

Effect of habitat fragmentation on genetic structure of terrestrial orchid *Cymbidium faberi* in the Qinling Mountains

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Abstract

Species dispersal patterns and population genetic structure can be influenced by large geographical features and habitat fragmentation. The Qinling Mountains are a major east-west mountain range and they are also the northernmost habitat of wild *Cymbidium faberi* in China. However, the impact of the Qinling Mountains and habitat fragmentation in the areas on genetic variation of *C. faberi* at population level is still poorly understood. Here, genetic analysis of *C. faberi* in the Qinling Mountains was conducted based on two chloroplast DNA sequences of 271 samples in 15 locations. Hierarchical analyses of molecular variance (AMOVA) and mantel test indicated that most of the genetic variance was within populations, genetic distance between populations was correlated with the geographical distance but not strong (mantel $r = 0.505$, $P = 0.011$). Spatial analysis of molecular variance (SAMOVA) indicated that the FCT reached the maximum value at $K = 2$ and then decreased, which supported a two-group genetic structure. Furthermore, the Extended Bayesian Skyline Plot revealed that the estimates of effective population size of *C. faberi* were under demographic equilibrium in the past but an apparent decline going from approximately 1 Ma towards the present. Moreover, we found that the genetic diversity of *C. faberi* in fragmented landscape was lower compared to continuous ones. Therefore, we concluded that the habitat fragmentation has restricted the gene flow of *C. faberi* by disrupting seed dispersal. Our findings may provide helpful references for understanding how humans shape the genetic structure and the importance of conserving wild orchids.

KEYWORDS

Cymbidium faberi, the Qinling Mountains, habitat fragmentation, genetic structure, chloroplast DNA sequences

1 | INTRODUCTION

The Qinling Mountains are a natural boundary between China's north and south stretching over 1,600 kilometers in east-west direction, which formed approximately during the Mesozoic and Cenozoic (Zhang, Cawood, Dong, & Wang, 2019). Due to most of the mountains are higher than 2000 meters and diversified topography, the mountains form an important boundary and are home to a diverse variety of wildlife, many of which are rare on earth. Recent phylogeographic and population genetic studies have showed that the Qinling Mountains probably served as a barrier for wild plants and animals, such as *Paeonia* (Yuan, Cheng, & Zhou, 2012; Xu et al., 2019), *Sinopodophyllum hexandrum* (Liu, Yin, Liu, & Li, 2014), *Actinidia chinensis* (Wang, Liao, & Li, 2018), and reptile (Yan, Wang, Chang, Ji, & Zhou, 2010; Huang et al., 2017). However, contrary to these studies, some other results suggested that the Qinling Mountains have no obvious impact on genetic structure (Zhan, Li, & Fu, 2009). This discrepancy may depend on the mechanisms by which the species survive and disperse, as well as how they respond to the surrounding environment.

Over the past few decades, habitat fragmentation occurred in the Qinling Mountains due to human's large-scale forest logging, building of roads and urbanization. The habitat fragmentation has resulted in low genetic

diversity and population structure not only for plants but also for other species, including golden snub-nosed monkey and giant panda (Huang et al., 2016; Ma et al., 2018). For most plants, habitat fragmentation often reduces gene flow and genetic diversity by disrupting the movement of seed (Sebbenn et al., 2011; Lander, Harris, Cremona, & Boshier, 2019; Ony et al., 2020). It was reported that seed dispersal play important roles in determining genetic variation patterns in fragmented landscapes (Browne & Karubian, 2018).

Cymbidium faberi is a terrestrial orchid enlisted in Appendix of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). It is a species with a distribution from Nepal, Northeast India to south of China, north to the Qinling Mountains (Flora of China Editorial Committee, 2009). It grows on rocky and scrubby slopes, usually in *Quercus variabilis* forests at altitudes of 700 – 3000 m. Like many other *cymbidium* species, *C. faberi* is a self-compatible allogamous species that depends on insect pollinators such as honeybees for seed production (Suetsugu, 2015). The dust-like seeds are often produced in huge numbers, and they usually lack endosperm and the embryo is undifferentiated. The empty space inside seeds and the trichomes on the endocarp make them well suited for wind dispersal (Arditti & Ghani, 2000; Gamarra, Ortúñez, Cela, & Merencio, 2018). Thus, topographic features such as the Andes seem not to have been much of a barrier to the dispersal of lowland epiphytic orchids *Cycnoches* (Perez-Escobar et al., 2017). However, a recent study of an epiphytic orchid found a seed dispersal barrier between northwestern and southeastern populations within Costa Rica (Trapnell et al., 2019).

To date, whether geological barriers such as the Qinling Mountains and habitat fragmentation in the areas have an impact on *C. faberi* remains unknown. Here, we analyzed 271 samples based on maternally inherited two chloroplast DNA sequences to evaluate the impact of Qinling Mountains and habitat fragmentation on genetic structure of *C. faberi*. The aims of this paper are: (1) to assess the level of genetic variation and spatial genetic structure of *C. faberi* in the Qinling Mountains; (2) to explore the demographic history of *C. faberi* in the Qinling Mountains; and (3) to try to explain the genetic structure based on species seeds dispersal ability and human activity that have influenced it.

2 | MATERIALS AND METHODS

2.1 | Sampling and DNA extraction

A total of 271 samples were collected from 15 sites in the Qinling Mountains of China (Figure 1). Of these locations, SZA and SYX are located in fragmented landscape, which are surrounded by roads, railway line and villages. Moreover, *Cymbidium goeringii*, *Cymbidium ensifolium*, *Cymbidium kanran* and *Cymbidium sinense* were collected as outgroups. DNA was extracted using Tiangen Extraction Kit (Beijing, China).

2.2 | Amplification of cpDNA and sequencing

PsbA–trnH and rpl14–rpl36 (Sang, Crawford, & Stuessy, 1997) selected from thirteen cpDNA primers were amplified by PCR. PCR were conducted under the following conditions: 94°C (5 min), and 35 cycles at 94°C (30 s) / 56°C (30 s) / 72°C (90 s), and then 72°C for 10 min. PCR was performed in a reaction volume of 20 µL containing 2µL of Taq buffer, 20ng of genomic DNA, 1 unit of Taq polymerase, 0.2 µmol/L of each primer and 0.2 mmol/L of each dNTPs. PCR products were checked on 1.5% agarose gels and sequenced at BGI (Beijing, China). Sequences were aligned using CLUSTAL X software (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997).

2.3 | Genetic diversity and population structure

Haplotype diversity (Hd) and nucleotide diversity (Pi) for each populations were calculated by DnaSP (Rozas & Rozas, 1995). The genealogical topology of haplotypes was constructed using Network software. F_{ST} values were calculated in Arlequin ver3.5 using conventional F-statistics (Excoffier & Lischer, 2010), the correlation between F_{ST} and geographical distance was examined by Mantel test in the “ecodist” R package (Goslee & Urban, 2007). Moreover, the spatial analysis of molecular variance was performed with Tamura molecular distance using SAMOVA version 2.0 (Dupanloup, Schneider, & Excoffier, 2002) combined with Arlequin. The group number ranged from 2 to 14, and the number of steps in each process was 100000. STRUCTURE bar plots were generated by DISTRUCT (Rosenberg, 2003).

2.4 | Demographic history and divergence time of haplotypes

Before investigating the change of *C. faberi* in population size, we estimated the divergence time of haplotypes firstly. About the origin of the Orchidaceae and *Cymbidium*, previous analysis suggested that the ancestor of orchids lived in 76 – 84 million years ago (Ma), and diversification in *cymbidium* is estimated to have begun 33.9 – 50 Ma (Ramírez, Gravendeel, Singer, Marshall, & Pierce, 2007); other analysis suggested that major diversification of the largest orchid subfamilies perhaps occurring during the cooler period at the end of the Eocene and into the Oligocene, an age for *cymbidium* clade is 28 or 31Ma (Gustafsson, Verola, & Antonelli, 2010); Givnish et al. (2016) suggested that Orchidaceae have arisen in Australia 112 Ma, and *Cymbidium* originated about 10 – 20 Ma. Ultimately, 33.9 – 50 Ma was chosen as the originated time of *cymbidium*.

The divergence time of *C. faberi* in the Qingling Mountains was estimated using BEAST Version 2.3.2 (Bouckaert et al., 2014); nucleotide substitution model (TVM+I+G) was chosen as the best fit to our data sets by AIC in Modeltest (Posada & Crandall, 1998) combined with PAUP* Version 4.0b10 (Swofford, 2002). Because the substitutions were expected to be constant and the rate of molecular evolution in the plastome is notably high in Orchidaceae, strict clock and a rate of 2.1×10^{-9} substitutions per site per year were implemented to calibrate the tree (Gaut, 1998). Markov chain Monte Carlo (MCMC) chain was run 100 million generations, then we combined the three independent runs using LogCombiner and check the effective sampling size values by Tracer; the first 25% of trees were discarded as burn-in using TreeAnnotator and the maximum credibility tree was generated by FigTree. Finally, we generated an Extended Bayesian Skyline Plot using BEAST.

3 | RESULTS

3.1 | Genetic diversity

The alignment of psbA-trnH and rpl14-rpl36 sequences was 1344 bp in length, with 49 haplotypes identified by 133 nucleotide substitution sites or indels. All haplotypes were deposited in GenBank (accession numbers: MT345628–MT345676). The haplotype geographical distribution of *C. faberi* in the Qinling Mountains was presented in Figure 1.

For all samples, estimates of haplotype diversity H_d ranged from 0.500 (SXX) to 0.952 (LSZ) with an average of 0.813, the lowest variance of haplotype diversity 0.500 and 0.661 were found in SXX and SZX; nucleotide diversity π varied from 0 to 3.80×10^{-3} with an average of 1.81×10^{-3} (Table S1), whereas nucleotide diversity values of SXX and SZX were moderate.

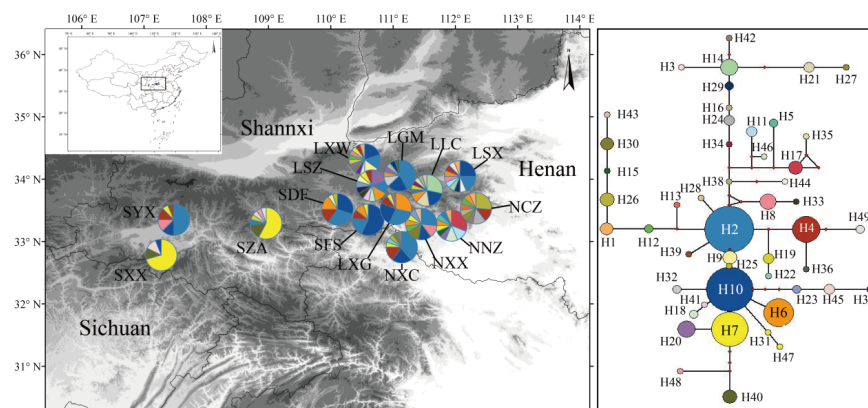


FIGURE 1 Sampling locations and haplotype geographical distribution of *Cymbidium faberi* in the Qinling Mountains. Haplotypes are represented in different colors. The area of circles in network corresponds to the

frequency of each haplotype

3.2 | Genetic differentiation and mantel test

Pairwise estimates of F_{ST} among the eastern populations varied from 0.0015 (between LSX and NXC) to 0.2152 (between SFS and NNZ) with an average of 0.0558, indicating low to moderate genetic differentiation among the eastern populations. The pairwise F_{ST} between SXX and eastern populations was generally higher, ranging from 0.2398 (between SXX and LXW) to 0.3809 (between SXX and SFS). The pairwise F_{ST} between SZA and eastern populations varied from 0.1597 (between SZA and LSZ) to 0.3203 (between SZA and SFS). There existed relatively high genetic differentiation between SXX, SZA populations and other populations. However, the average genetic differentiation between SYX located in middle section and populations located in eastern section was low ($F_{ST} = 0.0665$) (Figure 2).

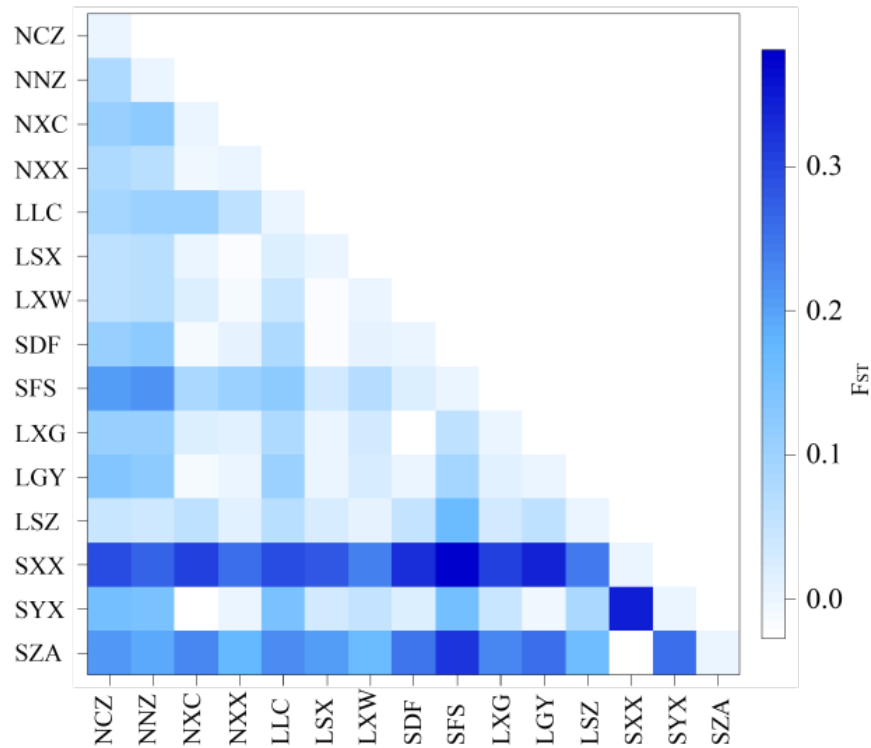


FIGURE 2 Pairwise F_{ST} among populations of *Cymbidium faberi* in the Qinling Mountains based on cpDNA

A mantel test of F_{ST} against geographical distance (in kilometers) was presented in Figure 3. A moderate but significant positive correlation between two variables was detected among populations of *C. faberi* (mantel $r = 0.505$, $P = 0.011$), which indicated that genetic distance was correlated with the geographical distance between populations but not strong.

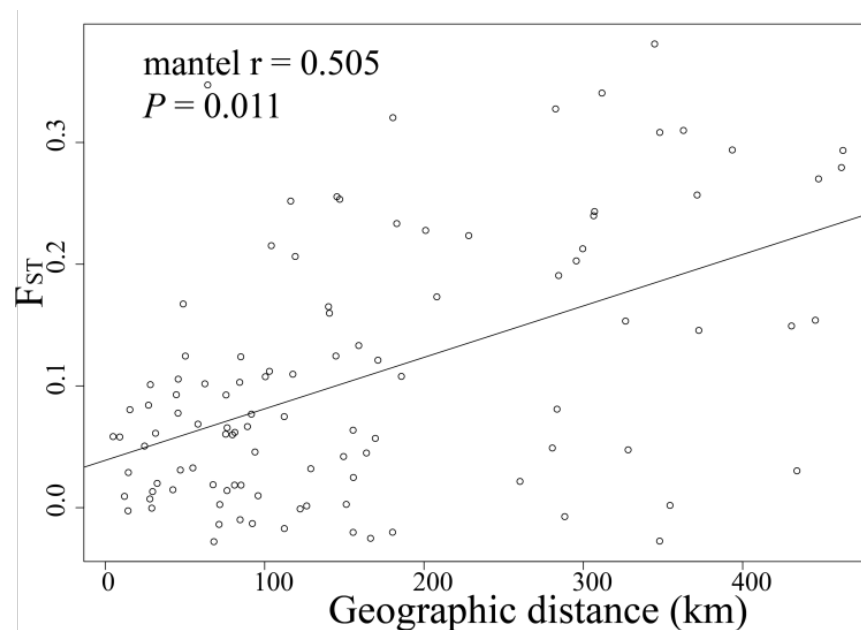


FIGURE 3 Mantel test between geographical distance (in kilometers) and genetic distance of *Cymbidium faberi* in the Qinling Mountains based on cpDNA

3.3 | Population genetic structure

AMOVA indicated that only 19.99% of the variation was observed between SXX, SZA and the middle-eastern populations (Table 1), whereas a high percentage of variation (75.47%, $P < 0.01$) was found within populations.

TABLE 1 Results of the analysis of molecular variance (AMOVA) based on cpDNA of *Cymbidium faberi* in the Qinling Mountains

Variance component	d.f.	Percentage of variation (%)	Φ -statistics	P -value
Among groups	1	19.99	$\Phi_{CT} = 0.199$	<0.01
Among populations within groups	13	4.54	$\Phi_{SC} = 0.056$	<0.01
Within populations	256	75.47	$\Phi_{ST} = 0.245$	<0.01

Results from SAMOVA indicated that F_{CT} reached a peak at $K = 2$ and then decreased (Tables S2), which supported a two-group genetic structure. SYX and populations in the eastern of the mountains were grouped together; whereas SXX and SZA were grouped together. The population genetic structures at $K=2, 3, 4$ using Bayesian clustering were shown in Figure 4.

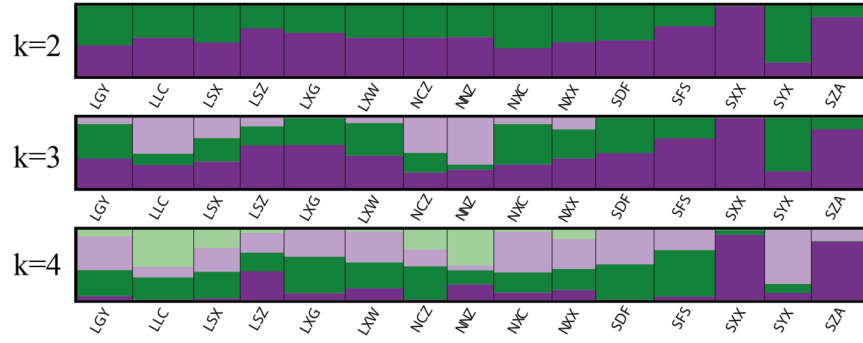


FIGURE 4 Estimated population structure based on cpDNA of *Cymbidium faberi* in the Qinling Mountains. STRUCTURE bar plots for K = 2 to 4. K = 2: group1 (SXX, SZA), group2 (NCZ, NNZ, NXC, NXX, LLC, LSX, LXW, SDF, SFS, LXG, LGY, LSZ, SYX); K = 3: group1 (SXX), group2 (SZA), group3 (NCZ, NNZ, NXC, NXX, LLC, LSX, LXW, SDF, SFS, LXG, LGY, LSZ, SYX); K = 4: group1 (SXX), group2 (SZA), group3 (LSZ), group4 (NCZ, NNZ, NXC, NXX, LLC, LSX, LXW, SDF, SFS, LXG, LGY, SYX); Black vertical lines identify the population boundary. Estimated K values are represented in different colors

3.4 | Estimations of divergence time and demographic history

Orchids are one of the world's oldest flowering plants, two chloroplast DNA sequences were used to estimate the divergence time of *C. faberi* in the Qinling Mountains, our estimates indicated *C. faberi* diverged from genus approximately 29.096 Ma (95% HPD = 15.573 – 42.864) during the Oligocene, as dated using BEAST (Figure 5). Of the 49 haplotypes, split between Hap7 and Hap 20 occurred about 0.7156 Ma, whereas Hap 1, Hap 6, Hap 9, Hap 13, Hap 30 and Hap41 were estimated to be of nearly Holocene ages.

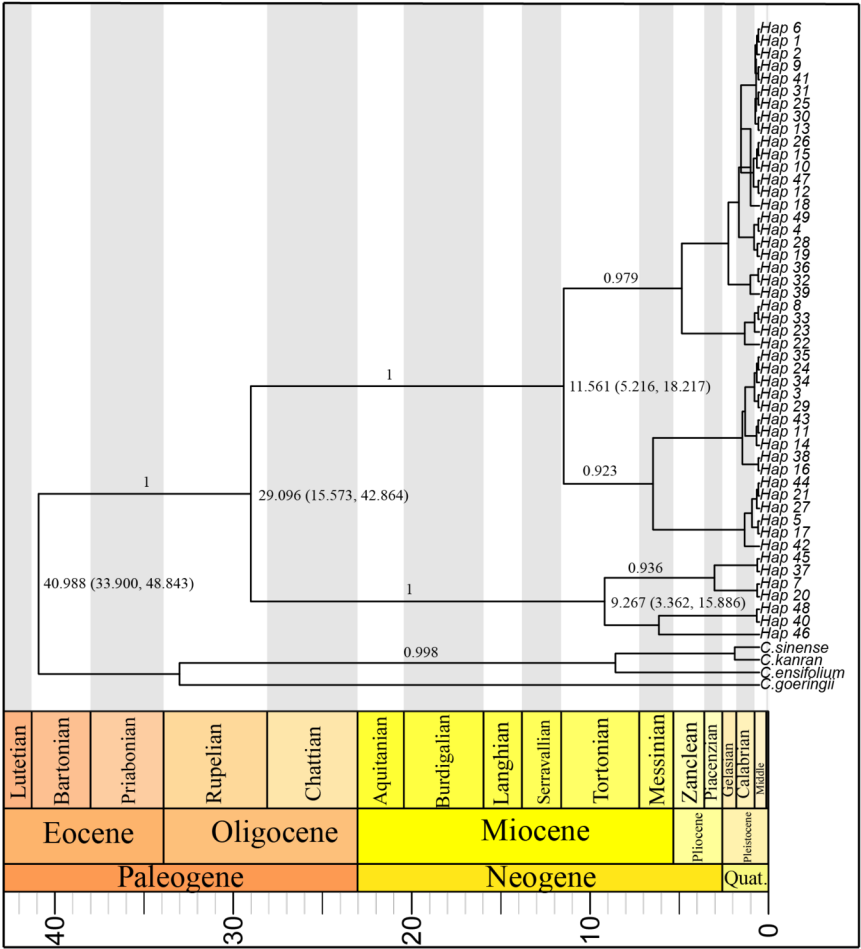


FIGURE 5 The maximum clade credibility tree of 49 haplotypes identified in *Cymbidium faberi* . Values above the main branches represent posterior probabilities, and 95% highest posterior density intervals are represented in brackets

Based on the estimations of divergence time, we generate the Extended Bayesian Skyline Plot, which revealed that the estimates of effective population size of *C. faberi* were under demographic equilibrium in the past but an apparent decline in population size going from approximately 1 Ma (Figure 6). Our data suggested that disturbance due to human activity may have contributed to the gradually reduced effective population size.

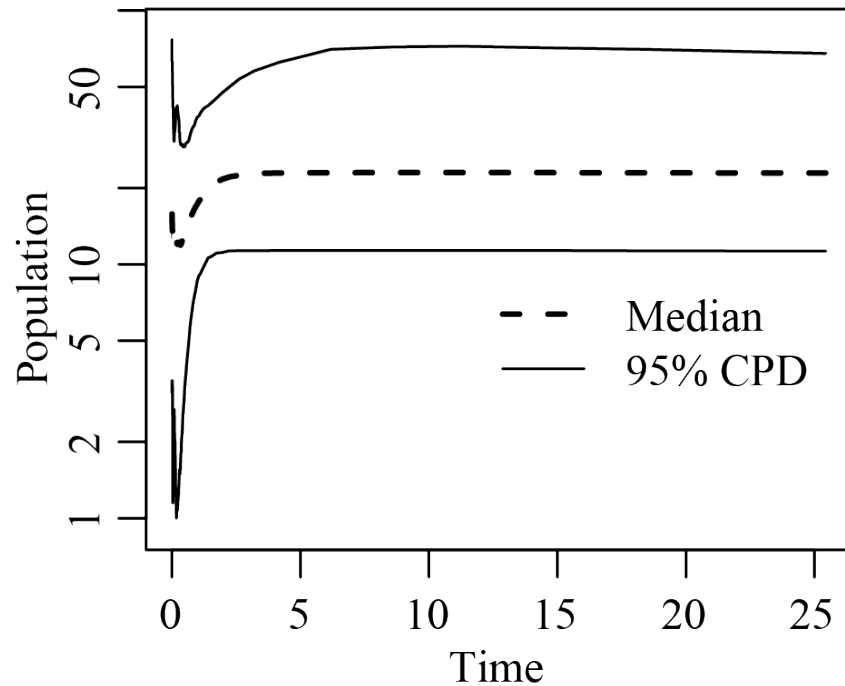


FIGURE 6 Extended Bayesian Skyline Plot of *Cymbidium faberi* in the Qinling Mountains. The X axis is the time before present (Ma) whereas the Y axis equals changes in the effective population size. The dashed line represents the median population size, and the solid lines indicate 95% central posterior density intervals

4 | DISCUSSION

4.1 | Effect of habitat fragmentation on population genetic structure

Habitat fragmentation has been identified as one of the threats to biodiversity of many terrestrial species. In fragmented habitats, the roads, villages and farmland surrounding the populations are generally considered to be barriers to gene flow, which often result in reduced genetic diversity, enhanced inbreeding within populations and genetic structure (Templeton, Robertson, Brisson, & Strasburg, 2001; Byrne, Elliott, Yates, & Coates, 2008; Vranckx, Jacquemyn, Muys, & Honnay, 2012; Betancourth-Cundar, Palacios-Rodríguez, Mejía-Vargas, Paz, & Amézquita, 2020). For most plants, habitat fragmentation often reduces gene flow and genetic diversity by disrupting the movement of seed (Sebbenn et al., 2011; Browne & Karubian, 2018; Ony et al., 2020).

Despite orchids have dust-like seeds, some studies investigating genetic structure of orchids in fragmented landscape have found a significant pattern (Cozzolino, Noce, Musacchio, & Widmer, 2003; Chung et al., 2014; Mallet, Martos, Blambert, Pailler, & Humeau, 2014; Minasiewicz, Znaniecka, Górniak, & Kawiński, 2018). The gene flow introduced via seed dispersal often depends on the morphological features of seeds. Orchid seeds are known to be small, light and easily transported by the wind, perhaps the habitat fragment cannot prevent the seeds from being transported for long distances. However, orchid seeds need the presence of a fungus to germinate and grow (McCormick, Whigham, Sloan, O'Malley, & Hodkinson, 2006), and the patches with variable fungi are shrinking and being replaced by unfavorable environment. A study in a landscape experiencing habitat fragmentation showed that although Chilean tree can produce a large number of seeds, seedling establishment was uncommon (Lander, Harris, Cremona, & Boshier, 2019).

Moreover, orchid species depend on insect pollinators such as honeybees for seed production and dispersal. In continual forest area, these pollinators are normally capable of flying one to several kilometers, so gene

flow via pollen was expected to be not restricted. In contrast, in a fragmented landscape, pollen dispersal will become very difficult. Suni & Brosi (2012) analyzed the genetic structure of two orchid bee species in fragmented habitat, and found genetic differentiation for two species was significant. Although biparentally inherited molecular data are not shown here, it is certain that pollen-mediated gene flow can be affected by habitat fragmentation.

Many forest areas in the Qinling Mountains have affected negatively by human disturbances. From 1984 to 2014, human activities greatly disturbed the forest in the mountains, and the degree of fragmentation has increased obviously (Cui et al., 2018). In our study, the populations SZA and SXX are surrounded by roads, railway line and villages, which inevitably lead to alterations in genetic structure of wild species. Our analysis revealed high genetic differentiation between *C. faberi* wild populations distributed within fragmented land and others in continuous forest. Furthermore, a decreased level of genetic diversity within SZA and SXX populations surrounded by roads was detected. This suggested that seed-mediated gene flow between SXX, SZA and other populations may be limited by habitat fragmentation. In contrast, extensive gene flow detected in populations located in eastern mountains can prevent or reduce genetic divergence, help maintain overall genetic variation by reducing genetic drift.

4.2 | Influence of topographic features on genetic structure

Topographic features often act as a physical barrier to gene exchange between populations of species (Pilot et al., 2006). A previous study of giant pandas has shown that natural barriers might have decreased gene flow between the Xiaoxiangling and Daxiangling regions (Zhu, Zhan, Meng, Zhang, & Wei, 2010). Moreover, using landscape genetic approaches, researchers investigated the population genetics of American mink, and found the Cairngorms Mountains may act as a major geographical barrier to gene flow among populations (Zalewski, Pierniey, Zalewska, & Lambin, 2009). A study by Wei et al. (2013) also showed that gene flow of *Euptelea pleiospermum* was limited by mountain ridges.

As mentioned before, Orchidaceae is often characterized by relatively low levels of genetic differentiation among populations because of long-distance dispersal afforded by minute size of seeds, and geographical features usually have no impacts on their spatial genetic structure. For example, Perez-Escobar et al. (2017) noted that the Andes seem not to have been much of a barrier to the dispersal of lowland epiphytic orchids. Consistent with the previous result, we also found the Qinling Mountains do not appear to be a geographic barrier of genetic exchange for terrestrial orchid *C. faberi*. However, another study of an epiphytic orchid found that seed flow among twelve populations in Costa Rican mountain ranges was comparatively limited (cpDNA, $F_{ST} = 0.443$), which possibly due to the directional prevailing winds (Kartzinel, Shefferson, & Trapnell, 2013).

4.3 | Genetic diversity of *C. faberi* and conservation implication

There have been efforts made to understand the impact of habitat fragmentation on genetic diversity of plant species, and genetic diversity of populations located in fragmented landscape was lower compared to those in continuous ones (Aguilar, Quesada, Ashworth, Herrerias-Diego, & Lobo, 2008; Dixo, Metzger, Morgante, & Zamudio, 2009), which can be explained by the increased genetic drift and inbreeding (Suárez-Montes, Chávez-Pesqueira, & Núñez-Farfán, 2016).

For orchid family, it is usually considered that it has high genetic diversity due to outcrossing and long-live, but fragmentation can modify this diversity. A previous study of terrestrial orchid *Cymbidium goeringii* found higher genetic diversity in continuous forests compared to fragment ones (Chung et al., 2014). Genetic diversity of *Phaius australis* in fragmented habitat was low across all populations (Simmons, Mathieson, Lamont, & Shapcott, 2018). Similarly, the genetic diversity analysis in our study of *C. faberi* also indicated that levels of population genetic diversity of fragmented populations was low (Hd was 0.500 and 0.661, respectively), but higher compared to an epiphytic orchid, *Epidendrum firmum*, which located in isolated bands of Neotropical mountain (mean Hd was 0.454) (Kartzinel, Shefferson, & Trapnell, 2013). Similar values for genetic diversity were also reported for orchid *Cypripedium calceolus* in a fragmented landscape where haplotype diversity was from 0 to 0.689 (Minasiewicz, Znaniecka, Górniak, & Kawiński, 2018).

Given the declined genetic diversity in fragmented populations, we recommend effective measures should be developed to the genetic conservation of the *C. faberi*. Primarily, selling of wild orchids is strictly prohibited without the trade permits. Additionally, raise local residents' awareness on the importance of biodiversity conservation through various activities is necessary to enhance species population persistence and evolutionary potential. Such information is essential for the proper design of conservation strategies in the Qingling Mountains.

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CONFLICT OF INTEREST

The authors declare no conflicts of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available at: <https://osf.io/xrd5f/>.

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SUPPORTING INFORMATION

Additional supporting information may be found in Supplementary material TABLE S1.docx and Table S2.docx.