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Genetic diversity and population structure of endangered *Neofinetia falcata* (Orchidaceae) in South Korea based on microsatellite analysis

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Population genetic assessment is essential for the conservation and management of endangered and rare plants. *Neofinetia falcata* is endangered epiphyte orchid and protected by law in Korea. In Korea, this species is only found on islands in the South Sea of Korea (including Jeju-do) and the southern coast of the Korean Peninsula. We developed nine microsatellite makers to assess the genetic diversity and population genetic structure of three populations of *N. falcata*. The genetic diversity at the species level was low, which can be attributed to inbreeding or fragmentation into small, isolated populations. A recent bottleneck was detected in one population, likely due to overcollection. *N. falcata* exhibited moderated levels of differentiation among populations, with the three populations were divided into two clusters based on genetic structure. The genetic diversity and structure of *N. falcata* are affected by restricted gene flow by pollen or seeds due to isolation and geographic distance. Strategies for *in situ* and *ex situ* conservation of this species are been proposed based on the results of our study.

Keywords: conservation, genetic diversity, microsatellite markers, *Neofinetia falcata*, population genetic structure

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Introduction

Neofinetia falcata (Thunb.) Hu is an epiphytic orchid that grows on tree trunks or rocks and is distributed in Korea, Japan, and China (Dressler, 1993; Chen and Jeffrey, 2009; Lee, 2011). This beautiful orchid contains a pleasant fragrance and is a symbol of wealth and nobility, which is why Japan began cultivating it in the 17th century (Yoon and Chung, 2011; Duttke *et al*., 2012). Due to the popularity, it has been collected extensively and is now threatened with extinction. *N. falcata* is known to be found at about 20 sites in Jeju-do and along the southern coast of the Korean Peninsula (Shin *et al*., 2009; National Institute of Biological Resources, 2012). Currently, this species has been classified as Critically Endangered (CR) on the IUCN Red List (National Institute of Biological Resources, 2012) and protected by law through the Ministry of Environment in Korea.

Endangered or rare plants, including orchids, have undergone habitat loss, over-collection, and fragmentation (Segelbacher *et al*., 2003; Michael, 2016). In particular, wild orchids are declining in numbers as a result of over-collection, habitat destruction due to human activities, and impact of climate change (Barman and Devadas, 2013; Michael, 2016; Tian *et al*., 2018; Xu *et al*., 2018). These factors induce genetic erosion from genetic drift, inbreeding, decrease in populations size, and reduced gene flow within small and isolated populations (Murren, 2002; David and Richard, 2003; Honnay and Jacquemyn, 2007; Hundera *et al*., 2013; Su *et al*., 2017). Genetic variability in small populations is vulnerable to environmental changes due to inbreeding, isolation, and bottleneck events, and this reduces the potential of small populations to respond to selective pressures (Spencer *et al*., 2000; Su *et al*., 2017). Appropriate conservation measures are needed of endangered to maintain genetic diversity. For population genetics research, it is important to understand the level of population differentiation in order to properly conserve endangered or rare plants.

Microsatellites are codominant molecular markers that

show a high level of polymorphism and contain repeat motifs of 1-6 nucleotides (Yun *et al*., 2011). They have been effectively utilized as a tool for genetic assessment of endangered or rare plants for conservation (Spencer *et al*., 2000; Escudero *et al*., 2003; Sun *et al*., 2011). Recently, the genetics of a wide range of endangered or rare plants were evaluated using microsatellite markers (Hou *et al*., 2012; Lavor *et al*., 2014; Miao *et al*., 2015; Kwak *et al*., 2017; Su *et al*., 2017).

N. falcata studies have primarily focused on the culture of seeds or cells for horticultural value (Chung, 1980; 1981; Ichihashi and Islam, 1999; Hahn and Paek, 2001; Mitsukuri *et al*., 2009; Han *et al*., 2010; 2013). As basic information for conservation, *N. falcata* is an anemochorous plant that is pollinated by the long-tongued hawkmoth (genus *Theretra*) (Kiyohara *et al*., 2012; Shimizu, 2012; Suetsugu *et al*., 2015). The phylogeny of *Neofinetia* and related genera has been extensively studied, but there is a lack of research towards genetic diversity using microsatellites (Duttke *et al*., 2012; Hidayat *et al*., 2012; Kim *et al*., 2014).

Understanding of genetic variation and population differentiation of *N. falcata* is essential for its conservation and restoration. However, the population genetic information of wild this species have not been studied. For this reason, we used next-generation sequencing (NGS) to develop microsatellites markers of *N. falcata*. We use these microsatellite markers to study the genetic structure, population differentiation, and gene flow between natural populations. These data provide baseline genetic information for conservation and management of this endangered species.

Materials and Methods

Plant materials and DNA extraction

N. falcata is difficult to collect because it commonly grows on steep cliffs and the number of natural populations is small. We chose sampling sites based on interviews with the national park official. Forty individuals of *N. falcata* were collected from Boksaeng Island (BS, n = 11), Geomun Island (GM, n = 6) and Galgot Island (GG, n = 23), located in the South Sea of Korea (Table 1; Fig 1). The leaves were dried on either silica gel or frozen in liquid nitrogen. Genomic DNA was extracted from the flesh of the leaves using the Wizard Genomic DNA Purification Kit (Promega, USA). The quality and

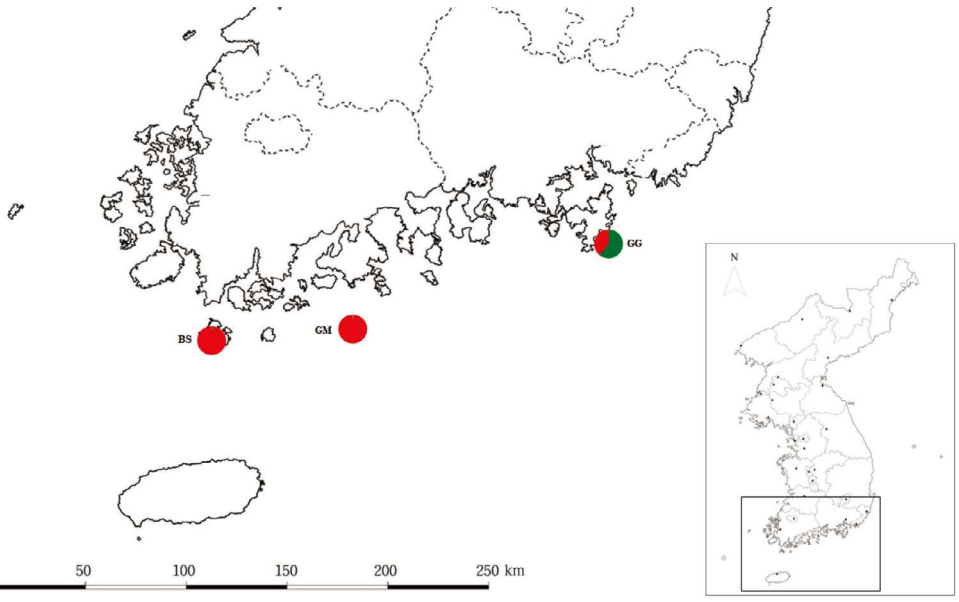

Fig. 1. Geographic distribution of *N. falcata* populations. Abbreviations are shown in Table 1. Pie charts represent assignment probability of belonging to each $K = 2$ clusters identified by STRUCTURE based on microsatellite allele frequencies, with probability values normalized using CLUMPP.

Table 1. Sample collection sites for populations.

Population code	No. individuals	Location
BS	11	Boksaeng Island, Wando-gun, Jeollanam-do Prov.
GM	6	Geomun Island, Wando-gun, Jeollanam-do Prov.
GG	23	Galgot Island, Geoje-si, Gyeongsangnam-do Prov.

concentration of DNA were assessed using 0.7% agarose gel electrophoresis and a spectrophotometer (Nanodrop, USA). DNA was stored at 4°C until used for later laboratory work.

Development of the microsatellite makers

DNA from one individual was used for NGS sequencing to screen for microsatellite fragments. Approximately 10 μg of genomic DNA was sequencing using the Roche 454 GS-FLX Titanium platform (454 Life Sciences, Branford, CT, USA) at the National Instrumentation Center for Environmental Management of Seoul National University (NICEM). The sequence reads were assembled using the Newbler software package (Roche Diagnostics, 454 Life Science). The sequences were scanned for di- and trinucleotide repeats using the "SSR_finder.pl" Perl program for the development of microsatellites (Shanker *et al*., 2007). The repeats were sorted according to the number of iterations, and primer pairs flanking each repeat were designed using Primer 3 to amplify fragments containing repeats of more than four iterations (Rozen and Skaletsky, 2000). The optimal primer size was set at 22 bp (range 18-26 bp), the optimal annealing temperature was set at 58°C (range 55-59°C), and the product size was selected to be 130-270 bp, with the remaining parameters left at the default settings.

PCR and microsatellite genotyping

Forty-eight designed primer pairs were tested for amplification using polymerase chain reaction (PCR) on two individuals. PCR was performed in a total volume of 20 μL containing 10-15 ng of DNA from 40 individuals of the three populations and 0.2 μM of both forward and reverse primer using AccuPower PCR Premix (Bioneer, USA). Conditions included initial denaturation at 94°C for 5 min; then 35 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 2 min; with a final extension at 72°C for 7 min. The amplified fragments and their sizes were visualized using QIAxcel (Qiagen) with the QIAxcel DNA Screening Kit (Qiagen). PCR amplifications of microsatellite loci were conducted in a final volume of 6 μL containing 1X QIAGEN Multiplex PCR Master Mix (QIAGEN, Germany), approximately 10 ng of template DNA, and 0.2 μM for both the reverse primer and labelled primer fluorescent (6-FAM, NED, PET and VIC). The PCR reaction was amplified using the following conditions: 5 min pre-denaturation at 95°C, followed by 35 cycles of 30 s at 94°C, 90 s at 57°C, and 72 s at 60°C, and a final extension at 60°C for 30 min. The amplified DNA fragments were analyzed using an ABI 3730XL (Applied Biosystems, USA), and genotypes were determined using the GeneMaker program ver. 1.85 (Softgenetics LLC).

Data analysis

The number of alleles (A), observed heterozygosity (H_o), and expected hetrozygosity (H_e) for each locus were calculated using ARLEQUIN ver. 3.5.1.2 (Excoffier and Lischer, 2010). Deviations from Hardy-Weinberg equilibrium (HWE) were tested using ARLEQUIN. Measures of the mean number of alleles per locus (N_a), number of effective alleles per locus (N_e), Shannon's information index (I), and inbreeding coefficient (F_{is}) were performed with GenAlEx ver. 6.503 (Peakall and Smouse, 2012). Allelic richness (R_s) and the number of private alleles (P_s) for a standard sample size were calculated using a rarefaction approach in ADZE (Szpiech *et al*., 2008). The possible loss of genetic variation through the bottleneck effect was tested using the BOTTLENECK ver. 1.2.02 (Piry *et al*., 1999). The Wilcoxon test was used to test the significance of heterozygote excess under the infinite allele model (IAM), the stepwise mutation (SMM), and two-phased models (TPM), as recommended by Piry *et al*. (1999). Population genetic structure was investigated using the STRUCTURE ver. 2.3.4 (Pritchard *et al*., 2000). The assumed K number of populations ranged from 1 to 20. Each run was performed using the admixture model and the burn-in period was set to 100,000 followed by 1,000,000 Markov chain Monte Carlo (MCMC) replicates, with 10 iterations per *K* to confirm stabilization of the summary statistics. The most appropriate *K* was determined by calculating ΔK following steps set forward by Evanno *et al*. (2005). All ΔK calculations were performed using the program STRUCTURE HARVESTER ver. 6.0 (Earl and vonHoldt, 2012). We combined the data of the interactions in the best *K* with the CLUMPP (Jakobsson and Rosenberg, 2007), then plotted the results using DISTRUCT (Rosenberg, 2004). Population differentiation was assessed by hierarchical analysis of molecular variance (AMOVA) using ARLEQUIN ver. 3.5.1.2.

Results

Isolation and characterization of microsatellite loci

From the NGS, we obtained 93 Mbp of sequence data (238,561 reads). The total number of contigs was 3,964 and number of singletons was 162,430. Di- and trinucleotide repeats were identified in 36,915 regions (dinucleotide repeats: 5,018; trinucleotide repeats: 1,928). The greatest number repeat motifs were 15 iterations of AT, CA, CT among dinucleotide repeats and 18 iterations of ATA among trinucleotide repeats.

Initial screening of the 48 primer pairs with two individuals resulted in 27 successfully amplified primer pairs. The 27 microsatellite primers were then evaluat-

Table 2. Characteristics of 9 microsatellite loci developed in *N. falcata*.

Locus	Primer sequences (5'-3')	Repeat motif	Fluorescent dye	Allele size range (bp)
NF1	F: GATGAATGGCCCTAAAACAATA R: TTTCTTTTATTTGTGTGTATGTTGAATCT	$(AAT)^{11}$	6-FAM	202-205
NF2	F: CCAACGCCTAACAAAATCAAG R: GAAGACTTGGGCTTATTGTATTA	$(ACA)^{15}$	6-FAM	286-300
NF3	F: AAATAAGTCTGTATCCCACTAAGA R: ATTGATTTGTTTTTGTATGGATTTT	$(TTG)^{10}$	NED	251-260
NF4	F: CTTAAATCAGAAGACTAACCATTAATTC R: GGTTTTCTTTTAAGGTTTTTGGA	$(AAT)^{12}$	NED	190-196
NF5	F: GCCGATATGTGATGCTTTAGTT R: CCTTGACTCACATACCC	$(ATA)^{11}$	6-FAM	169-172
NF6	F: CGTTTCACCTATTTAACCGAAG R: ACATCATTCTAGCTAAACAACATC	$(ATT)^{11}$	NED	190-196
NF7	F: TTACACGAATCACAAATCCAGA R: CTAAAACTTCTCCATTGTTCTTC	$(ATA)^{12}$	PET	151-169
NF8	F: TTATTAGACCAGGACATGGAGG R: CCCTTGAGCCTCCTTTAAT	$(TTA)^{11}$	PET	186-217
NF9	F: CAGATTTACAGGATTTGATAAATACG R: GACATGCACATCTTGATCACTC	$(TG)^{12}$	NED	182-193

Table 3. Genetic diversity values for 40 individuals of *N. falcata* across 9 microsatellite loci.

Locus	BS (N = 11)			GM (N = 6)			GG (N = 23)		
	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
NF1	1	–	–	1	–	–	2	0.30	0.51
NF2	2	0.27	0.51	1	–	–	3	0.26	0.59*
NF3	1	–	–	2	0.00	0.30	2	0.09	0.09
NF4	2	0.27	0.37	2	0.00	0.48*	3	0.35	0.48*
NF5	1	–	–	1	–	–	2	0.04	0.04
NF6	2	0.18	0.48	1	–	–	3	0.17	0.54*
NF7	1	–	–	1	–	–	2	0.48	0.41
NF8	2	0.36	0.31	3	0.33	0.32	5	0.26	0.70*
NF9	1	–	–	1	–	–	2	0.35	0.49
Average	1.44	0.27	0.42	1.44	0.11	0.37	2.67	0.26	0.43

A = number of alleles; H_o = observed heterozygosity; H_e = expected heterozygosity; N = number of individual sampled.
*Significant deviation from HWE after correction for multiple tests ($P < 0.005$).
– Monomorphic

Table 4. Genetic diversity indices of three populations based on 9 microsatellite loci.

Population	N	N_a	N_e	I	H_o	H_e	F_{is}	R_s	P_s
BS	11	1.444	1.307	0.260	0.121	0.177	0.261	1.408	0.160
GM	6	1.444	1.177	0.184	0.037	0.113	0.619	1.316	0.219
GG	23	2.667	1.921	0.680	0.256	0.419	0.279	2.396	0.828
Total	40	1.852	1.469	0.375	0.138	0.236	0.338	1.707	0.402

N = number of individuals; N_a = mean number of alleles per locus; N_e = number of effective alleles per locus; I = Shannon's information index; H_o = observed heterozygosity; H_e = expected heterozygosity; F_{is} = inbreeding coefficient; R_s = Allelic richness; P_s = number of private alleles

ed using all 40 individuals. Nine primer pairs yielded consistent and scorable genotypes (Table 2). In the BS population, the number of alleles (A) was two, and the observed heterozygosity (H_o) and expected heterozygosity (H_e) ranged from 0.18 to 0.36 and 0.31 to 0.51 (Table 3). In the GM population, the number of alleles (A) varied from 2 to 3, and the observed heterozygosity (H_o) and expected heterozygosity (H_e) ranged from 0.00 to 0.33 and 0.30 to 0.48. In the GG population, the number of alleles (A) ranged from two to five, and the observed heterozygosity (H_o) and expected heterozygosity (H_e) ranged from 0.04 to 0.48 and 0.04 to 0.70. One GM lo-

Table 5. AMOVA results showing the partitioning of genetic diversity among the three populations of *N. falcata*.

Source	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	2	26.885	0.5256 Va	26.84
Within populations	77	110.303	1.4325 Vb	73.16
Total	79	137.188	1.9581	

cus (NF4) and four GG loci (NF2, NF4, NF6, NF8) deviated from the Hardy-Weinberg equilibrium (HWE).

Genetic diversity

The values for genetic diversity for the 40 individuals of *N. falcata* are in Table 4. The mean number of alleles (N_a) and number of effective alleles (N_e) were 1.852 (1.444-2.667) and 1.469 (1.177-1.921), respectively. Observed heterozygosity (H_o) among all populations was 0.138 (0.037-0.256) and the average expected heterozygosity (H_e) was 0.236 (0.113-0.419). Inbreeding coefficients (F_{is}) ranged from 0.279 to 0.619 and were significantly negative for GM population. The allelic richness (R_s) and the number of private alleles (P_s) ranged from 0.316 to 2.396 and 0.160 to 0.828, respectively.

Genetic structure

AMOVA results showed substantial genetic variation in the sampled populations, with 26.84% of the total variation explained by differences among populations and 73.16% by differences within populations (Table 5). STRUCTURE analyses provided complementary methods for visualizing patterns of genetic similarity and differentiation among populations (Fig. 2). According to the Evanno method in STRUCTURE HARVESTER, the highest peak was detected at $K=2$, followed by peaks at $K=3$. For $K=2$, the first group consisted of 61% of the GG population (red), while the second group consisted of the the remaining population (BS, GM, 39% of GG) (green). These two genetic clusters from the Jeollanam-do population (BS, GM) were separated from the Gyeongsangnam-do population (GG) at $K=3$.

Genetic bottlenecks

Bottleneck analysis showed that two populations (GM, GG) did not experience a recent bottleneck event (Table 6). The GM and GG populations exhibited normal L-shaped allele frequency distributions. However, the BS population displayed evidence of a population bottleneck under IAM, TPM, and SMM, inferring this population has experienced a recent bottleneck event.

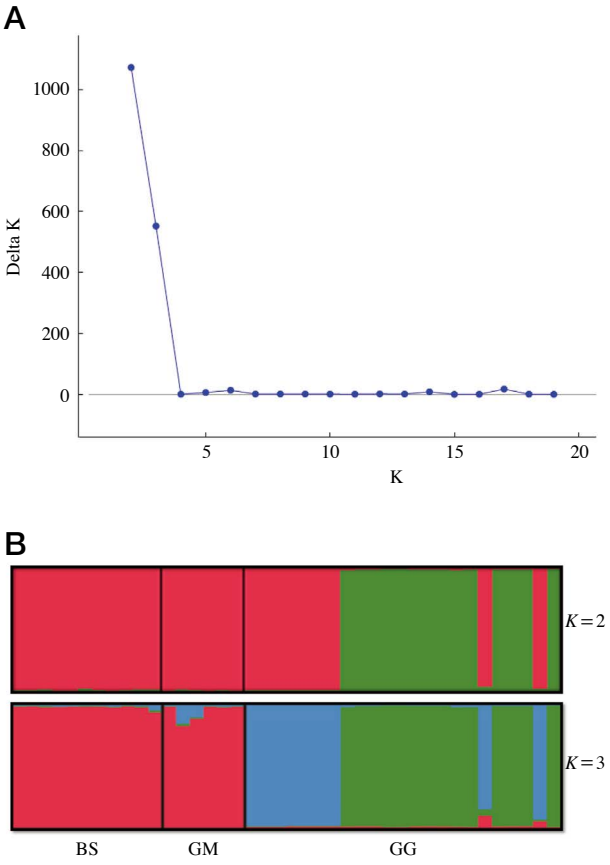

Fig. 2. Structure analyses for putative genetic clusters of *N. falcata*. A: Graphs of ΔK values to determine the ideal number of groups present in the accessions of *N. falcata*. B: Estimated genetic structure of the 3 populations of brinjal based on STRUCTURE analysis $K=2$ and $K=3$.

Discussion

We have developed a set of 9 polymorphic microsatellite markers for *N. falcata*. The nine microsatellite markers were useful for assessing genetic diversity and population structure for conservation. *N. falcata* has maintained a low level of genetic diversity as compared to other orchids that have been studied using microsatellite makers. The microsatellite heterozygosiy of *N. falcata* (mean $H_e=0.236$) was lower than the endangered or epiphytic orchids of *Encyclia tampensis* (mean $H_e=0.281$; Weremijewicz *et al*., 2016), *Cyrtopodium punctatum* (mean $H_e=0.272$; Weremijewicz *et al*., 2016), *Gastrodia elata* (mean $H_e=0.468$; Chen *et al*., 2014), *Dendrobium calamiforme* (mean $H_e=0.591$; Trapnell *et al*., 2015), and *Jumellea rossii* (mean $H_e=0.750$; Mallet *et al*., 2014). Allelic richness of *N. falcata* (mean $R_s=1.707$)

Table 6. Probabilities from Wilcoxon's test for mutation drift equilibrium (population bottlenecks) in the three populations of *N. falcata* under the infinite allele models (IAM), the stepwise mutation models (SMM) and two phase models (TPM) for microsatellite data using the program BOTTLENECK.

Population	Mutation-drift test			Mode shift test
	IAM	TPM	SMM	
BS	0.031	0.031	0.031	shifted
GM	0.875	0.875	0.875	L-shaped
GG	0.019	0.150	0.180	L-shaped

was also lower than *J. rossii* (mean $R_s = 7.98$; Mallet *et al*., 2014). The low levels of genetic diversity among *N. falcata* populations could be caused by inbreeding or fragmentation into small and isolated populations (Miao *et al*., 2014; 2015; Su *et al*., 2017).

Of the three *N. falcata* populations used in this study, GG exhibited high level of genetic and allelic diversity compared to the other two populations. The higher diversity of this population is likely influenced by the larger sample size (23 compared to 11 or 6 individuals). Genetic and allelic diversity of GM was lower than the other populations. The inbreeding coefficient (F_{is}) of GM was twice as high as the other two populations. As mentioned above, the low genetic and allelic diversity were affected by population size, gene flow, genetic drift, and inbreeding (Ellstrand and Elam, 1993; Frankham, 1996; Leimu *et al*., 2006). Our analysis suggests that BS was affected by a bottleneck (Table 6). The number of private alleles of BS was lower than the other two populations. Population that have experienced a recent bottleneck exhibit a larger decrease in number of alleles relative to the genetic diversity (Cornuet and Luikart, 1996). We hypothesize that BS has suffered from bottleneck due to unexpected exploitation.

The environment related to pollen and seed dispersal has significant impact on genetic diversity in plants (Murren, 2002; Muyegi *et al*., 2015; Tian *et al*., 2018). *Theretra* spp., long-tongued hawk moth, is known as potential pollinator of *N. falcata* (Suetsugu *et al*., 2015). The hawk moth can fly distances greater than 20 km (Wone *et al*., 2018). The dust-like seeds of orchids are dispersed long-distance by wind or water (Kiyohara *et al*., 2012; Shimizu, 2012; Tian *et al*., 2018). Nevertheless, recent studies demonstrated that seeds are transported short distances, remaining in the vicinity of the mother orchid, with rare long-distances dispersal (Mallet *et al*., 2014; Tian *et al*., 2018). The populations of *N. falcata* in this study are separated by approximately 70-100 km, and exhibit limited the pollen and seed dispersal. The restricted pollen and seed dispersal across isolated populations would cause low gene flow, decreasing the genetic diversity among population.

The genetic variation among *N. falcata* among populations was moderate (26.84%). This genetic variation among populations infers that long-distances gene flow occasionally occurs. The level of genetic variation among populations was correlated with geographic distance due to limited gene flow by pollen or seeds (Muyegi *et al*., 2015). Also, genetic structure is determined by gene flow and topoclimate with long-term stability (Zhang *et al*., 2017). Our analysis of genetic structure showed that the three populations could be separated in two genetic groups, according to geographic location.

N. falcata is threatened with extinction due to excellent horticultural value and habitat destruction by human activity. The low genetic and allelic diversity, along with the inference of a recent bottleneck, weakens the resilience of this species to environmental change, such as habitat destruction and climate change.

Thus, *in situ* and *ex situ* conservation efforts are necessary for this species. *In situ* conservation should be implemented to reduce the further loss of genetic diversity and preserve the current genetic structure. *Ex situ* conservation should also be considered given that using seeds at *ex situ* sites is a good strategy for conservation purposes (Johansen and Rasmussen, 1992). Seed storage will be essential to recover the extinction populations.

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