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Intraspecific divergences and phylogeography of *Panzerina lanata* (Lamiaceae) in Northwest China

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Climactic fluctuations during the Quaternary played a crucial role in genetic diversity and population genetic structure of many plant species in northwestern China. In order to understand the impact of climate change on herbaceous plants, we studied *Panzerina lanata* (Lamiaceae), a widely distributed species. Two chloroplast DNA intergenic spacers (*trnH-psbA* and *rpoB-trnC*) were used to sequence 269 individuals from 27 populations and seven haplotypes were identified. Genetic structure and demographic characteristics were estimated using AMOVA, neutrality tests, and mismatch distribution analyses. The divergence times between the seven haplotypes were estimated using Beast. Our results revealed high levels of total genetic diversity (H_T = 0.673±0.0869) and low levels of average within-population genetic diversity (H_S = 0.033±0.0214). The analysis of molecular variance indicated major genetic differentiation among the three groups: northern, central, and eastern group. The species distribution modeling and demographic analysis indicated that *P. lanata* has not experience a recent range expansion. The divergence time within *P. lanata* occurred between the early Pleistocene and the late Pleistocene, which coincides with aridification and the expansion of the deserts in northwestern China that resulted in species diversification and habitat fragmentation. In addition, we speculate that the deserts and the Helan Mountains acted as effective geographic barriers that led to intraspecific diversity.

Intraspecific divergences and phylogeography of *Panzerina lanata* (Lamiaceae) in Northwest China

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Abstract:

Climactic fluctuations during the Quaternary played a crucial role in genetic diversity and population genetic structure of many plant species in northwestern China. In order to understand the impact of climate change on herbaceous plants, we studied *Panzerina lanata* (Lamiaceae), a widely distributed species. Two chloroplast DNA intergenic spacers (*trnH-psbA* and *rpoB-trnC*) were used to sequence 269 individuals from 27 populations and seven haplotypes were identified. Genetic structure and demographic characteristics were estimated using AMOVA, neutrality tests, and mismatch distribution analyses. The divergence times between the seven haplotypes were estimated using Beast. Our results revealed high levels of total genetic diversity (H_T = 0.673±0.0869) and low levels of average within-population genetic diversity (H_S = 0.033±0.0214). The analysis of molecular variance indicated major genetic differentiation among the three groups: northern, central, and eastern group. The species distribution modeling and demographic analysis indicated that *P. lanata* has not experience a recent range expansion. The divergence time within *P. lanata* occurred between the early Pleistocene and the late Pleistocene, which coincides with aridification and the expansion of the deserts in northwestern China that resulted in species diversification and habitat fragmentation. In addition, we speculate that the deserts and the Helan Mountains acted as effective geographic barriers that led to intraspecific diversity.

Keywords: Intraspecific divergences; Aridification; Desert expansion; *Panzerina lanata*; Northwest China.

1. Introduction

Studies on plant phylogeography in China have mainly focused on endemic or endangered species of the Hengduan Mountains and the adjacent Qinghai Tibetan Plateau, which are highly species and generic rich areas (Cun & Wang, 2010; Gao et al., 2016; Yan, Yang & Tang, 2013; Yang, Zhou & Li, 2011). Since the Hengduan Mountains and the Qinghai Tibetan Plateau were not directly affected by ice sheets, most studies have shown that phylogeographic patterns no large-scale contraction-expansion occurred, which was mainly influenced by climate fluctuations during the Quaternary, resulting in intraspecific divergences and regional range expansion (Guo et al., 2010; Liu et al., 2012). However, recent research on plant phylogeography in northwestern China has expanded (Meng et al., 2015; Wang et al., 2016; Zhang et al., 2017). The arid northwestern China includes the entire Xinjiang Region, the Hexi Corridor of Gansu, the Caidamu Basin of Qinghai, and western parts of the Helan Mountains in Inner Mongolia (Alxa Desert) (Dang & Pan, 2001). In these areas, the phylogeography was mainly influenced by climatic fluctuations in the Pleistocene. During the Pleistocene, northwestern China was mainly impacted by extreme aridification and low temperatures, which caused the expansion of desert. Aridification and desert expansion have been shown to have a significant impact on the phylogeography of many species in northwestern China (Meng et al., 2015; Wang et al., 2016).

The continued aridification and desert expansion in the Pleistocene resulted in species divergence, species diversity, and habitat fragmentation as well as led to the distribution of the montane forest plants on both sides of the desert in northwest China (Ma, Zhang & Sanderson, 2012; Meng & Zhang, 2011; Wang et al., 2016; Xu & Zhang, 2015b). Previous studies on these regions have mainly concentrated on shrubs, with fewer studies on herbaceous plants (Ge et al., 2011; Shi & Zhang, 2015; Su, Lu & Zhang, 2016; Su et al., 2015; Xu & Zhang, 2015b). Herbaceous plants are more sensitive to climate oscillation; therefore, we selected *Panzerina lanata* (Linnaeus) Sojak (Lamiaceae) as a suitable model to understand the genetic structure and response to Quaternary climatic fluctuations in the arid northwest China.

The genus *Panzerina* contains two species that are mainly distributed in the desert and desert grassland areas of central Asia and has been described in the Flora of China (Li & Hedge, 1994). *Panzerina lanata* is a perennial herb with medicinal properties that is mostly distributed in the sandy desert steppes of Inner Mongolia, Gansu, Ningxia, and Shanxi. Previous studies on *P. lanata* have mainly focused on biological characteristics, chromosome research, plant taxonomy, floral analyses, and pharmacology, but its genetic diversity and phylogeography lack sufficient studies.

In the cpDNA fragments, intraspecific genetic variation was common and had profound insight into the evolution process within and among populations (Jordan, Courtney & Neigel, 1996). We used chloroplast DNA sequences to infer the phylogeographic structure of *P. lanata* with the aim to: (1) reveal the genetic diversity and genetic structure of *P. lanata* and (2) determine if the intraspecific divergence of *P. lanata* corresponds to the climatic fluctuations of the Pleistocene.

2. Materials and methods

2.1 Population sampling

A total of 269 individuals from 27 natural populations of *P. lanata* were collected, covering almost the entire distribution area in northwest China (Table 1). In every population, 4–14 individuals were collected. In order to avoid clones or close relatives, the distance between individuals in each population was at least 30 m. Fresh leaves were collected in the field and immediately dried on silica gel until DNA extraction. Voucher specimens are deposited in the Herbarium of Xinjiang Institute of Ecology and Geography, Chinese Academy of Science (XJBI). *Leonurus turkestanicus* and *Lagochilus ilicifolius* were included as outgroups in this analysis (Wu & Li, 1982).

2.2 DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted following the CTAB method (Doyle & Doyle, 1987). The cpDNA intergenic spacers *trnH-psbA* and *rpoB-trnC* had high levels of variation in the species and were easy to amplify. Other primers tested (*psbK-psbI*, *rpl32-trnL*, *rps16-trnK*, *trnV*, and *ycf6-psbM*) had low levels of variation and amplification was difficult for *P. lanata*, thus these regions were not used. PCR samples consisted of a total volume of 25 μL, containing 2.5 μL of 10×PCR buffer, 2.5 μL of 25 mM $MgCl_2$, 2.0 μL of 2.5 mM dNTP mixture, 1 μL of each primer, 0.125 μL of Taq polymerase, and 1 μL of template DNA. Samples were amplified as follows: 95 °C for 4 min, 36 cycles of denaturation at 94 °C for 30s, annealing at 52 °C for 30s, extension at

72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were detected on 1.0% agarose gel, purified using the QIAquick Gel Extraction Kit (Qiagen), and sequenced using an ABI Prism 3730 Genetic Analyzer by Shanghai Sangon Biological Engineering Technology & Service, Shanghai, China.

The DNA sequences were edited in SeqMan (Lasergene, DNASTAR Inc., Madison, Wisconsin, USA) and aligned using BioEdit (Hall, 1999). All sequences are deposited in Genbank.

2.3 Genetic diversity and population structure

The haplotype diversity (H_d) and nucleotide diversity (π) for the following: all samples, each population individually and every group were conducted using Arlequin 3.5 (Excoffier & Lischer, 2010). SAMOVA version 1.0 (Dupanloup, Schneider & Excoffier, 2002) was used to subdivide the geographical structure of all populations. We set $2 \leq K \leq 12$ until the F_{CT} values reached the maximum, and when a single population was clustered into one group, the combination was excluded (Beatty, Provan & Comes, 2013; Iwasaki et al., 2012). Using Arlequin 3.5, molecular variance analyses (AMOVA) were used to estimate genetic variation among three groups, among populations within groups, and within populations, which was calculated with a significance test based on 1,000 permutations. Permut version 1.0 (Pons & Petit, 1996) was used to estimate genetic differentiation (G_{ST}, N_{ST}) and calculate the average within population gene diversity (H_S) and total gene diversity (H_T) with 1,000 permutation tests. These two parameters (N_{ST}, G_{ST}) were used to test if phylogeographic structure existed. If the N_{ST} was significantly larger than the G_{ST}, this indicated the presence of phylogeographic structure. The phylogenetic relationship among the haplotypes was implemented in Network version 5.0 following the median-joining (MJ) algorithm (Bandelt, Forster & Röhl, 1999).

2.4 Demographic history and divergence time analyses

In order to test whether all *P. lanata* populations and groups divided by the SAMOVA experienced demographic expansion, Tajima's *D* (Tajima, 1989) and Fu's F_S (Fu, 1997) were calculated using Arlequin 3.5 with 1,000 permutation tests. In addition, a mismatch distribution analysis was used to test for demographic expansion for all populations and groups. In general, unimodal distribution indicates that the population has undergone a recent expansion, whereas bimodal or multimodal distribution indicate that the population was stable. We also estimated the sum of squared deviations (SSD) and the raggedness index of Harpending (HRag) (Harpending, 1994) between the observed and expected mismatches in Arlequin 3.5 with 1,000 permutation tests. The significance of *P* was examined if the populations experienced expansion.

We estimated the divergence time between different lineages of *P. lanata* using Beast version 1.6.1 (Drummond & Rambaut, 2007). Due to the lack of fossil evidence, the cpDNA substitution rates of *P. lanata* were estimated based upon the reported substitution rate ($1.0–3.0\times10^{-9}$ s/s/y) of most angiosperms (Wolfe, Li & Sharp, 1987). We use the GTR substitution model and Markov Chain Monte Carlo (MCMC) with a coalescent tree prior. The MCMC chains were run for 10,000,000 generations and sampled every 1,000 generations. The effective population sizes (ESS＞200) were checked in Trace version 1.5. The maximum clade credibility tree was implemented using TreeAnnotator version 1.6.1 with a burn-in of 1,000 trees. Finally,

trees were edited in FigTree version 1.3.1.

2.5 Species distribution inference

In order to estimate the distribution ranges of *P. lanata* at present and during the Last Glacial Maximum (LGM; *ca*. 21,000 before present), we inferred the potential distribution area using the ecological niche model. The potential distribution simulations were calculated using the maximum entropy method, which was implemented in MAXENT 3.3.1 (Phillips, Anderson & Schapire, 2006). Geographical information about *P. lanata* was gleaned from field collection records and the Chinese Virtual Herbarium (http://www.cvh.org.cn/). A total of 36 points were used in these modelling analyses. We used 19 bioclimatic variables from the WorldClim database (http://www.worldclim.org/download) to estimate the current potential distribution. The LGM distribution was constructed following the Model for Interdisciplinary Research on Climate (MIROC) (Hasumi & Emori, 2004) and the Community Climate System Model (CCSM) (Collins et al., 2006). The AUC value was used to estimate the goodness of fit between the model and the training data.

3. Results

3.1 Genetic variation and haplotype distribution

The total length of the aligned *rpoB-trnC* and *psbA-trnH* sequences was 1,512 bp. In the combined data, we identified seven haplotypes (H1–H7) (Table 1) and 17 polymorphic sites (14 substitutions and three indels) from 269 individuals collected from 27 populations (Table 2). The total haplotype diversity (H_d) was 0.6691 with a within population variation of 0–0.5333. The total nucleotide diversity (π) was 0.0077, ranging from 0–0.0130. The BYT2 population had higher haplotype diversity and nucleotide diversity than the other populations (Table 1).

From the 27 populations sampled, only three populations had two haplotypes; all other populations had a single haplotype. In the seven haplotypes identified among our *P. lanata* samples, H1 was widely distributed and was the only haplotype in most populations. H5 was distributed in four populations, of which three populations had only H5. H2, H3, and H7 were distributed in three different populations, respectively. H4 and H6 were specific haplotypes found in BYHT and BYT2, respectively (Table 1; Fig. 1).

3.2 Genetic structure

According to the SAMOVA, all populations of *P. lanata* were divided into three groups: (1) the northern group (populations 1–3), (2) the central group (populations 4–11), and (3) the eastern group (populations 12–27).

Among all of the populations, the total genetic diversity was high (H_T = 0.673±0.0869), while the average within-population genetic diversity was relatively low (H_S = 0.033±0.0214) (Table 4). The level of genetic differentiation among the populations were high (G_{ST} = 0.950±0.0308, N_{ST} = 0.922±0.0559), showing that genetic differentiation mainly occurred among populations. N_{ST} was not significantly greater than G_{ST} ($p > 0.05$), indicating that no obvious phylogeographic structure is present in *P. lanata*. In the central group, the total genetic diversity was high (H_T = 0.753±0.0476), and the average within-population genetic diversity was low (H_S = 0.021±0.0208). The level of genetic differentiation among populations was high (G_{ST} = 0.972±0.0263, N_{ST} = 0.755±0.1630). By contrast, the total genetic diversity (H_T = 0.137±0.1122)

and the average within-population genetic diversity (H_S = 0.046±0.0348) of the eastern group were both low. The hierarchical analysis of molecular variance (AMOVA) demonstrated that 89.87% ($p<0.001$) of the total variation mainly occurred among the three groups (northern, central, and eastern populations), and the total variation among populations within groups was 5.22%. The total variation within populations was 4.91% (Table 3). The F_{ST} (0.9509, $p<0.001$) and F_{CT} (0.8987, $p<0.001$) values from the AMOVA also indicated that the largest genetic differentiation was among the three groups.

3.3 Divergence time and demographic analysis

Among all of the populations, Tajima's D and Fu's F_S ($p>0.05$) were positive and insignificant, indicating that *P. lanata* has not experienced a recent expansion (Table 5; Fig. 2). The P-value was greater than 0.05 for the SSD (0.1433, $p>0.05$) and Hrag (0.1417, $p>0.05$) values. In the central group, the Tajima's D and Fu's F_S ($p>0.05$) were insignificant and positive indicating that the group has not experienced recent expansion. This was also confirmed by the P-values ($p<0.05$) of SSD, Hrag, and the multimodal mismatch analysis (Table 5). In the eastern group, Tajima's D (-1.8814, $p<0.05$) was and significantly negative, with the P-values of SSD and Hrag were both greater than 0.05. In addition, the mismatch distribution was unimodal, indicating that the populations experienced a regional-scale expansion (Table 5; Fig. 2). In the northern group, H5 was the sole haplotype thus it was impossible to further analyze the population expansion of this group.

Based on the Beast analysis, the divergence time between the seven haplotypes occurred in the early Pleistocene (2.02 Mya) to the late Pleistocene (0.09 Mya) (Fig. 3), which is consistent with the aridification and desert expansion that occurred during the Pleistocene in northwest China.

3.4 Current and future species distributions

The AUC (area under the curve) values of *P. lanata* for the training data and test data were 0.997/0.997 (the current model) and 0.997/0.997 (under the MIROC climate model). The higher AUC values indicated that the model was more suitable for the current distribution and the potential distribution during the LGM. In accordance with the potential distribution during the LGM and the present day, the results revealed that the current distribution of *P. lanata* had contracted. The distribution within the central region (Inner Mongolia, Shaanxi, Gansu, and Ningxia) remained stable, a small-scale contraction occurred in northern Inner Mongolia, and the distribution within the eastern region (Shanxi, Hebei, etc.) showed a large contraction (Fig. 4a, b).

4. Discussion

4.1 Genetic diversity of *P. lanata*

We found the total genetic diversity of *P. lanata* to be high across all populations. In contrast, the average within-population genetic diversity was low. The value of genetic differentiation (G_{ST} = 0.950) was higher than the average reported for other angiosperms (G_{ST} = 0.637) (Petit et al., 2005), indicating strong genetic differentiation among populations. The total genetic diversity of *P. lanata* (H_T = 0.673) was similar to that of *Allium mongolicum* (H_T = 0.693), but lower than that of *Lagochilus ilicifolius* (H_T = 0.925), which all of them are herbaceous plants

in the northwest of China. The average within-population genetic diversity (H_S = 0.033) of *P. lanata* was lower than *Allium mongolicum* (H_S = 0.180) (Meng & Zhang, 2011; Zhang et al., 2017). This is mainly due to its morphology and the influence of gravity on seed dispersal, resulting in a severely restricted gene flow between populations, as the seeds can only be spread over short distances (Meng & Zhang, 2011).

High levels of genetic variation and unique haplotypes are usually associated with centers of plant diversity or potential refugia, whereas regions of recent colonization have low levels of genetic variation (Li et al., 2010; Meng & Zhang, 2011; Stewart et al., 2010). The central group had the highest genetic diversity and haplotype diversity, which also have other unique haplotypes. The Helan Mountains are a diversification center for species, thus a high level of genetic diversity can be expected. This diversity center is supported by other species distributed in the area (Meng & Zhang, 2011; Shi & Zhang, 2015). In the eastern group, haplotype H1 was widely distributed in most populations and the most haplotypes for all populations except HQH and BYT2. During the range expansion process, the founder effect should be responsible for less genetic diversity, often causing a single prevailing haplotype (Hewitt, 2000; Xu & Zhang, 2015a; Zhang, Volis & Sun, 2010). Therefore, the low genetic diversity in the eastern group should be attributed to the founder effect.

4.2 Intraspecific divergence of *P. lanata*

All populations of *P. lanata* were divided into three groups by SAMOVA subdivisions. In the eastern group, the haplotype H1 was dominant and H2, H5, and H6 were rare haplotypes. The central group was dominated by the haplotypes H2, H3, and H7 and H4 was rare. The northern group only had the haplotype H5. The haplotypes were unique among the three groups except for H2 and H5, suggesting a division between the three groups. AMOVA analyses showed that the variation among the three groups contributed to the total variation, indicating that there was a restricted gene flow among the three groups.

Geographic isolation and climate oscillations resulted in regional genetic differentiation of species and population fragmentation (Guo et al., 2010; Hewitt, 2004; Ye et al., 2018). Climate fluctuations during the Pleistocene resulted in increased aridification, with cold dry climatic conditions that caused desert expansion and in turn promoted the diversification, speciation, and habitat fragmentation of some desert species in northwest China. According to the Beast analysis, the divergence times for the seven haplotypes ranged from about 2.02 to 0.09 Mya, indicating that the divergence time of populations of *P. lanata* occurred in the early to late Pleistocene. The persistent aridification in the early Pleistocene led to diversification of *P. lanata*. This species showed a fragmented distribution, which may be attributed to the development of deserts, causing regional-scale differentiation.

In addition, the northern and eastern groups are separated by the Hobq Desert, Mu Us Sandy Land, and Ulan Buh Desert, geographical barriers that may limit the gene flow among the three groups, causing genetic differentiation within the species. The central group is located around the Helan Mountains. Previous studies have shown that the Helan Mountains acted as migration corridors for recolonization after ice ages as well as a geographical barrier (Meng et al., 2015; Zhang et al., 2017). Therefore, we speculate that deserts and the Helan Mountains may act

as geographical barriers restricting the long-distance dispersal of *P. lanata* seeds, eventually resulting in isolation and differentiation of the populations in these three regions (northern, eastern, and central).

4.3 Demographic history of *P. lanata*

According to the neutrality test and mismatch analysis, this species has not experienced a recent expansion. However, by geographical subdivisions, the eastern group has experienced a recent expansion. Previous studies have shown that plants have lower genetic diversity and a single, widely distributed haplotype in regions with rapid expansion (Hewitt, 1996; Shi & Zhang, 2015; Zhang & Zhang, 2012). The eastern group has a low number of haplotypes and limited genetic diversity. H1 is widely distributed and is the only haplotype for most populations in the eastern group, indicating that the region has experienced an expansion. This result is also confirmed by the mismatch analysis and neutrality test. Since the founder effect and anthropogenic over-exploitation have led to the loss of genetic diversity in *P. lanata* in the eastern group.

In comparison to the distribution of *P. lanata* during the LGM, the present distribution of *P. lanata* has contracted. However, the distribution of *P. lanata* in the central area has remained relatively constant, probably due to the higher annual precipitation in central Inner Mongolia and eastern Gansu than in northern Inner Mongolia, allowing for more suitable habitat. Based on its current distribution, the eastern region (Shanxi and Hebei) has severely contracted. Records from the Chinese Virtual Herbarium and Flora of China indicate that *P. lanata* is distributed in Shanxi and Hebei. However, the distribution during the LGM predicted that there should have been some populations of *P. lanata* in these provinces, which requires further investigation.

5. Conclusions

Our results show that all populations of *P. lanata* has not experienced recently range expansion and has intraspecific divergences that is likely due to habitat fragmentation. The fragmented distribution was mainly influenced by Quaternary climate fluctuations, especially the continued aridification and desert expansion that occurred during the Pleistocene. In addition, deserts and the Helan Mountains serve as geographic barriers that hinder the gene flow among populations, resulting in the genetic differentiation of populations of *P. lanata*.

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Ethics statement

The sampling sites described in this study were not protected and special permits are not required when field sampling. The study species was also not listed as a protected plant.

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

All sample collections were conducted under the permission of the Grassland Station of Alashan, Left Banner, Inner Mongolia.

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