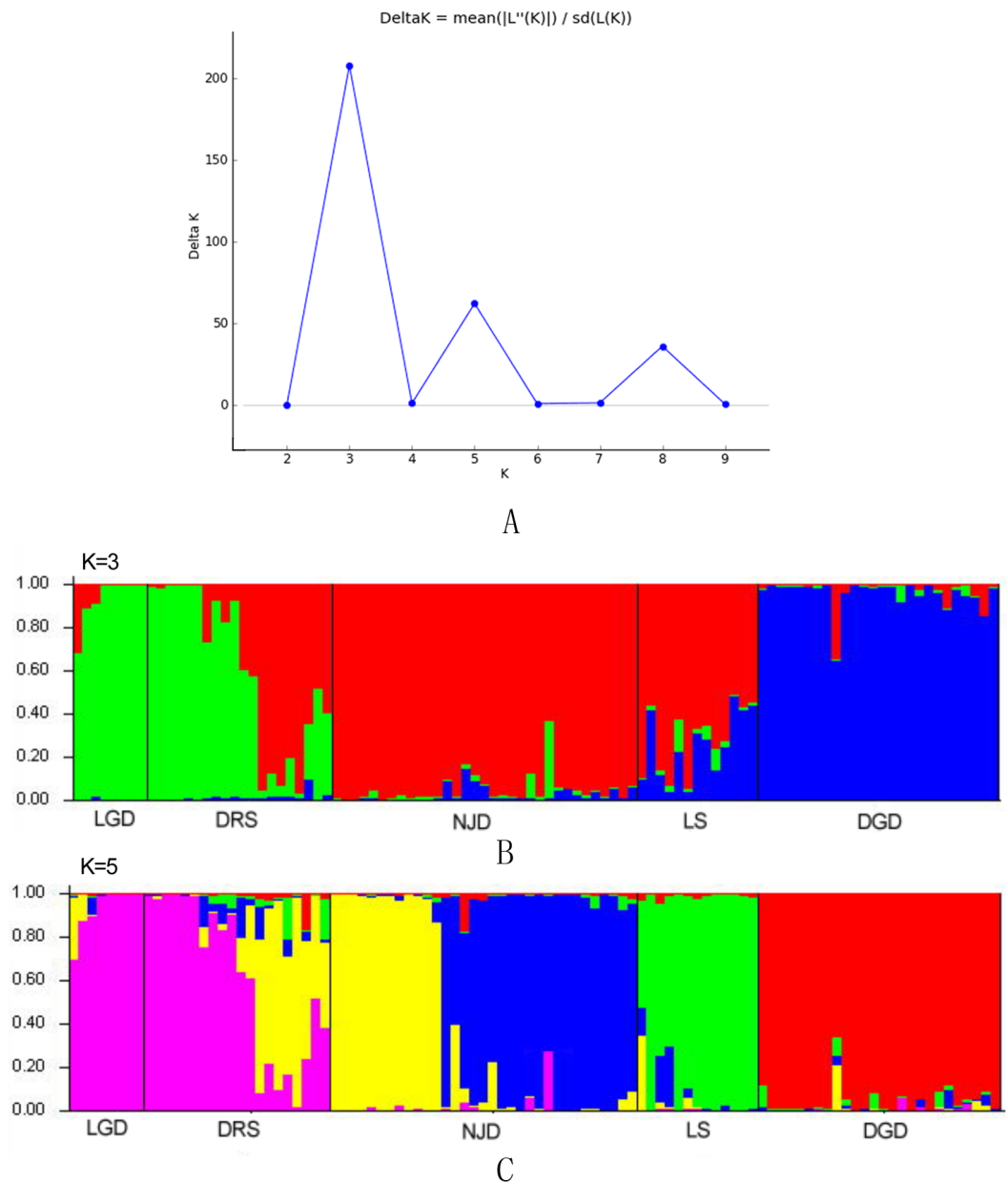
The results of the Bayesian clustering analysis of the genetic structure showed that the populations of *E. macrophylla* best fit three genetic groups, and when  $K = 5$ , the delta  $K$  value was also large (Fig. 5A). When  $K = 3$  (Fig. 5B), the LGD population and half of the DRS samples clustered into the first groups, further indicating that the two populations were closely related. Most samples from the LS population, half of the samples from the DRS population and all the samples from the NJD population clustered into the second group, and a portion of the samples from the LS and DGD populations formed the third group. When  $K = 5$  (Fig. 5C), the LGD population and half of the DRS samples clustered into the first group, half of the DRS samples and the NJD samples indicated in yellow in the figure clustered into the second group, the NJD samples indicated in blue in the figure formed the third group, all samples from the LS population clustered into the fourth group, and all samples from the DGD population clustered into the fifth group. The LGD, LS, and DGD population pedigrees were simple, and the samples from the islands tended to cluster together. However, the NJD and DRS populations were different. The NJD population formed two subpopulations when  $K = 5$ : a northwestern group (indicated in yellow) and a southeastern group (indicated in blue). The DRS population differentiated into two subpopulations when  $K = 3$  and  $K = 5$ : Nos. 9–18 and Nos. 19–28, respectively.

### Gst and Nm of the two NJD and DRS subpopulations

The Nm between the NJD and DRS subpopulations was 2.6084 and 2.0843, respectively, the G<sub>st</sub> was 0.1609 and 0.1935, respectively. The subpopulations presented strong gene flow but high genetic differentiation.

## DISCUSSION

Frankham (1997) compared and analyzed the allelic diversity of 202 groups of land and island populations of various species, including those of mammals, birds, fish, reptiles, insects, and plants: in 165 cases (81.7%), the genetic diversity of island populations was lower than that of continental populations, with an average decrease of 29%. The average PPL via the CDDP markers in five populations of the island plant species *E. macrophylla* was 48.928%, the  $N_e$  was 1.1801,  $H$  was 0.1149, and  $I$  was 0.1848. All these numbers are far below those of populations of the continental species *Camellia japonica* (PPL = 86.11%,  $N_e = 1.4775$ ,  $H = 0.2940$ ,  $I = 0.4459$ ) (Juan, 2018) and *Paeonia suffruticosa* (PPL = 72.1%,  $N_e = 1.2389$ ,  $H = 0.1623$ ,  $I = 0.2682$ ) (Li, 2013) estimated on the basis of CDDP marker data. Moreover, compared with the rich genetic diversity of another species within the same genus (*Elaeagnus mollis*) (Qin, Zhang & Yan, 2006), the genetic diversity of *E. macrophylla* is low, which is consistent with the results of Frankham. As an endangered coastal plant species, *E. macrophylla* has a lower genetic diversity than



**Figure 5** Population structure analysis and Delta-K values. (A) Delta-K values; (B) population structure analysis,  $K = 3$ ; (C) population structure analysis,  $K = 5$ .

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its continental relatives and other continental species have. For island species, breeding characteristics, dispersal capability, and effective population size are often considered important factors affecting genetic diversity (Frankham, 1997; Weller, Sakai & Straub, 1996). *E. macrophylla* is a typical bisexual flowering plant species that produces flowers that have a small diameter and that produce nectar; thus, this species relies mainly on small insects as pollinators for cross-pollination (Zang, 2012). The  $H_t$  of *E. macrophylla* was lower than the average value of insect-pollinated plants ( $H_t = 0.2019$ ; Hanwick &

*Godt, 1990*), indicating that its insect-borne pollination has been affected to some extent. Owing to the large sea breeze on the island, insects can live only in groups, which affects their range of activities. As a result, pollen transmission is limited to a very small range, and random fixation of alleles and limited gene exchange leads to poor population expansion, thus reducing genetic diversity (*Hamrick & Nason, 1996; Hamilton & Miller, 2002*). As another important carrier of gene flow, seeds are also essential for the natural regeneration and expansion of plant populations and for increasing their genetic diversity (*Hamilton & Miller, 2002*). According to previous studies, *E. macrophylla* can produce fruit naturally in the wild, and the fruit is sweet (*Zang, 2016*). The fruit is heavily favored by birds, and some birds that feed on these fruits can spread the seeds. Unfortunately, owing to the influence of insect pollination, the seed setting rate in the wild is low. Moreover, owing to increased amounts of human activities, especially the vigorous development of tourism, island birds are becoming increasingly rare, which further restricts the spread of seeds and affects the genetic diversity of populations. Compared with that of *C. japonica* and *E. mollis*, the distribution of populations of *E. macrophylla*, an island species, is small. *E. macrophylla* occupies a fragile habitat and has a narrow distribution range, making it more vulnerable than continental species to extinction (*Francisco-Ortega et al., 2000*). Island segregation means fragmentation of habitats, which leads to the maintenance of small populations in fragmented habitats; as such, genetic diversity of endangered species may be lost because genetic drift causes allele loss and because inbreeding frequencies are increasing (*Zhang & Jiang, 1999; Emerson, 2002*).

In the present study, the  $H_t$  and  $H_s$  of *E. macrophylla* were 0.1706 and 0.1149, respectively. Compared with endangered and Chinese secondary protected plants (*Fu & Jin, 1992*), including *R. rugosa*, in previous CDDP-based ( $H_t = 0.2770$ ,  $H_s = 0.1522$ ) studies (*Jiang & Zang, 2018*), *E. macrophylla* in the present study showed low diversity. Similarly, the diversity values in the present study were lower than those estimated via CDDP markers for the plant species *C. japonica* ( $H_t = 0.2874$ ,  $H_s = 0.2518$ ; *Juan, 2018*).  $G_{st}$  is calculated as the ratio of between-population genetic variance to the total variance among populations (*Wright, 1965*). The  $G_{st}$  of the five populations of *E. macrophylla* was 0.3263, indicating that 32.63% of the variation existed among the populations. Genetic differentiation of the *E. macrophylla* populations was significant on the basis of Nei's  $G_{st}$  classification criteria for genetic differentiation (low,  $G_{st} < 0.05$ ; medium,  $G_{st} = 0.05 \sim 0.15$ ; and high,  $G_{st} > 0.15$ ) (*Nei, 1978*). This genetic differentiation value was greater than the average value of 23 species (28.06%) of the Carina Islands (*Francisco-Ortega et al., 2000*). Furthermore, the genetic differentiation between *E. macrophylla* populations was relatively high, and the prevention of gene flow, genetic drift, and inbreeding was the main cause of genetic differentiation among populations (*Starkin, 1987; Ouborg, Piquot & Groenendaal, 1999; Manel et al., 2003*).

$N_m$  refers to the process by which a biological individual disperses from its place of origin, followed by the exchange of genes between populations. Such exchange may occur between biological populations of the same species or between different species and is essential to the evolution of many plant populations (*Grant, 1991; Gerber et al., 2014*). The populations of *E. macrophylla* displayed little gene flow ( $N_m = 1.0325$ ), and the UPGMA

clustering analysis of samples revealed no hybridization among individuals from different localities. Structure analysis ( $K = 5$ ) revealed that most of the populations had a simple pedigree, and the genetic exchange between each pair of populations was low. These results were mainly due to the geographical isolation of the islands (mainly barriers posed by seawater), which limited the range of dispersal by pollen- and seed-dispersing birds (Kwon & Morden, 2002). For the *E. macrophylla* populations, the shortest distance is between the populations of LS and DGD (8,100 m); it is difficult for small pollinators to spread pollen across islands separated by vast seas. In addition to pollen, seeds play an important role in the spread of gene flow. One important aspect of seed movement is its role in the initial founding of a population (Chung et al., 2002). In a study of *N. sericea*, owing to seeds being dispersed over long distances by birds, numerous seedlings and juveniles (mostly aged <5 yr) of *N. sericea* were dispersed within a range of approximately 480 m (Hakdongri on Keojae Island) and 680 m (Naechori on Oenaro Island), respectively, from their maternal plants (Chung et al., 2002). The fruit of *E. macrophylla* is a drupe-shaped nut with a seed and rich flesh (Zang, 2012). After birds feed and digest their food, they can excrete seeds in their feces. This type of transmission is called intra-animal transmission, which carries seeds far away and promotes gene exchange (Petit et al., 2003). It is difficult to directly observe how far birds can spread seeds in the wild, but the retention time of seeds in the digestive tract of fruit-eating birds can be used to determine the potential propagation distance and the ability to reach the appropriate breeding ground (Manson & Stiles, 1998). The retention time of fruits of *Sorbus pohuashanensis* in the digestive tract of birds were found to be approximately 20 min. The first stopping point after feeding was mostly located between 5 and 10 m from the female plants, but the birds could have had many various landing points within 20 min, which might spread the seeds to distant areas (Zhang et al., 2010). However, birds are affected by factors such as seed size, feed intake, digestion, and excretion; thus, it is very difficult to distribute seeds between distances over sea areas greater than 8000 m or farther. Last, water currents are also a major medium of genetic exchange between islands (Kwon & Morden, 2002). Zhang et al. (2007) studied the differences in genetic variation between the species *E. emarginata*, *I. integra* and *Machilus thunbergii*. Clustering analysis revealed that the reason for the intermixing of individuals among *E. emarginata* populations was that seeds floating with ocean currents promoted gene exchange among populations, while those of *I. integra* and *M. thunbergii* did not. Therefore, the genetic differentiation of *E. emarginata* is lower than that of *I. integra* and *M. thunbergii*. The fruits of *E. macrophylla* fall into the sea because of sea breezes, but the spread of seeds by ocean currents to allow genetic exchange between populations is a rare event, as submersion quickly reduces the germination capability after a few days (Angélique & Debussche, 2000). When soaked in seawater, seeds of *I. integra* also presented a germination rate close to zero (Leng et al., 2005). Our sample clustering map revealed no hybridization among individuals, indicating that the genetic exchange between the populations is limited. Therefore, it is very difficult for *E. macrophylla* to achieve genetic exchange via seeds floating by currents. For *E. emarginata*, the reason for success may be that the distance between the islands is short and the seeds do not lose their ability to germinate after floating.

The results of the Bayesian clustering analysis showed, that when  $K = 3$ , the same gene pool exists between different populations. On the basis of limited gene flow and high genetic differentiation between populations, we speculate that the reason for this may be that *E. macrophylla* inherits the gene pool of its ancestors, such that populations with distant geographic distances also are part of the same gene pool.

As opposed to the high genetic differentiation between different island populations, individuals on islands are often grouped into a single group, indicating that individuals in each population have relatively close kinship, which may be related to the small island range, similar habitats within the population, and strong gene flow (*Sahuquillo & Lumaret, 1995; Carlos, Emerson & Oromi, 2000*); however, the DRS and NJD populations are exceptions. According to the UPGMA clustering and STRUCTURE analysis results, the DRS population differentiated into two subpopulations: Nos. 9–18 and Nos. 19–28. Gene flow ( $Nm = 2.0843$ ) was strong between the two subpopulations, but there was high genetic differentiation ( $G_{st} = 0.1935$ ). Because gene flow was not blocked and the habitats were similar, we speculate that the cause of this result was that the DRS population contains two gene pools, and complete gene introgression has not yet occurred in the two subpopulations under the condition of a gene flow of 2.0843. When  $K = 3$ , the NJD population did not exhibit differentiation; when  $K = 5$ , the NJD population differentiated into two subpopulations: a northwestern group and a southeastern group. The gene flow between the two subpopulations was determined to be 2.6084, indicating strong genetic exchange between the two subpopulations of NJD. However, there was still some genetic differentiation between the two subpopulations ( $G_{st} = 0.1609$ ). After possible causes such as the prevention of gene flow were excluded, the reason may be related to differences in habitats within the population. The NJD nature reserve has numerous islands and reefs with meandering shorelines, headlands, and numerous bays. There are many types of coastal beaches, such as mudflats, gravel beaches, and rocky reefs, and NJD is at the intersection of the Taiwan Warm Current and the Jiangsu and Zhejiang Coastal Currents. The flow system is complex, so the habitat is complex (*Xiao, 2007*). The species of marine shellfish algae in this area not only are abundant but also have characteristics beneficial to temperate zones and tropical zones. Moreover, an obvious regional “fracture distribution” phenomenon is occurring. It is rare that three species with characteristics beneficial to different climate zones (i.e., tropical, subtropical and temperate zones) coexist in the NJD sea area at the same time; for example, typical tropical species such as *Oliva emicator* can survive in the NJD above 27°N, which is sufficient to demonstrate the particularity of the NJD habitat (*Xiao, 2007*). The two subpopulations are separately located in the northwestern and southeastern regions of NJD, and may exhibit some genetic differentiation because of habitat differences. However, our current evidence is insufficient, and additional research is needed.

In the comparison of the genetic diversity index of 22 endemic plant species on the Canary Islands, the average genetic diversity index of island species with relatively large populations (number of individuals >2,500;  $H = 0.1460$ ) was significantly greater than that of island species with relatively small populations (number of individuals <100;  $H = 0.0970$ ) (*Francisco-Ortega et al., 2000*). The average diversity index of each population

of *E. macrophylla* was (from large to small) NJD (0.1580)>DRS (0.1290)>DGD (0.1222)>LS (0.1208)>LGD (0.0446), with an average value of 0.1149. The average genetic diversity index of the NJD population is much greater than that of the LGD population. The values of the other three populations are relatively similar. The NJD population is the largest, the DRS, DGD, and LS populations are similar in size, and the LGD population is declining. Human disturbances such as excessive logging, habitat destruction, and the introduction of exotic species are considered to be the main causes endangering island species (Atkinson, 1989; Frankham, 1997; Raven, 1998), which are manifested mainly as effective population decline, increased frequencies of inbreeding, loss of genetic diversity, decline in survival competitiveness, etc. (Ferson & Burgman, 1995; Frankham, 1997; Mengens, 1998). NJD is far from mainland China, is relatively closed, and experiences relatively limited exchange with the mainland, so there is little anthropogenic damage. Moreover, the island area is large, and the genetic background is complex, so the island presents high genetic diversity and a large population. LGD is a famous tourist destination in China, and the coastline is developing rapidly. Moreover, LGD has a small area, and its populations have similar genetic backgrounds and a single habitat; as such, loss of genetic diversity and populations declines have occurred.

### Conservation of *E. macrophylla* diversity

Am in situ conservation method was proposed because the conservation of sufficient natural population numbers and sizes to prevent a reduction in genetic diversity is urgently needed. The best strategy for in situ conservation of genetic diversity during an endemic is the preservation of natural habitat (Francisco-Ortega et al., 2000). In this study, the NJD population displayed relatively high genetic diversity and should, therefore, be a priority for in situ conservation. The LGD population had the lowest genetic diversity and the smallest population size; the site of this population should be protected as the most urgent site. Furthermore, natural protection areas should be established to conserve and restore the habitat and populations, the awareness of local residents and tourism management personnel should be heightened, and the populations should be increased by artificial cultivation and subsequent management; for example, seeds collected from other populations could be sown, branches could be collected for cuttings, gene barriers could be broken by appropriate species regression, and the genetic diversity of populations could be increased. Moreover, to achieve effective conservation of germplasm resources, efforts are needed to carefully plan and construct pollen banks and gene banks for *E. macrophylla*.

## CONCLUSIONS

The present study is the first genetic investigation of *Elaeagnus macrophylla* using conserved DNA-derived polymorphism markers to investigate the distribution and genetic variation. The results showed that conserved DNA-derived polymorphism markers can be effectively used to study the genetic diversity of *Elaeagnus macrophylla* populations and revealed that *Elaeagnus macrophylla* populations have low genetic diversity and high genetic differentiation. The low levels of gene flow between populations are the main cause of

the high levels of genetic differentiation. On the basis of these findings, some conservation measures for *Elaeagnus macrophylla* are proposed.

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### Competing Interests

The authors declare there are no competing interests.

### Author Contributions

- Yi Wang conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Yan Ma conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Bingyu Jia performed the experiments, prepared figures and/or tables, and approved the final draft.
- Qichao Wu performed the experiments, prepared figures and/or tables, collection of experimental samples, and approved the final draft.
- Dekui Zang conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Xiaoyan Yu performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

## Data Availability

The following information was supplied regarding data availability:

Raw data is available in the [Supplementary Files](#).

## Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.8498#supplemental-information>.

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