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Massive Extraction of the Orchid *Laelia speciosa* (HBK) Schltr. for Trading in Local Markets Affect Its Population Genetic Structure in a Fragmented Landscape in Central Mexico

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Abstract

Laelia speciosa is an orchid species listed as threatened of extinction in the Mexican standard NOM-059. Wild populations of *L. speciosa* have been declining due to fragmentation of its habitat and massive extraction for trading in local markets in Mexico. In this study, we aimed to evaluate the evolutionary potential of *L. speciosa* within a fragmented landscape of ca. 4000 km^2 in the Cuitzeo basin, State of Michoacán. We sampled 15 populations throughout the Cuitzeo basin and amplified eight nuclear microsatellite markers to assess genetic diversity, structure, and connectivity and to test for evidence of recent bottlenecks. Surprisingly, *L. speciosa* populations showed high genetic diversity, with values ranging from moderate to high compared with those reported for other orchid species. Also, the analysis of molecular variance and R_{ST} results indicated the existence of low genetic differentiation, favored by its cross pollination habit which facilitates the maintenance of gene flow and that have been observed in other orchid species. Wright's within-population inbreeding (F_{IS}) was positive in all cases, denoting a heterozygosity deficit, with moderate-to-high values. Fragmentation may also lead to inbreeding due to either increased self-fertilization or mating between related individuals within remnant fragments. The *L. speciosa* populations examined showed evidence suggesting that some populations had recently gone through a bottleneck. We also observed that all the *L. speciosa* populations had a moderate effective population size. The history of *L. speciosa* in the Cuitzeo basin suggests that both fragmented and non-fragmented populations may have been recently subject to moderate reductions in effective population size, large enough to affect their allelic diversity, F_{IS}, but not their H_E. Such reductions may have been caused by episodic environmental fluctuations or resulted from the recent founding of some of the populations. The effective population size can be used as an indicator of habitat quality, and this was confirmed for the *L. speciosa* populations, which have undergone a drastic decline due to environmental changes, habitat destruction, and illegal collection. The ultimate goals of conservation are to ensure the continuous survival of populations and maintain their evolutionary potential by preserving natural levels of genetic diversity. Great efforts should be made to preserve this species' extant populations and their habitats to prevent further population reductions and preserve its overall genetic basis. Collection of this orchid should be banned and robust legal protection measures should be enforced through local authorities.

Keywords

forest fragmentation, effective population size, genetic bottlenecks, genetic diversity, *Laelia speciosa*, orchids

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Introduction

The Orchidaceae is one of the most species-rich plant families in the world, with an estimation of over 27,000 species (Chase, 2005; Dressler, 1990). However, orchids also include the highest proportion of threatened or extinct species of all plant families (Swarts & Dixon, 2009). In general, orchids are habitat specialists with distinctive requirements for biotic interactions and environmental quality. Most orchid species depend on highly specific biotic interactions with both mycorrhizal fungi (Ávila-Díaz, Garibay-Orijel, Magaña-Lemus, & Oyama, 2013; Rasmussen & Rasmussen, 2009; Smith & Read, 2008) and pollinators (Chung, Chong-Wook, Myers, & Chung, 2007; Van der Pijl & Dodson, 1996) to complete their life cycle. Even minor environmental changes can affect such interactions with significant negative impacts on plant performance that might lead to population decline and eventually local extinction (Honnay, Jacquemyn, Bossuyt, & Hermy, 2005; Rasmussen & Rasmussen, 2009). It is therefore important, from the conservation standpoint, to understand the genetic consequences of the decline in population size of orchids (Izawa, Kawahara, & Takahashi, 2007; Swarts & Dixon, 2009). Habitat fragmentation may induce genetic changes in remnant plant populations, including erosion of their genetic variability and acceleration of the genetic divergence between them through reduced gene flow or increasing genetic drift (Jacquemyn, de Meester, Jongejans, & Honnay, 2012; Lowe, Boshier, Wars, Bacles, & Navarro, 2005; Schaberg, DeHayes, Hawley, & Nijensohn, 2008; Young & Pickup, 2010). Genetic erosion could reduce the adaptive flexibility of populations to respond to environmental changes (Farwig, Braun, & Bohning-Gaese, 2008; Griffiths, Wessler, Lewontin, & Carroll, 2008; Jump & Peñuelas, 2006).

Some 1,106 species and subspecies of orchids, distributed in 159 genera, occur in Mexico. One outstanding feature is the high level of endemism: about 444 of the known orchids species occurring in Mexico are endemic to this country (Soto-Arenas, 1996). This makes the Mexican flora one of the most endemic-rich among tropical countries (Dressler, 1990). Today, approximately 180 orchid species are listed in the official Mexican standard NOM-059-SEMARNAT (Semarnat, 2010) under some category of risk, including the species *Laelia speciosa* (HBK) Schltr. Among epiphytic orchid species, *L. speciosa* is considered one of the most beautiful flowers, and its wild populations are intensively collected in Mexico (Halbinger, 1993; Soto-Arenas, 1996). In Mexico, it inhabits the oak forest of the Sierra Madre Occidental and Sierra Madre Oriental, the southern part of the Central Mexican plateau, and the Trans-Mexican Volcanic Belt, at elevations ranging from 1440 to 2500 m asl (Halbinger & Soto, 1997). The inflorescence displays one to three large colorful flowers, 10 to 16 cm in diameter, widely open with the petals and sepals almost on the same plane, which may be pale pink to lilac pink often with purple stripes along the margins (Van der Pijl & Dodson, 1996). *L. speciosa* is pollinated by *Bombus pennsylvanicus, B. sonorous*, and *B. ephippiatus* (Neiland & Wilcock, 1998). Due to the massive extraction and habitat fragmentation, wild populations of *L. speciosa* have been declining to the extent that this species is now listed as threatened in the Mexican standard NOM-059-SEMARNAT (Ávila-Díaz & Oyama, 2007; Semarnat, 2010; Soto-Arenas, 1996), see Figure 1. A rough estimation states that some 1,500 flowers and 6,000 plants or segments of plants are extracted every day during the blooming season to be traded in local markets in the State of Michoacán in central Mexico (Ávila-Díaz & Oyama, 2007). The use of this orchid is closely related to commercial and cultural activities. Artisans in several towns in the state of Michoacán extract mucilage from the pseudobulbs to make handcrafts called ''cane paste figurines'' (Miranda, 1997). Large amounts of flowers are also used in religious ceremonies, a practice that has severely reduced its natural populations (Ávila-Díaz & Oyama, 2007). Other factors contributing to the decline of *L. speciosa* populations include the plants' slow growth and poor seed formation as well as the fragmentation of their habitat in the basin caused by human activities such as agriculture, grazing, wildfires, and deforestation (Ávila-Díaz & Oyama, 2007).

With that jeopardizing scenario, we could expect a negative effect of intensive extraction along with the destruction of the pine-oak forests which have possibly been triggering the extensive fragmentation in their populations. The majority of population genetic studies have been focused on both epiphytic and terrestrial orchids and was mainly based on isozymes (Ávila-Díaz & Oyama, 2007; Borba, Felix, Solferini, & Semir, 2001; Murren, 2003; Trapnell, Hamrick, & Nason, 2004) and microsatellites (Da Cruz, Selbach-Schnadelbach, Lambert, Ribeiro, & Borba, 2011; Muñoz, Warner, & Albertazzi, 2010; Pinheiro et al., 2012; Stone, Crystal, Devlin, Downer, & Cameron, 2012; Swarts, Sinclair, Krauss, & Dixon, 2009; Vargas, Parra-Tabla, Feinsinger, & Leirana-Alcocer, 2006). Despite the molecular marker used, the few studies in orchids indicated that epiphytes enjoy some of the dispersal advantages of trees (e.g., greater potential for gene flow derived from longer-distance dispersal of pollen and seeds; Borba et al., 2001; Flores-Palacios & García-Franco, 2003; Trapnell & Hamrick, 2004), which may attenuate the genetic impacts of habitat fragmentation.

In this study, we aimed to assess the genetic diversity and population structure of the threatened orchid *L. speciosa* using microsatellite markers. In particular, we are interested to know the levels of inbreeding and

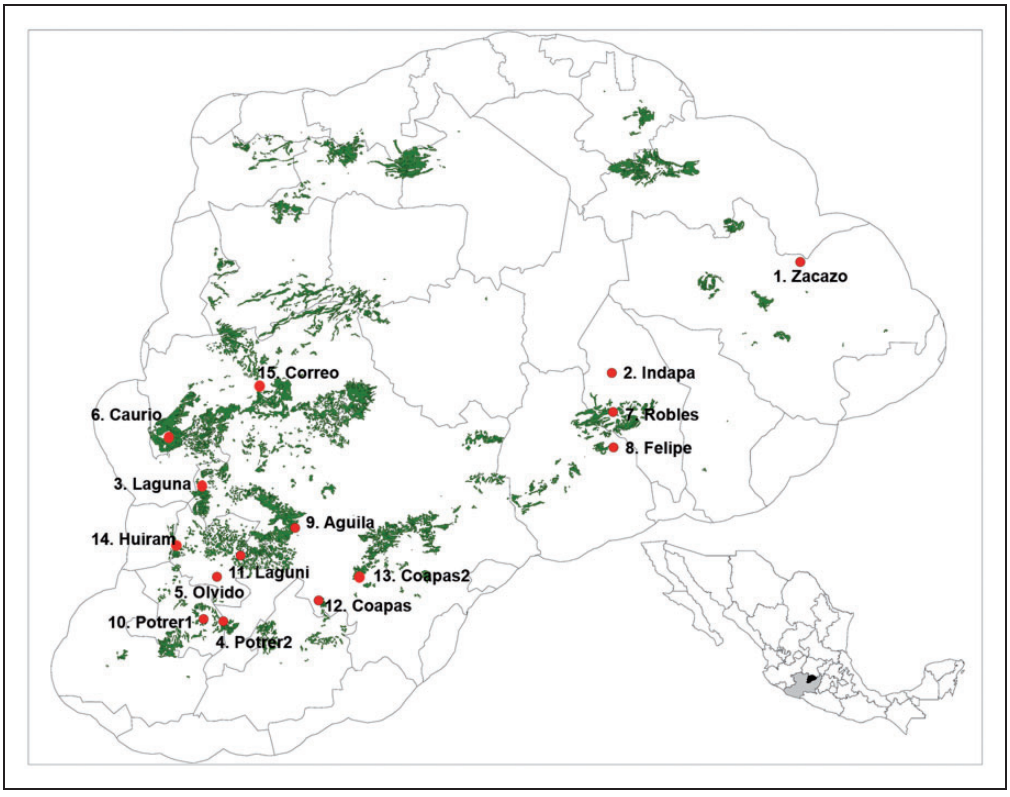

Figure 1. Map showing the delimitation of 15 populations of *L. speciosa* sampled in the Cuitzeo Basin, Michoacán, Mexico. We include, information about the forest fragment of oak forest remained in the area to illustrate the pattern of fragmentation on the host species *L. speciosa* within the Cuitzeo basin in green color. The miniature map of Mexico highlighted the Michoacán state and the Cuitzeo Basin.

population bottlenecks, gene flow, and connectivity, as a consequence of massive extraction for trading in a fragmented landscape.

Methods

Collecting Sites and DNA Amplification

Samples of *L. speciosa* were collected in 15 forest fragments in the Cuitzeo basin, Michoacán (Table 1, Figure 1). We collected 12 to 20 individual plants from each population; the plants sampled were growing on *Quercus deserticola* trees separated from each other by at least 30 m. Samples were frozen until DNA extraction. Genomic DNA was extracted from 100 mg of fresh leaf material using the protocol proposed by Lefort and Douglas (1999). Eight nuclear microsatellite loci were selected and amplified by means of multiplex polymerase chain reaction (PCR; Cortés-Palomec, McCauley, & Oyama, 2008). Based on allele size, annealing temperature, and fluorescent labels, the PCR amplification products were sorted into three groups of primers. The first group included the primer pairs for Lspe8 and Lspe12; the second group those for Lspe1, Lspe4, and Lspe7; and the primer pairs for Lsp6 and Lspe10 were included in the third group (Cortés-Palomec et al., 2008). The PCR was performed using the QIAGEN Multiplex PCR kit (QIAGEN) in a 5-µl volume containing 1X Multiplex PCR Master Mix, 2 µM of each primer, dH_2O, and 20 ng of template DNA (Cortés-Palomec et al., 2008). The thermal cycling conditions consisted of 35 cycles of 94°C for 1 min, annealing for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 10 min. Multiplex PCR products were combined with a GeneScan-500 LIZ size standard and the analyses performed on an ABI-PRISM 3100 Avant sequencer (Applied Biosystems). Fragments were analyzed and recorded using the Peak Scanner program 1.0 (Applied Biosystems).

Genetic diversity

We tested for the presence of null alleles, large-allele dropout, and errors due to stuttering in the microsatellite data using the MICRO-CHECKER 2.2.3 program (Van Oosterhout, Weetman, & Hutchinson, 2006) with 10^2 bootstrap simulations and a 95% confidence interval (CI). Deviations from the Hardy-Weinberg equilibrium, such as excess heterozygosity, or F_{IS} deficit, were tested by means of a Markov-chain approach (with 10^3

Table 1. Locality Name, Sample Size, Geographical Coordinates (Degrees), Mean Number of Effective Alleles per Locus (N_e), Mean Observed Heterozygosity (H_O), Mean Expected Heterozygosity (H_E), and the Inbreeding Index (F_{IS}) for 15 Populations of *L. speciosa* From Cuitzeo Basin, Michoacán.

		Genetic diversity				
Locality	*N*	Coordinates	N_e	H_O	H_E	F_{IS}
1. Zacazonapan (Zacazo)	15	19° 53′ /100° 46′	6.87 (2.29)	0.710 (0.23)	0.717 (0.10)	0.009 (0.05)
2. Indaparapeo (Indapa)	15	19° 44′ /100° 57′	7.25 (2.71)	0.715 (0.25)	0.719 (0.14)	0.006 (0.07)
3. Lagunas (Laguna)	20	19° 35'/101° 23	7.00 (2.92)	0.635 (0.30)	0.751 (0.11)	0.160 (0.09)
4. Potrerillos 2 (Potrer2)	16	19° 30′ / 101° 4′	8.50 (2.92)	0.628 (0.20)	0.787 (0.12)	0.208 (0.05)
5. El Olvido (Olvido)	15	19° 32′ /101° 25′	9.62 (3.37)	0.593 (0.23)	0.815 (0.08)	0.279 (0.07)
6. Caurio de Gpe. (Caurio)	15	19° 37′ / 101°29′	7.62 (1.84)	0.714 (0.23)	0.770 (0.07)	0.074 (0.07)
7. Puente Roble (Robles)	16	19° 43′ /100° 54′	7.35 (3.77)	0.589 (0.19)	0.745 (0.13)	0.215 (0.06)
8. San Felipe (Felipe)	16	19° 42′ /100° 58′	7.62 (2.44)	0.664 (0.29)	0.755 (0.09)	0.124 (0.09)
9. Cerro Aguila (Aguila)	19	19° 36′ 101° 19′	7.00 (2.20)	0.588 (0.29)	0.723 (0.07)	0.191 (0.08)
10. Potrerillos 1 (Potrer1)	12	19° 42′ / 101° 5′	6.87 (2.64)	0.682 (0.24)	0.779 (0.08)	0.130 (0.04)
11. Lagunillas (Laguni)	20	19° 35′ /101° 23′	7.75 (2.91)	0.603 (0.30)	0.710 (0.12)	0.154 (0.09)
12. C. Coapas (Coapas)	16	19° 32′ / 101° 7′	6.25 (2.60)	0.534 (0.36)	0.692 (0.15)	0.234 (0.06)
13. C. Coapas 2 (Coapas2)	16	19° 32′ / 101°17′	6.50 (2.56)	0.536 (0.31)	0.743 (0.13)	0.285 (0.05)
14. Huiramba (Huiram)	16	19° 32′ / 101° 7′	7.12 (3.60)	0.602 (0.28)	0.741 (0.10)	0.194 (0.06)
15. El Correo (Correo)	13	19° 32′ / 101°17′	6.50 (2.20)	0.565 (0.29)	0.757 (0.09)	0.262 (0.07)

Note. Standard errors are included in parenthesis. The abbreviated names of every population are in parenthesis.

dememorization steps, 10^2 batches, 10^3 iterations per batch) using the GENEPOP 4.1 software (Raymond & Rousset, 1995). For each of the 15 populations of *L. speciosa*, we estimated the following genetic diversity parameters: number of alleles per locus (*A*), observed heterozygosity (H_O), and expected heterozygosity (H_E), using the GENETIX 4.02 software (Belkhir, Borsa, Chikhi, Raufaste, & Bonhomme, 2004).

Genetic Structure and Bayesian Admixture Analysis

To have a clear scenario about the genetic assignment, we run two Bayesian approaches, Geneland and Structure, to test if the genetic assignments are the same or differ in the number of genetic cluster detected. The first approach, Structure, was tested to infer the genetic ancestry of each individual plant (Falush, Stephens, & Pritchard, 2003; Hubisz, Falush, Stephens, & Pritchard, 2009; Pritchard, Stephens, & Donnelly, 2000). STRUCTURE 2.3.4 software (Falush et al., 2003; Hubisz et al., 2009; Pritchard et al., 2000) uses a Bayesian clustering model to determine the proportion of each individuals' ancestry originating from different populations (Evanno, Regnaut, & Goudet, 2005). The optimal number (*K*) of groups was determined by varying *K* from 1 to 10 and running the analysis 10 times for each *K* value to find the maximum posterior likelihood (Ln*P* (D)). Each run was performed using 50^4 burn-in periods and 10^6 Markov Chain Monte Carlo (MCMC) repetitions after burn-in. We used an admixture model that allows correlated allele frequencies without any prior information. Following Evanno et al. (2005), we determined the most likely value of *K* based on the maximum value of ΔK as implemented in the Structure Harvester 0.6.9 software (Earl & von Holdt, 2012). In the second approach, we run the spatial cluster model implemented in the GENELAND package (Guillot, Mortier, & Estoup, 2005) of the R 3.2.3 program (R Development Core Team, 2016). Different sets of parameters (MCMC, thinning and burn-in) were used in different test runs, in order to find the optimal parameters. Following the recommendation of the user's manual, the first step was replicated 10 times, allowing K to vary from 1to 10 clusters and using the Markov chain Monte Carlo (MCMC) repetitions were set at 100,000, thinning was set at 100, and the burn-in period was set at 200, to know the effective number of genetic clusters in the data set. Then, we set ten long runs MCMC iterations were performed, with K fixed to the maximum value of the logarithm of posterior probability of the data (PPD), using 5×10^6 MCMC iterations, and the other parameters unchanged. We calculated the mean logarithm of posterior probability of the data (PPD) for each of the 10 runs and selected one with the highest PPD (Guillot et al., 2005; Guillot, Santos, & Estoup, 2008). To ensure that the run was long enough, we obtained 10 different runs and compared the parameter estimates (*K*, individual population membership, maps). The pairwise population genetic differentiation R_{ST} was estimated using the infinite allele model (IAM), performing 10^4 permutations in the

ARLEQUIN 3.5.1.2 software (Excoffier & Lischer, 2010). A hierarchical test of population structure was conducted using the stepwise mutation model (SMM) with analysis of molecular variance (AMOVA) in ARLEQUIN 3.5 (Excoffier & Lischer, 2010). We conducted a hierarchical analysis to estimate genetic variance components between groups, between populations within groups, and within populations, using the assignation of groups obtained by Structure and Geneland. Statistical significance was tested using 10^4 permutations. The genetic exchange between *L. speciosa* populations was assessed by estimating the Bayesian-scaled long-term effective population size (*Ne*) and migration rate (m) with the MIGRATE 3.5.1 software (Beerli & Felsenstein, 2001). For all the analyses, the starting value of the chain was set to 20^6 visited and 16 recorded genealogies, following a burn-in period of 50^3 iterations.

To identify geographic and genetic breaks between *L. speciosa* populations, we used the Monmonier's maximum difference algorithm with the BARRIER version 2.2 software (Manni, Guerard, & Heyer, 2004). BARRIER creates a map of the sampling locations from their geographical coordinates. Barriers are then represented on the map by identifying the maximum values within the population-pairwise genetic distance matrix. We used a pairwise matrix of average square distances (Goldstein, Ruiz-Linares, Cavalli-Sforza, & Feldman, 1995; Slatkin, 1995) estimated for the 15 populations of *L. speciosa*. Resampling random subsets of individuals within populations with the MSA program (Dieringer & Schotterer, 2003) provided 100 bootstrap replicate distances that were used to achieve statistical significance for the predicted barriers.

Changes in population size

We used the BOTTLENECK 1.2 software (Piry, Luikart, & Cornuet, 1999) to detect population bottlenecks, using the three genetic groups previously identified with Structure. Recent bottlenecks can be identified as a population where rare alleles are the first to be lost, then the mean number of alleles per locus is reduced accordingly. In contrast, heterozygosity is less affected, producing a transient excess in heterozygosity relative to that expected based on the resulting number of alleles (Cornuet & Luikart, 1996; Luikart & Cornuet, 1998). For this test, we used 90% stepwise and 10% multistep mutations and performed 10^4 iterations of the Wilcoxon's signed-rank test with the SMM, the IAM, and the two-phase mutation (TPM) model.

Additionally, we estimated the effective size of 15 populations of *L. speciosa* using the LDNe software (Waples & Do, 2008). This program implements the bias-correction method developed by Waples (2006) to obtain Ne from a sample of S individuals. We set Pcrit = 0.02 (i.e., alleles with a frequency <0.02 are excluded), which generally provides a good balance between accuracy and bias (Waples & Do, 2008). CIs for Ne were calculated with the chi-square approximation implemented in LDNe (Waples & Do, 2008).

Results

Genetic Diversity

No evidence of null alleles was found in any of the sample-loci combinations. The tests for errors due to stuttering and large-allele dropout yielded negative results in all cases. Values of the genetic diversity parameters estimated in 15 populations of *L. speciosa* ranged from moderated to high in Zacazo, Indapa, and Caurio ($Ne = 6.87$–9.62, $H_O = 0.635$–0.714); Laguna, Laguni, Potrer2, Felipe, Potrer1, and Huiram ($Ne = 6.87$–7.62, $H_O = 0.602$–0.682); and in Olvido, Robles, Aguila, Coapas, Coapas2, and Correo ($Ne = 6.25$–7.0, $H_O = 0.534$–0.589), see Table 1. Wright's inbreeding coefficient within populations (F_{IS}) showed positive values in all cases, denoting a heterozygote deficiency (Table 1); F_{IS} values were low in Zacazo, Indapa, and Caurio ($F_{IS} = 0.009$–0.279); slightly high in Laguna, Laguni, Potrer2, Felipe, Potrer 1, and Huiram ($F_{IS} = 0.124$–0.215); and high in Olvido, Robles, Aguila, Coapas, Coapas2, and Correo ($F_{IS} = 0.154$–0.285). The gene exchange levels detected were moderate to high in all the populations examined (Table 2).

Genetic Structure and Bayesian Admixture Analysis

We applied two complementary Bayesian clustering algorithms, namely Structure (Falush et al., 2003; Pritchard et al., 2000) and Geneland (Guillot et al., 2005), to infer population structure (i.e., a number of clusters, *K*) and to assign individuals probabilistically to populations based on individual multilocus. Both approaches shown similar results over 10 replicated runs tested varying *K* from 1 to 10 to get the highest probability. In terms of the number of genetic clusters, both approaches, according to the maximum posterior likelihood (LnP (D)) and the maximum ΔK value, showed that $K = 3$ is the optimum number of genetic groups, see Figure 2. Also, Structure and Geneland approaches confirm in the majority of the assignment of each individual to each genetic cluster (Figures 3, 4, and 5). For instance, in the bar plot and the pie charts in Figures 3, 4, and 5 show the distribution of ancestry proportions in each collection site. Cluster 1 (e.g., red in Structure and cluster 1 Geneland) is widespread across the Cuitzeo basin and includes the Zacazo, Indapa, Laguna, Potrer2, Olvido, and Caurio populations, except for Laguna that shift for Laguni in Geneland. Cluster 2 (e.g., green in Structure and cluster 3 Geneland) includes the populations of Robles, Felipe,

Table 2. Levels of Genetic Exchange Estimated With the Program MIGRATE.

Nm	Zacazo	Indapa	Laguna	Potrer2	Olvido	Caurio	Robles	Felipe	Aguila	Potrer1	Laguni	Coapas	Coapas2	Huiram	Correo
Zacazo	–	1.030	1.084	1.084	0.749	0.948	1.638	1.129	0.460	0.596	0.460	1.220	0.596	1.302	1.292
Indapa		–	1.114	1.059	0.965	0.716	1.007	0.919	0.504	1.301	0.996	1.044	0.610	1.546	1.271
Laguna			–	0.906	1.422	1.483	0.579	0.347	1.576	0.301	1.020	1.228	1.298	0.896	1.111
Potrer2				–	1.272	0.683	1.222	1.492	0.927	0.611	0.953	0.489	0.880	1.174	0.871
Olvido					–	0.640	1.415	0.893	0.926	0.745	1.018	0.856	0.951	0.855	1.257
Caurio						–	0.840	0.896	0.679	0.714	0.921	1.464	0.569	0.759	1.456
Robles							–	0.741	1.036	0.765	0.609	0.683	1.080	0.743	0.742
Felipe								–	1.747	1.193	1.341	0.436	1.183	1.310	1.136
Aguila									–	1.022	0.992	0.992	1.561	0.572	0.210
Potrer1										–	0.896	0.944	0.895	1.044	1.276
Laguni											–	1.336	0.681	0.632	1.451
Coapas												–	0.601	1.307	1.700
Coapas2													–	1.282	1.549
Huiram														–	0.522
Correo															–

Note. Directional pairwise migration rates among 15 *L. speciosa* populations from Cuitzeo basin, Michoacán. Donating and receiving populations are below and above the diagonal, respectively. Migration rates are given as the value of effective number of migrants per generation (*Nm*).

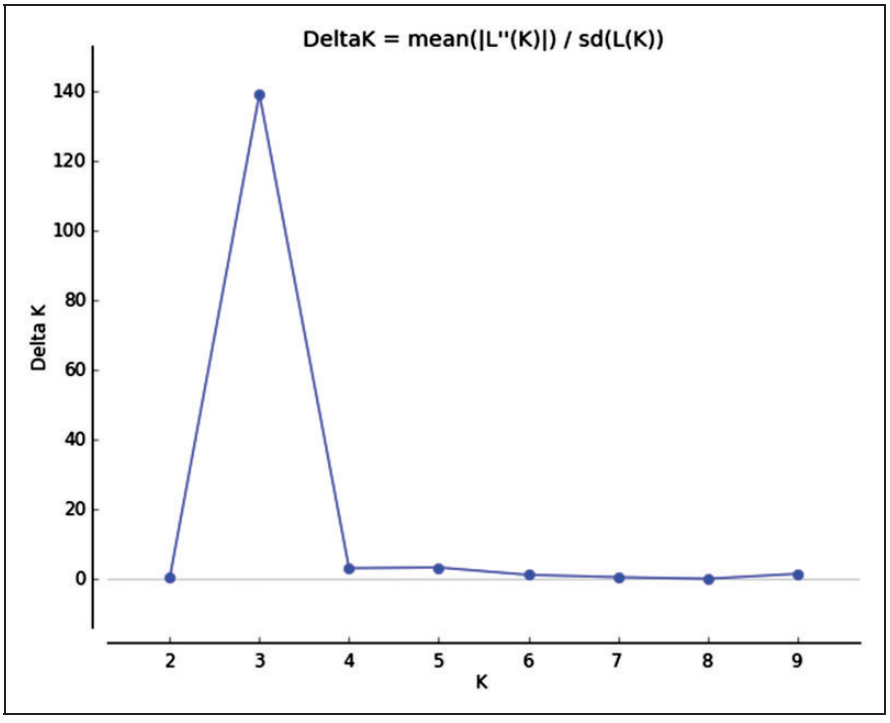

Figure 2. Estimated number of populations from Structure and Geneland analyses. Structure values of *K* plotted against *K*, the peak indicates the most probable number of genetic groups given the data using Structure Harvester program. Geneland plot of the number of populations simulated from the posterior distribution obtained with GENELAND.

Aguila, and Potrer1, which are consistently structured across the landscape. Cluster 3 (e.g., blue in Structure and cluster 2 in Geneland) has a wider distribution and includes the populations of Correo, Coapas, Coapas2, Laguni, and Huiram (Figures 3, 4, and 5) except for Laguni that was changed for Laguna in Geneland. Geneland and Structure also differs in the presence of admixture (not noticeable by Geneland), as an evidence

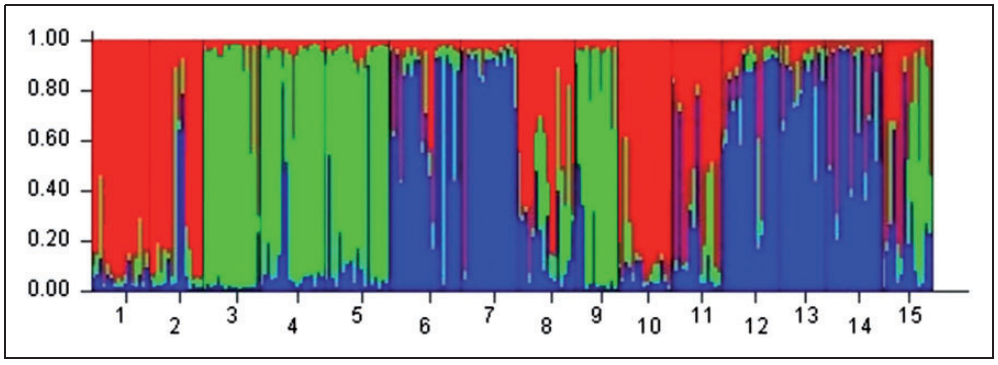

Figure 3. Genetic assignment of individuals and populations according to the Bayesian method implemented in the program STRUCTURE. Each thin horizontal line represents an individual and the proportion of each color is the proportion of ancestry derived from each of the three main genetic groups (K 3) inferred. Green, red, and blue genotypes are representing the genetic ancestry groups corresponding to *L. speciosa* populations. Populations are separated by black lines.

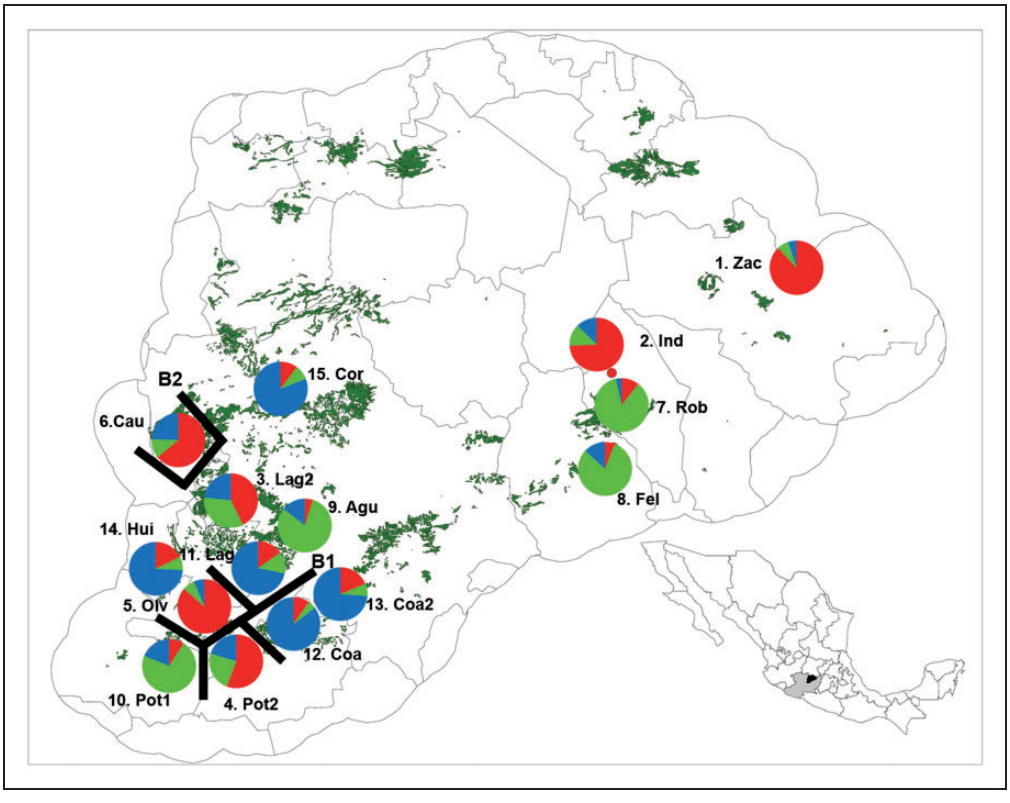

Figure 4. Each pie chart represents the proportions in each population of the three genetic groups as assigned by the program STRUCTURE. Green, red, and blue genotypes are representing the genetic ancestry groups corresponding to *L. speciosa* populations. Genetic discontinuities (bold lines B-1 and B2) obtained with Monmonier's maximum difference algorithm on genetic distances derived from microsatellite allele frequencies.

of gene exchange, particularly in the populations of Indapa, Caurio, Laguna, Potrer1, and Potrer2, which have ancestry coefficient (Q) values ranging between 0.732 and 0.848 (Figures 3 and 4).

The AMOVA was performed using both genetic assignation from Structure and Geneland. Results showed that most of the variation (95.7 and 95.6%) occurred within *L. speciosa* populations; the variation between groups accounted for 3.29% and 3.51%, and the variation between populations within groups accounted for 1.02% and 0.87% (Table 3). Similarly, there was a moderate pairwise genetic differentiation between some

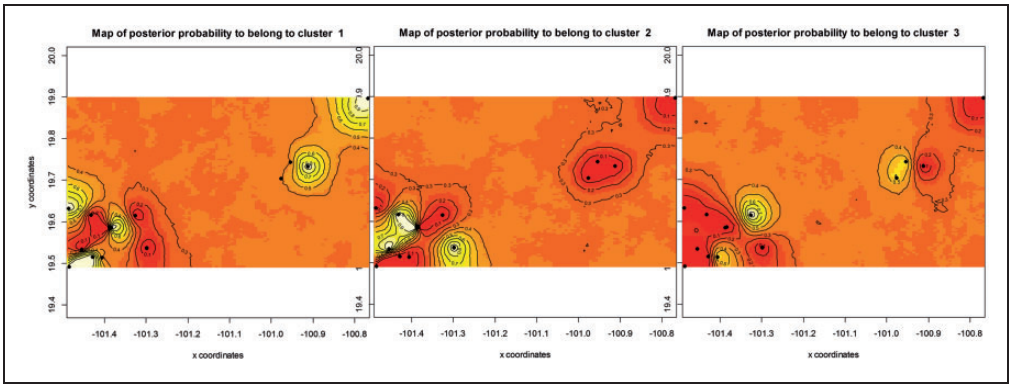

Figure 5. Maps of Geneland individual assignments to clusters for $K = 3$ (scale units in latitude, longitude). The three plots represent the assignment of pixels to each cluster: cluster 1, cluster 2, and cluster 3. The highest membership values are in light yellow and the level curves illustrate the spatial changes in assignment values. The plot is based on the highest probability run at that value of *K*.

Table 3. Analysis of Molecular Variance (AMOVA) Performed on the nSSR Data and Using R_{ST} for the Three Group Genetic Clusters Obtained by Means of STRUCTURE and by GENELAND for Populations of *L. speciosa*.

	Source of variation	SS	Variance components	Percentage of variation	Fixation index
R_{ST}					
	Among groups	4772.52	12.286	3.29	$\Phi_{CT} = 0.032^{***}$
	Among populations within groups	5717.44	3.8067	1.02	$\Phi_{SC} = 0.010^{***}$
	Within populations	162782	357.763	95.70	$\Phi_{ST} = 0.043^{***}$
	Total	173272.1	373.856		
R_{ST}					
	Among groups	4976.41	13.125	3.51	$\Phi_{CT} = 0.035^{***}$
	Among populations within groups	5513.55	3.258	0.87	$\Phi_{SC} = 0.009^{***}$
	Within populations	162782	357.763	95.62	$\Phi_{ST} = 0.043^{***}$
	Total	173272.1	374.146		

Note. Tests were based on 10^4 random permutations.
***statistically significant values ($p < .01$).

populations, as indicated by the R_{ST} values observed (Table 4): Aguila/Olvido ($R_{ST} = 0.149$), Coapas/Aguila ($R_{ST} = 0.143$), Caurio/Coapas2 ($R_{ST} = 0.082$), Laguna/Olvido ($R_{ST} = 0.107$), and Caurio/Correo ($R_{ST} = 0.082$). Finally, a low differentiation was found between the following population pairs: Indapa/Caurio ($R_{ST} = 0.006$), Zacazo/Coapas ($R_{ST} = 0.014$), and Laguni/Felipe ($R_{ST} = 0.028$), see Table 4.

The analysis performed using 100 bootstrap replicates of the average square distance matrices revealed two barriers (with over 50% bootstrap support) between the 15 populations of *L. speciosa* studied (Figure 4). The most significant barrier, with 95% bootstrap support, is a complex break separating the southwestern part from the central part of the Cuitzeo basin. This indicates that, despite the admixture revealed by the Structure analysis, some populations have become isolated such as the Potrer1, Potrer2, Olvido, Coapas, Coapas 2, Laguni, Laguna, and Aguila (Figure 4). The second barrier, with 89% bootstrap support, sets apart the Caurio population (located in the northwest part of the basin) from the rest of the *L. speciosa* populations.

Changes in population size

Results from the analyses to test for evidence of recent bottlenecks (excess heterozygosity) using the IAM, the TPM, and the SMM are shown in Table 5. No significant results were found with the IAM and TPM. However, the SMM showed significant ($p =$ .001, .003, .001) evidence of mild bottlenecks in populations of the three genetic groups, particularly in loci Lspe 4, 6, and 14, and also

Table 4. Pairwise Population Genetic Differentiations Using the Matrix of Slatkin Linearized R_{ST} (SMM) Among Localities of *L. speciosa* in Cuitzeo Basin, Michoacán, Over 10^4 Replicates in the Arlequin 3.5 Software (Schneider et al., 2005).

R_{ST}	Zacazo	Indapa	Laguna	Potrer2	Olvido	Caurio	Robles	Felipe	Aguila	Potrer1	Laguni	Coapas	Coapas2	Huiram	Correo
Zacazo	–														
Indapa	0	–													
Laguna	0	0.002	–												
Potrer2	0	0	0	–											
Olvido	0.013	0	0.019	0.017	–										
Caurio	0.019	0.006	0.058	0.033	0.050	–									
Robles	0.053	0.044	0.045	0.036	0.080	0.015	–								
Felipe	0.048	0.053	0.028	0.024	0.067	0.028	0	–							
Aguila	0.118	0.113	0.098	0.086	0.149	0.049	0.009	0	–						
Potrer1	0.004	0.016	0.002	0.005	0.034	0.028	0.012	0	0.001	–					
Laguni	0.068	0.040	0.032	0.030	0.107	0.050	0	0	0.007	0	–				
Coapas	0.014	0.082	0	0.001	0.014	0.141	0.097	0.081	0.143	0.046	0.079	–			
Coapas2	0.007	0.033	0	0.005	0.006	0.082	0.091	0.071	0.142	0.043	0.083	0	0		
Huiram	0	0	0	0	0.025	0.024	0.023	0.011	0.063	0.002	0.009	0	0.002	–	
Correo	0	0.023	0	0	0.007	0.082	0.090	0.078	0.131	0	0.059	0	0	0	–

Table 5. Bottleneck Analysis for *L. speciosa* Populations in the Cuitzeo Basin Using Wilcoxon Rank Test Under Infinite Allele, Stepwise Mutation, and Two-Phase Model.

Models	Group 1	Group 2	Group 3
IAM	0.524	0.628	0.371
TPM	0.310	0.875	0.962
SMM	0.001***	0.003***	0.001***
LDNe	122.1	349.2	414.2

Note. Parameters for TPM: variance = 10%, proportion of SMM = 90%, estimation based on 10^4 replications. P = probability; IAM = infinite allele model; TPM = two phase model; SMM = stepwise mutation model. Also, we include the results obtained for the estimation of the population effective size for the three genetic groups, values obtained with the program LDNe.

** and *** indicate significant deviation from equilibrium as value less than 0.05 and 0.01.

significant evidence of excess heterozygosity in the *L. speciosa* populations suggesting that they have recently gone through a bottleneck. The estimates of effective population size (N_e) obtained using LDNe for the *L. speciosa* populations showed that the third genetic group had the highest value ($N_e = 441$ individuals), followed by the second ($N_e = 349$) and the first ($N_e = 122$) groups. All N_e estimates had a high Jackknife support and a good CI (Table 5).

Discussion

Genetic Diversity

Habitat fragmentation, coupled with the massive extraction of individuals from natural populations, has reduced the genetic diversity and population size of these plants, with the ensuing reduction in population connectivity (Honnay et al., 2005; Jump & Peñuelas, 2006). In this study, we examined the genetic structure of populations of *L. speciosa*, an orchid species that is threatened by the indiscriminate extraction of specimens for trading in local markets. Surprisingly, the *L. speciosa* populations examined showed moderate to high levels of genetic diversity, as determined from nuclear microsatellites. Recent studies based on microsatellite variation have also found high genetic diversity (H_O: 0.0–0.728) in the rare orchid *Isotria medeoloides* (Stone et al., 2012), the critically endangered spider orchid *Caladenia huegelii* (H_O: 0.587–0.766; Swarts et al., 2009), and in two *Ophrys* species (H_O: 0.76–0.91 to 0.77–0.92; Mant, Peakall, & Schiestl, 2005; Soliva & Widmer, 2003). Most of these species share similar characteristics with *L. speciosa*, such as being epiphytic, long-lived, cross-pollinated, and long-distance seed dispersal. Furthermore, widespread species such as *L. speciosa* tend to exhibit a higher genetic diversity compared with threatened or endemic species (Li & Jin, 2007; Mathiasen, Rovere, & Premoll, 2007; Qian, Wang, & Tian, 2013; Yu, Yang, Sun, & Liu, 2011). This may be the result of historical patterns of genetic variation in *L. speciosa*, for example, from a formerly wide-ranging distribution to becoming rare only recently. These patterns suggest that the massive extraction of individuals of this species has not yet had a noticeable effect on gene frequencies and heterozygosity or in the alleles that cause genetic drift in populations (Aguilar, Quesada, Ashworth, Herrerías-Diego, & Lobo, 2008; Schaberg et al., 2008). Similarly, Wright's inbreeding coefficients within populations (F_{IS}) were positive in all cases, denoting heterozygote deficiency (see Table 1). Fragmentation may also lead to inbreeding due to either increased self-fertilization or

mating between related individuals within remnant fragments (Chung et al., 2014; Honnay et al., 2005; Mathiasen et al., 2007; Swarts & Dixon, 2009). In this case, inbreeding seems to have been due to the activity of pollinators, as they were reported visiting nearby forest fragments more often than distant ones (Bacles, Lowe, & Ennos, 2004; Smith-Ramírez & Armesto, 2003). The ecological features of *L. speciosa* could also contribute to an increased gene flow via pollinators or long-distance dispersal between populations and suggest that isolated trees might serve as stepping-stones for gene flow between populations (Barrett & Kohn, 1991; Herrera-Arroyo et al., 2013; Soliva & Widmer, 2003; Tremblay & Ackerman, 2001).

Some of the populations of *L. speciosa* exhibited evidence of having recently gone through a mild-bottleneck, but only with the SMM model. Based on the well-documented history of *L. speciosa* in Michoacán (Miranda, 1997), we hypothesized that these populations have been subject to massive extraction and habitat fragmentation for at least 100 years. Studies on conservation genetics have put forward a rule to define the effective population size necessary to prevent genetic damage (Barrett & Kohn, 1991). An effective population size of 50 is considered the minimum necessary to maintain sufficient genetic variability, while 500 individuals are required to offset effective drift (Barrett & Kohn, 1991; Brzosko, Wróblewska, & Talalaj, 2004; Honnay, et al., 2005; Jacquemyn et al., 2012; Tremblay & Ackerman, 2001). Despite the massive decline in abundance caused by drastic environmental change, habitat destruction, and illegal collection, the populations of *L. speciosa* still have moderate to high values of effective population size. The wide distribution range and the cross-pollinated syndrome of *L. speciosa* seem to have been partly responsible for its high genetic diversity and moderate-to-high effective population size.

Genetic Structure and Bayesian Admixture Analysis

The AMOVA showed that most of the variation in *L. speciosa* occurs within populations. This is consistent with the results obtained by Da Cruz et al. (2011); based on the genetic structure of plants, Li and Ge (2006) suggested that cross-pollinated plants tend to have a greater diversity within populations and very little between them.

The distribution of ancestry proportions clearly showed a substantial connectivity between populations across the Cuitzeo basin. Nevertheless, we identified breaks in the continuity of gene flow such as the first barrier, which separates populations located in the southwestern from those in the central part of the Cuitzeo basin. We also observed some populations that have become isolated by a complex barrier; this was also evident in the map of oak forests remaining in this region (Figure 4). Geographical restrictions and a low gene flow between populations were also indicated by the second barrier that separate populations located in the northwestern from those in the central part of the basin. This pattern reflects the effect of habitat fragmentation on the patchy distribution of populations across the Cuitzeo basin. Pine and oak trees have almost disappeared from some localities of this basin due to their value as timber for construction or as a source of charcoal, an important economic activity in the region (Aguilar, Guilardi, Vega, Skutsch, & Oyama, 2013).

Small forest fragments that might still remain between larger populations would possibly function as vegetation corridors where isolated trees could function as stepping stones for gene flow between populations, as is the case in *Quercus castanea* in the same basin (Herrera-Arroyo et al., 2013). The history of *L. speciosa* in the Cuitzeo basin points to the fact that both fragmented and continuous populations have been recently subject to moderate reductions in effective population size, which have been large enough to affect allelic diversity (Barrett & Kohn, 1991; Lowe et al., 2005). Such reductions may have been caused by episodic environmental fluctuations (e.g., drought or geographical restrictions) or resulted from the recent founding of some of the populations (Chung et al., 2014; Forrest, Hollingsworth, Hollingsworth, Sydes, & Bateman, 2004; Murren, 2003). One likely explanation could be the effect of historic long-distance dispersal, which can maintain diversity through colonization. Thus, diversity is maintained at the regional rather than at the population level, with a moderate differentiation between populations (Bialozyt, Ziegenhagen, & Petit, 2006; Trapnell & Hamrick, 2004). Exponential population growth in newly colonized sites can also preserve genetic diversity near the leading edge, as rare alleles are less likely to be lost to drift (Excoffier, Foll, & Petit, 2009). Loveless and Hamrick (1984) suggest that genetic differentiation is the result of the ability of the species to disperse pollen and seeds; this holds true in orchids as they are wind dispersed (Chung et al., 2007; Gustafsson & Sjögren-Gulve, 2002; Li & Ge, 2006) and are capable of long-distance dispersal (Ackerman & Ward, 1999; Arditti & Ghani, 2000; Neiland & Wilcock, 1998). Therefore, it is quite possible for orchids to disperse across considerable distances of up to several kilometers (Flores-Palacios & García-Franco, 2003; Sharma, Clements, & Jones, 2000; Trapnell & Hamrick, 2004). Furthermore, *Bombus pennsylvanicus, B. sonorus*, and *B. ephippiatus* have been documented as potential pollinators of *L. speciosa* (Van der Pijl & Dodson, 1996). Other studies have concluded that, in orchids, population genetic structure is strongly influenced by the behavior of pollinators (Chung et al., 2014; Cozzolino & Widmer, 2005; Neiland & Wilcock, 1998; Qian et al., 2013; Sharma, Jones, & French, 2003; Tremblay, Ackerman, Zimmerman, & Calvo, 2005).

Implications for Conservation

The life-history characteristics and growth form of *L. speciosa*, such as its epiphytic habit, allow it to occupy a three-dimensional space on the branches, trunks, and canopy of trees, whereas terrestrial herbs can only occupy a two-dimensional setting. As a consequence, epiphytes enjoy some of the dispersal advantages of trees (e.g., greater potential for gene flow derived from longer-distance dispersal of pollen and seeds; Borba et al., 2001; Flores-Palacios & García-Franco, 2003; Trapnell & Hamrick, 2004), which may attenuate the genetic impacts of habitat fragmentation. Because of this, the effects of the extensive extraction that *L. speciosa* populations are currently facing will become evident in subsequent generations. We found evidence of a moderate effect on the inbreeding levels, the recent bottlenecks detected in the three groups, and the effective population size of isolated populations. The ultimate goal of conservation is to ensure the continued survival of populations and the maintenance of their evolutionary potential by preserving natural levels of genetic diversity (Godt & Hamrick, 1995; Izawa et al., 2007). Great efforts should be made to preserve extant populations of *L. speciosa* and its habitats to prevent further population reductions and preserve its overall genetic basis (Barrett & Kohn, 1991; Chung et al., 2014; Jacquemyn et al., 2012). Collection of this orchid should be banned and robust legal protection measures should be enforced through local authorities. It might be necessary to transfer mature plants between populations as an effective way to increase genetic diversity (Qian et al., 2013; Swarts et al., 2009). We also recommend the maintenance of a germplasm bank for ex-situ conservation of *L. speciosa* (Izawa et al., 2007; Li & Ge, 2006) and its extensive cultivation for future cultural and religious uses by people in local communities.

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