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Authors: Li, Zongyan, Li, Jing, and Li, Minyang

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Short Communication

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Effect of Human Disturbance on Genetic Structure of Rare and Endangered *Paphiopedilum micranthum* Implied the Habitat Status

Zongyan Li[1,2], Jing Li[1], and Minyang Li[3]

Abstract

Seven populations of *Paphiopedilum micranthum* from Southeast China were used to assess the influence of human disturbance on genetic structure through analysis with sequence-related amplified polymorphism technology. The results indicated that there was high genetic diversity at species level ($p = 81.25\%$; $I = 0.3709$) and a significantly higher differentiation level compared to that those of other outcrossing orchid species, and that moderately disturbed populations sustained higher genetic diversity indexes than the natural populations. This study revealed that human disturbance and population size did not significantly affect the populations' genetic diversity but aggravated their differentiation. This may suggest that the habitat had a much greater influence on genetic variation.

Keywords

Paphiopedilum micranthum, genetic diversity, genetic differentiation, human disturbance, habitat status

Introduction

The genus *Paphiopedilum* is currently listed as one of the most endangered categories in the *Orchidaceae* family due to its unsustainable collection and discontinuous distribution in the tropical and subtropical regions of Asia (Cribb, 1998). *Paphiopedilum micranthum* is only found in the karst area of southeast Yunnan, southwest Guizhou, northern and western Guanxi in China, and northern Vietnam (Chen, 1999). It is listed as first-class protected plant and was forbidden to be sold according to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (Cribb, 1998).

Previous studies had focused on pollination-related biological characteristics, plant geography and evolution (Banziger, 1994; Cribb et al., 2002; Ma et al., 2016). A preliminary study showed that the species had low genetic diversity at population level, whereas it had a significantly high population differentiation level (Li et al., 2002). Disturbance regimes are increasingly recognized as drivers of changing biodiversity patterns that cause temporally fluctuations in the distributions and the abundances of species (Davies et al., 2016). Anthropogenic disturbance could have a complicated influence on the population structure (Banks et al., 2013). Some evidence has indicated that disturbance at different scales could trigger genetic degradation within populations (Soares et al., 2019). Population diversity, especially for the rare and endangered species, is easily affected by the timing, frequency, and intensity of disturbance (Babai & Molnar, 2014; Nakahama et al., 2016). The genotypic diversity of *Populus tremuloides* is related to the intensity of disturbance in North

[1]Southwest Landscape Engineering Technology Institute of State Forestry Bureau, Southwest Forestry University, Kunming, People's Republic of China
[2]Functional Flower Center, Southwest Forestry University, Kunming, People's Republic of China
[3]College of Horticulture and Landscape Architecture, Southwest University, Chongqing, People's Republic of China

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Corresponding Author:
Zongyan Li, Southwest Forestry University, Bailong Road 300, Kunming, Yunnan 650224, People's Republic of China.
Email: lizyan74@sina.com

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American boreal forests (Latutrie et al., 2019). Generally , intermediate levels of disturbance improve the genetic diversity of clone plants, providing greater recruitment of new genes into the population (McMahon et al., 2017). However, Tunaitien et al. (2017) found that population diversity parameters of *Erigeron annuus* did not significantly differ between stable habitats and disturbed regions. A recent meta-analysis based on 92 case studies revealed that anthropogenic disturbance had a negative effect on allelic richness, but not on genetic diversity (Gonzalez et al., 2019). Regarding the correlation between disturbance and genetic structure, some reported that the environmental disturbance variables at different scales were not associated with the spatial genetic structure or genetic divergence in palm populations (Soares et al., 2019).

The limestone area in southeastern Yunnan is thought to be the origin and evolutionary center as well as the largest trading center of *P. micranthum* in the world (Luo et al., 2003). However, few studies are available that show the genetic structure, especially in the region with overcollection. Consequently, there is no information on the impacts of human disturbance on the genetic structure of *P. micranthum*.

Sequence-related amplified polymorphism technology has been used as successful molecular markers for detecting a population's genetic structure (Babaei et al., 2014). The objective of this study was to assess the influence of human disturbance on genetic structure of seven populations of *P. micranthum* to promote a better environmental strategy.

Methods

According to the frequency and intensity of seedling collection in observed field sites, human disturbance was categorized into three levels: natural (no disturbance), light and moderate. It could be defined as light when overall picking for blooming individuals occasionally happened, and moderate disturbance was identified when the same collection continuously occurred about once per year. In total, 167 seedlings of seven populations were drawn randomly from *P. micranthum* collection areas in three counties (Wenshan, Maguan, and Malipo) of Southeast Yunnan, China, representing two natural populations (WSDZ and MLPTB), two light disturbance populations (WSXBZ and MGGLQ), and three moderate disturbance populations (WSYLJ, MGJHQ, and MGMG) respectively. The relative information was shown in Table 1. The geographical locations of the seven populations were showed separately in the State map and the map of China (Figure 1). The sampling-distance of individuals was more than 2 m away from each other to avoid clonal specimens. A field survey was conducted on the habitat types of the individuals including the herbaceous companion species.

Genomic DNA was extracted from the powdered tissue using a modified cetyltrimethyl ammonium bromide method (Doyle & Doyle, 1987). The DNA quantify was examined by a 0.8% agarose gel electrophoresis.

Primers were selected for further analysis provided that the reproducible and unambiguous bands could be produced. The sequences of ten sequence-related amplified polymorphism primer combinations were referred to Li and Quiros (2001), Ferriol et al. (2003), and Zhang et al. (2008), and the amplified results were listed in Table 2. All primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd.

Polymerase chain reaction (PCR) amplification was performed with a 20 µl reaction mixture containing 100 ng of template DNA, 1.5 mM of $MgCl_2$, 0.3 mM of each dNTP, 0.5 µM of each primer, and 1.5 U of Taq DNA polymerase with Eppendorf Master cycler Gradient PCR (Eppendorf Corp., Hamburg, Germany). The reaction procedure was referred to Ferriol et al. (2003). The PCR products were separated with 6% denaturing polyacrylamide gels using a 50 bp

Table 1. Information of Collection Site of *P. micranthum* Populations.

Code	NS	Disturbance levels	*Na*	*Ne*	*H*	*I*	*PPB* (%)
MGMG	23	Intermediate	1.344	1.198	0.116	0.175	34.38
MGJHQ	20	Intermediate	1.462	1.276	0.161	0.240	46.18
MGGLQ	30	Light	1.507	1.287	0.169	0.254	50.69
WSDZ	25	Natural	1.507	1.239	0.146	0.225	50.69
WSYLJ	19	Intermediate	1.538	1.290	0.174	0.263	53.82
WSXBZ	19	Light	1.469	1.260	0.155	0.234	46.88
MLPTB	31	Natural	1.528	1.278	0.166	0.252	52.78
Population			1.479	1.261	0.155	0.235	47.92
Species			1.813	1.403	0.242	0.371	81.25

Note. Code presented seven different geographical populations. MGMG = MaguanMagu; MGJHQ = MaguanJiahanqing; MGGLQ = MaguanGulinqing; WSDZ = WenshanDouzui; WSYLJ = WenshanYanliujin; WSXBZ = WenshanXiaobazi; WSTB = MalipoTianba; NS = number of samples. *Na* = Observed number of alleles; *Ne* = Effective number of alleles; *H* = Nei gene diversity; *I* = Shannon's information index; *PPB* = the percentage of polymorphic band.

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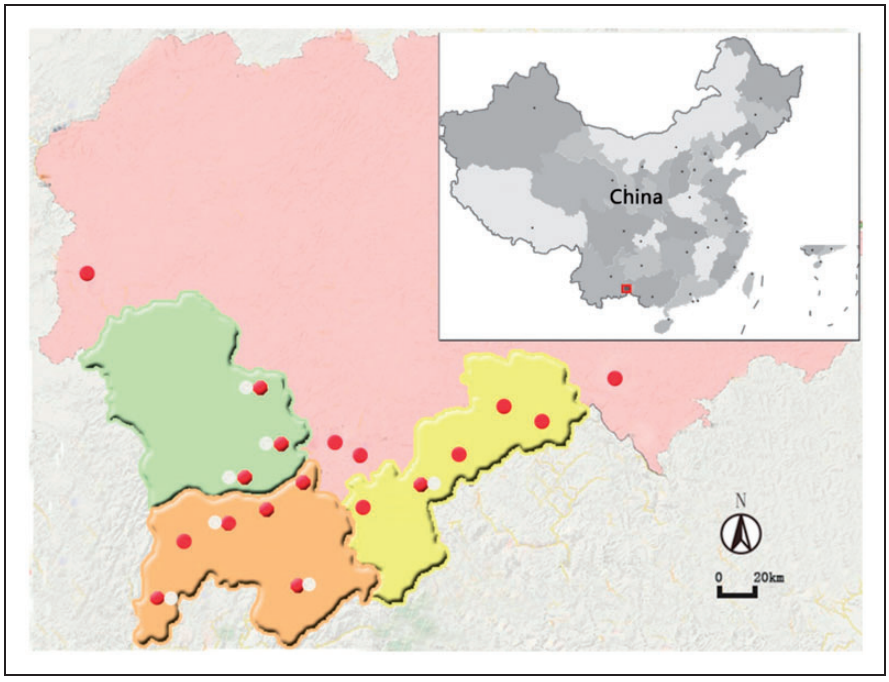


Figure 1. Map Showing the Geographical Distribution of *P. micranthum* Populations in Southeast Yunnan of China. Green, yellow, and orange area represent Wenshan, Malipo, and Maguan county, respectively. Red dots are all populations sites in Yunnan province recorded in historical documents. White dots mean seven sampled populations. Red square shows the location of sampled counties in China map.

Table 2. Sequence-Related Amplified Polymorphism Primer Sequences Used in Genetic Diversity Analysis and Amplified Results of *P. micranthum*.

Code	Forward primer (5′-3′)	Code	Reverse primer (5′-3′)	Primer combination	Total bands	POL bands	*PPB* (%)
me1	TGAGTCCAAACCGGATA	em1	GACTGCGTACGAATTAAT	me1/em4	30.0	20.0	66.67
me2	TGACTCCAAACCGGAGC	em3	GACTGCGTACGAATTGAC	me1/EM5	37.0	31.0	83.78
me3	TGAGTCCAAACCGGAAT	em4	GACTGCGTACGAATTTGA	me 2/em1	36.0	31.0	86.11
me4	TGACTCCAAACCGGACC	Em9	AGGCGGTTGTCAATTGAC	me3/Em9	33.0	32.0	96.97
me5	TGACTCCAAACCGGAAG	EM2	ACTGCGTACGAATTCTGC	me4/EM3	30.0	24.0	80.00
ME6	TGAGTCCTTTCCGGTAA	EM3	ACTGCGTACGAATTCGAC	me4/em3	20.0	16.0	80.00
ME7	TGAGTCCTTTCCGGTCC	EM5	ACTGCGTACGAATTCAAC	me5/em3	24.0	17.0	70.83
				ME6/ EM2	22.0	19.0	86.36
				ME7/ em1	34.0	26.0	76.47
				ME7/EM2	22.0	18.0	81.81
				Total	288	234	81.25

Note. Pol bands = polymorphic bands; *PPB* = the percentage of polymorphic band; POL = polymorphic band.

DNA ladder standard (Promega Corp., Wisconsin, United States) as the reference and were visualized by silver staining.

The DNA band was numbered as *present* (1) or *absent* (0) for each primer pair. All genetic parameters were calculated by using POPGENE 32 (version 1.32), including Observed number of alleles (*Na*), Effective number of alleles (*Ne*), Shannon's information index (*I*), Nei's genetic distances, Coefficient of gene differentiation (*Gst*), Percentage of polymorphic band (*PPB*), and Gene flow (*Nm)* (Nei, 1973). The analysis of molecular variance and Mantel test were estimated by GenALEX software (Peakall & Smouse, 2006). Spearman correlation coefficient (ɤ) was performed to analyze the influence of human disturbance on five genetic-diversity indexes based on SPSS 17.0 software (Statistical Program for Social Sciences, SPSS Inc., United States).

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Table 3. The Correlation Based on Effect of Disturbance Level and Population Size on Diversity Parameters Revealed by Spearman Coefficients.

Item	Samples size	Disturbance	*Na*	*Ne*	*H*	*I*	*PPB*
Samples size	1	−.639	.164	0	0	0	−.164
Disturbance	−.639	1	−.381	.076	.076	.076	−.381
Na	.164	−.381	1	.757[a]	.757[a]	.757[a]	1.000[b]
Ne	0	.076	.757[a]	1	1.000[b]	1.000[b]	.757[a]
H	0	.076	.757[a]	1.000[b]	1	1.000[b]	.757[a]
I	0	.076	.757[a]	1.000[b]	1.000[b]	1	.757[a]
PPB	.164	−.381	1.000[b]	.757[a]	.757[a]	.757[a]	1

Note. Any two of parameters showed a significant or extremely significant correlation with each other. *Na* = observed number of alleles; *Ne* = effective number of alleles; *H* = Nei gene diversity; *I* = Shannon's information index; *PPB* = the percentage of polymorphic band.
[a]Correlation is significant at the .05 level (two-tailed).
[b]Correlation is significant at the .01 level (two-tailed).

Results

Divergent karst habitats of P. micranthum

P. micranthum was generally distributed in the subtropical secondary evergreen broad-leaved forests or shrubs from the upper areas to the summit of the northern slope of the limestone mountains. According to its diverse habitats, the species could be classified terrestrial (scattered in litter layers under trees or shrubs), epiphytic, or hemi-epiphytic (growing on eolian deposits on rocks or in rock cracks). Moreover, the individuals were often accompanied by other orchids and mosses in the half-shady partially shaded and moist environment, where the orchid species richness was high, and included genera such as *Pholidota, Coelogyne*, *Panisea*, *Bulbophyllum*, and *Anoectochilus*.

Genetic diversity at population level

The highest values of *PPB* and *Nei's* genetic diversity (*H*) were observed in population WSYLJ with 53.82% and 0.1735, respectively (Table 1). Compared with the five diversity indexes, there was little difference between the natural populations and lightly disturbed populations. However, moderate disturbance had a complicated effect on the genetic diversity. Population WSYLJ maintained higher genetic diversity than the two natural Populations (WSDZ and MLPTB) despite its smaller size. Conversely, another intermediate disturbance population, MGMG had lower genetic diversity than the other six populations. The results also revealed that it maintained a higher level of genetic diversity at species level ($PPB = 81.25$; $I = 0.371$; $H = 0.242$).

Significant correlations between disturbance level and genetic diversity

The Spearman coefficient (ɤ) from SPSS analysis indicated that the disturbance level showed a changeable correlation with five genetic diversity parameters varying from −0.381 to 0.076, and that the population size had a nonlinear impact on them with ɤ values ranging from −0.164 to 0.164. However, none of the disturbance levels had a significant correlation with any of five diversity indexes (Table 3).

Genetic differentiation among seven populations

The analysis of molecular variance further revealed that 33.73% of the total genetic variation was at the population level (Table 4.). The coefficient of gene differentiation (*Gst*) showed that approximately 64.32% of the genetic variation came from individuals within the populations. Both revealed that there was significant genetic differentiation among populations.

The smallest genetic distance among the seven populations was between WSDZ and WSYLJ, and the largest one was found between WSXBZ and MGMG varying from 0.066 to 0.192. The Mantel test showed that there was no significantly positive correlation between the phylogenetic relationship and geographical distance ($r = .299$, $p = .188 < .01$).

Discussion

In summary, the population genetic structure and its variation mainly depended on the breeding and mating system, life history, natural selection, human disturbance, gene flow, and genetic drift (Ge, 1997; Meffe & Carroll, 1997). Outcrossing species and those with long life spans tend to be more genetically diverse and have less genetic differentiation among their populations (Hilde & Lgor, 2000). The high genetic diversity at species level of *P. micranthum* was partly determined by its mating system and long-life history. Similar results have been found for *Cymbidium goeringii* and *Phragmipedium longifolium* (Chung et al., 2014; Melania et al., 2010). In addition, natural selection and human disturbance were

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Table 4. Analyses of Molecular Variance at Different Hierarchical Level Based on Sequence-Related Amplified Polymorphism.

Source	*Df*	SS	MS	Est. Var.	Per. Var. (%)	*p*
Among pops	6	2,151.908	358.651	13.972	33.73	<.001
Within pops	160	4,392.332	27.452	27.452	66.27	<.001
Total	166	6,544.240		41.424	100	

Note. *df* = degrees of freedom; SS = sum of squares; MS = mean square error; Est. Var. = estimated variance; Per. Var. = percentage of variance; *p*: probability.

two of prominent and extrinsic factors that affected population genetic structure.

Currently, the influence of disturbance on genetic structure is increasingly controversial in population ecology (Banks et al., 2013). Human disturbance is generally thought to be the most important factor threatening population genetic variation. Disturbance regimes could aggravate habitat fragmentation, influencing seed dispersal and accelerating the natural selection process (Fant et al., 2016). Theoretically, declining population size and habitat fragmentation could result in a loss of genetic diversity and genetic differentiation due to a limited gene flow, especially for in narrowly distributed populations (Reusch et al., 2005). Conversely, others have argued that disturbance is taken as a driver of biodiversity patterns (Fajardo et al., 2016). Disturbance on tundra was beneficial for separating willow clones and improving sexual recruitment, which caused higher levels of population genetic diversity (Huebner et al., 2019). Environmental disturbance might affect some parameters of population genetic diversity, such as the fixation index, and the number of alleles (Soares et al., 2019).

In this study, moderate disturbance had different effects on the population genetic diversity of *P. micranthum*. One population (WSYLJ) maintained the highest level of genetic diversity, but the other (MGMG) had the lowest level in all populations. *P. micranthum* is known to propagate both sexually and vegetatively, forming clonal groups to enlarge the population size. Sexual recruitment and low clone expansion will help to improve population genetic diversity. Therefore, a population with a large size does not indicate high genetic diversity. This was proven by Erichsen's finding that in the exhaustively sampled populations, the genetic diversity index could not reach a maximum value because the recruitment strategies included both sexual and clonal individuals (Erichsen et al., 2019). Sexual propagation of orchids depends on the amounts of seeds that can be stored in the soil and maintain higher viability for long periods. The reason for the high genetic diversity in *P. micranthum* populations immediately after disturbance presumably resulted from the lack of a decrease in sexual recruitment because of the previously stored seedbank. This result suggested that a reduction in the seedbank output due to unsustainable collection would not appear in the short term. Some studies have reported that population genetic diversity has a delayed response to recent anthropogenic disturbance, especially for some perennial plants (Aavik et al., 2019).

More evidence proved that biotic disturbance had little influence on the genetic diversity of clonal populations' genetic diversity. When the population diversity of *Elytrigia atherica* subjected to two management regimes (under gazed and undisturbed environments) were compared, no distinct difference was found (Veeneklaas et al., 2011).

However, human disturbance would inevitably lead to a greater population differentiation among many species, such as *Phragmites australis* and *Prosopis alba* (Bessega et al., 2016; Fant et al., 2016).

The complex responses to habitat disturbance were closely related to the plants' life-span, mating system and life-form. Long-lived trees and self-incompatible species were more susceptible to a changing habitat (Gonzalez et al., 2019).

Adaptive evolution and neutral evolution are generally regarded as two important sources of gene variation. Generally speaking, the habitat is one of most important evolutionary force. The influence of disturbance on genetic structure results from the imposed environmental and demographic changes, which are closely related to the species responses to habitat suitability, leading to accelerate its evolutional process (Banks et al., 2013). Therefore, some presumed that genetic differentiation could be associated with founder effects or different selection pressures under disturbed habitats (Tunaitien et al., 2017). The population structure of *Ranunculus ficaria* under heterogenic habitats provides a good example of the impact of selection pressure. Higher population diversity was found in disturbed meadow plots than in disturbed forestry (Reisch & Scheitler, 2009).

The present correlation analysis revealed that moderate disturbance did not cause a serious decline in the genetic diversity of *P. micranthum*. Soares et al. (2019) found that disturbance was not always associated with population spatial genetic structure or genetic divergence. The results of this study were basically consistent with Soares' finding. Whereas *P. micranthum* maintained a significantly higher differentiation level ($Gst = 0.357$) than that of three populations ($Gst = 0.203$) in Guizhou (Li et al., 2002). Theoretically, the average genetic differentiation coefficient (*Gst*) of outcrossing

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populations could reach 0.23 (Hilde & Lgor, 2000). We speculated that this apparent differentiation could be attributed to the selection pressure caused by human disturbance and habitat heterogeneity. First of all, natural selection would lead to adaptive evolution to increase genetic differentiation. Because of its unique biological characteristics, *P. micranthum* relies on root symbiotic fungi to provide it with sufficient mineral nutrients. This helped it develop strict habitat selectivity, which could grow under partially shaded, wet environments with full litter layers to form various ecotypes, such as terrestrial, epiphytic, and hemiepiphytic. Second, environmental changes caused by human disturbance would shape the divergence of selective evolution. Simultaneously, the community structure and habitat had great influences on demographics and seed dispersal. The gene flow is usually classified as high level ($Nm \geq 1.0$), moderate level ($0.99 \geq Nm \geq 0.25$), and low level ($Nm < 0.25$) based on the parameter scale (Govindaraju, 1988). The limited gene flow ($Nm = 0.902$) among seven populations might be that the abusive collection unavoidably caused habitat destruction and fragmentation, which lowered the level of gene flow and aggravated population differentiation. In addition, the Mantel test implied that genetic drift should be responsible for the high genetic differentiation.

Implications for Conservation

Therefore, the above mentioned analyses demonstrated that the habitat was the main factor that led to high genetic differentiation. This research provided insight into the effect of disturbance on population genetic structure in clonal plants distributed in karst heterogenic habitats. The results informed species conservation to a certain degree. Avoiding high genetic differentiation among populations of *P. micranthum* should be focused on methods for restoring the ecological status as soon as possible. In situ conservation could not only prevent the habitat from further fragmentation, but also benefit the relevant fungi and pollinators. The unsustainable collection of *P. micranthum* resources should be strictly forbidden especially during the blooming period, to promote higher gene flow.

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ORCID iD

Zongyan Li https://orcid.org/0000-0001-5435-5569

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