Diversification and vicariance of desert plants: Evidence inferred from chloroplast DNA sequence variation of *Lagochilus ilicifolius* (Lamiaceae)

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**A B S T R A C T**

To document arid Northern China as a diversification center for desert plants, and to better understand the mechanisms of desert taxa diversification, we used five cpDNA spacers (*trn-L-trnF, rps16, psbA-trnH, psbK-psbI* and *trn-S-trnG*) to investigate *Lagochilus ilicifolius* in all distribution areas, including Northern China, Mongolia and Russia. Phylogenetic analyses showed that *L. ilicifolius* comprises two distinctive lineages, one distributed in China, and another in Mongolia–Russia. Our data confirmed that arid Northern China, is a distinctive area with many endemic genera. Biogeographic inferences, based on a Bayesian uncorrelated lognormal model together with molecular dating, suggested that the main diversification within the species occurred in the Pleistocene (ca. 1.38–0.3 Ma), resulting from the transition of the climate of Eurasia to a dry-cold pattern as well as the desertification caused the vicariance of desert plants.

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1. Introduction

To understand factors that influence organismal diversity is a fundamental goal in biology (Darwin, 1872; Simpson, 1961); and to appreciate the drivers and mechanisms of species diversification and persistence is of central interest to biogeography, evolutionary biology and conservation genetics (Lexer et al., 2013). The global distribution of plant diversity and endemism is uneven, with concentrations of species in the Andes, the Atlantic coastal forests of Brazil, the southern tip of Africa, the East Indies and the eastern Himalayas (Mutke and Barthlott, 2005). Thus, in China, many plants studies have focused on the Sino-Japanese Floristic Region of East Asia where harbors species more abundantly and ranks prominently among diversity centers of the world’s temperate regions, which was referred to as the core of the Himalayan hotspot, is one of the greatest concentrations of biodiversity in the world due to its high level of species diversity and generic richness (Myers et al., 2000). These regions of high endemism were the most important glacial refugia for their Tertiary representatives (*‘relics’*)
throughout the Quaternary ice–age cycles (Qiu et al., 2011). But arid regions in China, especially Alxa–Helan Mountain Range, are considered one of eight diversification centers within China, which contain a number of endemic genera (Wu et al., 2010; Zhao and Zhu, 2003). This region, designation is supported by Maxent and Domain species distribution model simulations of the ranges of 13 genera, indicated the Alxa of Inner Mongolia to be the most noticeable endemic area (Ma et al., 2012). However, this enrichment of diversification and endemism in arid zones has not drawn much attention; and only a few studies have called for conservation for its desert taxa, such as for Ammopiptanthus (Ge et al., 2005), Tetraena mongolica (Ge et al., 2011), Helianthemum songaricum (Su et al., 2011) and Gymnocarpos przewalskii (Ma and Zhang, 2012).

Central parts of the Qinghai-Tibet Plateau (QTP) might have been uplifted to present heights in early Miocene, which compelled Tethys Ocean retreated from Central Asia, establishing the beginning of an arid climate (Guo et al., 2002; Sun et al., 2010). That aggravated climatic aridification and cooling (Clark et al., 2005; Fang et al., 2005) in the Pleistocene, which sharply increased aridity in the Chinese deserts (Ding et al., 2005; Fang et al., 2002). Also, it changed the hydrology and climate of these regions, played a significant role in determining the local geographic distribution and evolutionary history of plant species (Meng and Zhang, 2011, 2013; Su and Zhang, 2013). As a dynamic ecosystem, the deserts interaction among vicariance, dispersal and habitat shift, often promotes diversification of desert plants (Meng et al., 2014). Plants, as primary producers, also play a key role in maintaining these ecosystems. Desert ecosystems currently cover large Earth’s land surface and are continuously expanding. Desertification and ongoing deterioration in arid, semi-arid regions have recently focused attention on the urgent need to protect desert ecosystems. Greater comprehension of the diversification and vicariance of desert plants may provide important insights into the processes of aridification and desertification of these regions, because evolutionary history might have preserved the genetic imprint in these processes.

Evolutionary history of L. ilicifolius has suggested that diversification was consistent with aridification and desert expansion during the middle Pleistocene; and Helan Mountains might be suggested as the diversification center of the species (Meng and Zhang, 2011). However, previous study of L. ilicifolius was limited to populations in China and lacked distribution areas from Mongolia and Russia, and conclusions were inferred from only two chloroplast DNA (cpDNA) intergenic spacers. In addition to Northern China, L. ilicifolius occurs in southwestern Siberia (Tuviskaya), southeast Mongolia and Leningrad (presently, St. Petersburg) (Komarov, 1954)(Fig. 1). So, the evolutionary history and biodiversity of L. ilicifolius will be better understood if we give full consideration to plants in all distribution areas, including China, Mongolia and Russia. Here, we used an integration of phylogenetic, biogeographic, and molecular dating methods to reconstruct the evolutionary history of L. ilicifolius. The distribution of this species spans desert steppe and grassland, sandy areas as well as semidesert thickets on gentle slopes, which offers a unique opportunity to disentangle the evolutionary processes underlying botanical evolution in the arid regions. So, we investigate sequence variation of five chloroplast cpDNA spacers in samples of L.

Fig. 1. Distribution map of Lagochilus ilicifolius showing the habitat areas in Northern China, Mongolia and Russia. (A) Adult nature plant of L. ilicifolius, (B) flower, (C) flower bud, (D) seeds and (E) special feature of flower of a different individual morphology.
**2. Materials and methods**

### 2.1. Taxon sampling

Our sampling within the species *L. ilicifolius* attempted to include as wide a geographic diversity as possible, covering its distributional range including China, Mongolia and Russia. From the viewpoint of morphology, *Lagochilus* and Panzeria are derived from the genus *Leonurus* (Wu et al., 2003). Thus, *Leonurus turkestanicus*, *Leonurus glaucescens*, and Panzeria alashanica were used as outgroups in the analyses. Natural individuals from 14 populations (localities in Inner Mongolia, Ningxia, Shaanxi and Gansu) and outgroups were collected in China during field investigation and collections in 2010. Some plant materials of Mongolia and Russia were obtained from herbarium specimens (Table 1 and Table 2). In the field work, young and healthy leaves were sampled randomly, and quickly dried with silica gel in the field and stored frozen until DNA extraction. Voucher specimens were deposited in the Herbarium of Xinjiang Institute of Ecology and Geography, Chinese Academy of Science (XJBI). All the materials and GenBank accession numbers are presented in Tables 1 and 2.

### 2.2. Methods

#### 2.2.1. DNA extraction, amplification and sequencing

Total genomic DNA was extracted from silica gel-dried leaf tissue using modified cetyltrimethylammonium bromide (CTAB) protocol (Cullings, 1992). Isolated DNA was amplified using five cpDNA regions; *trnL-trnf* (Taberlet et al., 1991), *rps16* (Shaw et al., 2005), *psbA-trnH* [psbA-trnH-f (Sang et al., 1997), psbA-trnH-r (Tate and Simpson, 2003)], *psbK-psbl* (Lahaye et al., 2008) and *trnS-trnG* (Shaw et al., 2007) were performed using the published primers of the cited authors (Table 3). Polymerase chain amplification (PCR) and cycle sequencing followed the methods described in the previous study (Meng and Zhang, 2013). PCR products were examined by gel electrophoresis using a 0.8% agarose gel in a 0.5 \* TAE (pH 8.3) buffer, and stained with EB (ethidium bromide) to confirm single products. PCR products were purified using QIAquick Gel Extraction Kits. The same primers were used for sequencing, at Sangon Biotech (Shanghai) Co., Ltd., China.

DNA sequences were edited using SeqMan (Lasergene, DNASTAR Inc., Madison, Wisconsin, USA), and consensus sequences were obtained from each individual. Multiple sequence alignment was carried out in Clustal X 1.81 (Thompson et al., 1997), and refined and adjusted manually. Regions of ambiguous alignment were excluded from the analyses.

#### 2.2.2. Phylogenetic analyses

Five cpDNA spacers were concatenated and analyzed as one sequence in phylogenetic analyses. The combined data set was analyzed under maximum parsimony (MP) in PAUP* 4.0 b10 (Swofford, 2002) and Bayesian inference (BI) relying on MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). For MP analysis, we used heuristic searching, starting trees obtained via stepwise addition tree bisection reconnection branch swapping, steepest descent, and the MulTrees and Collapse options in effect, which enables us to contemplate: 1) is there a diversification center of desert plants in the arid zone of China? 2) did the progressive aridification in arid zones determine the diversification and vicariance of desert species, such as *L. ilicifolius*?

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with no upper limit for the number of trees held in memory; support values for all nodes (on a 50% majority rule bootstrap rule consensus tree). The default options in MrBayes were used for chain heating and mixing. We discarded a burn-in of the first 2,000,000 generations and used 20,000 trees from the posterior distribution to obtain a majority rule consensus tree.

2.2.3. Divergence time estimation

Bayesian relaxed method was implemented in BEAST 1.5.4 (Drummond and Rambaut, 2007) to explore divergence time. BEAUti interface was used to create an input file for BEAST, in which a general time reversible (GTR) nucleotide substitution model with gamma + invariant sites was applied using uncorrelated lognormal model. Ten million generations of the MCMC chains were run, sampling every 1000th. After discarding the first 1000 trees as burn in, the samples were summarized in the maximum clade credibility tree using TreeAnnotator 1.4.8 (Drummond and Rambaut, 2007), with the posterior probability limit setting to 0.5, and summarizing mean node heights. Final trees were evaluated and edited in FigTree 1.3.1 (Rambaut, 2007). Statistical support for the clades was determined by assessing the Bayesian posterior probabilities. In the estimation of divergence time, we used published nucleotide substitution rates of 0.28% M yr$^{-1}$, in accordance with the substitution rate of cpDNA spacers in previous study (Meng and Zhang, 2011, 2013). Because there are neither Lagochilus fossil records nor specific substitution rates available by which to calibrate a molecular clock, these estimates are provisional and should be interpreted with caution, which provide approximations that allow us to hypothesize possible scenarios under the lineages have diverged.

2.2.4. Biogeographic analyses

Although many biogeographic analyses have used dispersal-vicariance analysis (DIVA) (Ronquist, 1997) to infer ancestral distributions (Baird et al., 2010; Harris et al., 2013), DIVA requires fully bifurcated trees. Because our Bayesian trees were not fully resolved, we used RASP 1.1 that implements the S-DIVA (statistical dispersal-vicariance analysis) method but allows uncertainties in phylogenetic trees (Yu et al., 2010).

All L. ilicifolius species were coded as the current distribution according to the phylogenetic tree. Based on the extant distributions of the species in the Bayesian tree, we defined three areas to assess the historical biogeography of the L. ilicifolius clade, Mongolia to Novosibirsk (A), St. Petersburg (previously Leningrad) (B), and North China (C). We used Bayesian trees from the phylogenetic analyses (10,000 trees, excluding the remote outgroup of Leonurus) as input for S-DIVA. The condensed tree was computed using these 9000 trees (excluding the burn-in 1000 trees); the 'maxarea' was set to three and state frequencies were estimated.

3. Results

3.1. Phylogenetic relationships

Five cpDNA region matrix exploited two different analyses, the maximum parsimonious (MP) tree and Bayesian inference (BI) tree yielded essentially identical topologies, but only the Bayesian analysis tree with parsimony bootstrap (BP) values in
the MP analysis and Bayesian posterior probability (PP) values were presented here for higher resolution (Fig. 2). *L. ilicifolius* was strongly supported as monophyletic (PP = 1.00, BP = 100%) with two well-supported groups on the phylogenetic tree: Clade A, including taxa in Mongolia and Russia (PP = 1.00, BP = 94%); Clade B with the taxa in Chinese arid lands (PP = 1.00, BP = 98%) (Fig. 2). In the analysis, Clade A supported two subclades: A-1 (PP = 1.00, BP = 93%) and A-2 (PP = 1.00, BP = 85%), which represented taxa from Mongolia to Novosibirsk, and St. Petersburg, respectively.

### 3.2. Temporal and spatial diversification

The chronogram divergence times from the dating analyses at the intraspecies level (Fig. 3) showed that initial separation of *L. ilicifolius* clades was in late Pliocene. Within Clade A, species of Mongolia–Russia, the divergence time of the Mongolian-Novosibirsk species (A-1) was in the Pleistocene at ca. 1.38 Ma; that of the St. Petersburg species (A-2) was at ca. 1.72 Ma; diversification of Clade B occurred at ca. 1.80 Ma, respectively. The most probable ancestral area and highest posterior probability values of ancestral area reconstructions were inferred from statistical dispersal-vicariance analysis (S-DIVA), indicating that the most recent common ancestor had likely already spread in China and the western regions of Mongolia–Russia (Fig. 3) at the time of divergence of the main clades.

### 4. Discussion

#### 4.1. Diversification of *L. ilicifolius*

Our results, based on the combined analyses of five plastid regions, support the monophyly of intraspecific phylogeny in *L. ilicifolius* (Fig. 2), which comprises two clades: Clade A from the Mongolian taxa extending to Novosibirsk and St. Petersburg; Clade B from Northern China. The previous study was hampered by a lack of taxa from Russia and Mongolia, although the results suggested that the diversification among *L. ilicifolius* lineages in Northern China occurred during the middle Pleistocene (Meng and Zhang, 2011). When we re-examined taxa representing the additional distribution areas, there still seemed to have been rapid diversification in the Pleistocene (Fig. 3).

During Pleistocene and Holocene, environments in the Mongolian Altai and western Mongolia varied between arid and semi-arid conditions due to climatic fluctuations (Grunert and Lehmkuhl, 2004). The extent of Pleistocene ice in the Russian
Altai is still under debate, however, Pleistocene glaciers in the eastern Altai and especially in the northern part of the Mongolian Altai has been proven. Glaciers were restricted to several isolated mountain systems (Lehmkuhl et al., 2004). The more limited extent of present and Pleistocene glaciers in the eastern part of the Russian and Mongolian Altai was the result of decreasing precipitation from west to east. Moreover, Pleistocene climatic in Siberia during cold-arid stages was recorded by loess deposition in a uniform palaeoclimatic pattern (Chlachula, 2003). Quaternary aridification, expansion of the Arctic ice-sheet driven by ongoing global cooling; successive uplifts in the Himalayan–Tibetan complex enhanced the summer monsoon and brought wetter climates to India and Southeast Asia. But the moisture could not reach the Asian interior, because the uplifted Himalayan–Tibetan topography blocked airflow from the south (Guo et al., 2002). Meanwhile, uplifts in the QTP strengthened the winter monsoon, causing additional mid-latitude to high-latitude Asian interior cooling (An et al., 2001; Rea et al., 1998). As a result, the interior of Asia, including Northern China, Mongolia and Siberia, became increasingly dry and cold during the Pleistocene, which strongly affecting the biota within these regions. As a drought tolerant plant, \emph{L. ilicifolius} might have been driven to rapid diversification in the mountains or at the edges of deserts as an adaptation to the environment.

4.2. Vicariance

Molecular dating and ancestral area reconstruction indicate that \emph{L. ilicifolius} originated from the interior of Eurasia (Fig. 3), which might appear to be in line with the hypothesis that \emph{Lagochilus} originated and differentiated along the coasts of Tethys Ocean (Wu et al., 2003). In addition, aridification in the Pleistocene did promote the diversification and vicariance of \emph{L. ilicifolius} (Meng and Zhang, 2011, 2013). The common ancestors of the \emph{L. ilicifolius} clades had likely spread from Mongolia–Russia into adjacent western China by the start of the Mid-Pliocene. The lineages rapidly diversified through the Pleistocene (Fig. 3) as the climate of Eurasia transitioned to a dry-cold pattern.

Vicariance had a significant role in the diversification of \emph{L. ilicifolius} either. Dramatic crustal deformation induced by the Indo-Asian collision contributed to the intensifying drought in the interior of Eurasia. Alongside climatic shift, massive mountains and deeply carved valleys appeared and the deserts expanded, which acted as barriers to distribution and resulted in differentiation that subsequently gave rise to three subclades. The effects of climate-driven vicariance are evident in other arid zone taxa, such as the cryptic vicariance in the historical assembly of a Baja California Peninsular Desert biota (Garrick et al., 2009; Riddle et al., 2000). But few studies have been carried out on the vicariance of species adapted to open areas (arid zones) interspersed in the interior of Eurasia. For \emph{L. ilicifolius} in Northern China, the physiographical heterogeneity of the Helan Mountains area may have given rise to geographical and probably ecological isolation (Meng and Zhang, 2011).

Fig. 3. Time tree of \emph{Lagochilus ilicifolius}. The tree topology, derived from BEAST analyses of all samples. Branch colors reflect biogeographic designations (for species at tips) and ancestral area reconstruction. The upper circles indicate the results of S-DIVA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Phylogenetic relationships indicate that Clade A and Clade B are two distinctive lineages (Fig. 2). It is interesting that the clades have a genetic break between the geographic regions of China and Mongolia–Russia. DIVA analysis demonstrates that *L. ilicifolius* was likely to have originated from an arid adapted ancestor and then has been restricted to a more narrow ecosystem niche in response to drastic environment changes (extremely dry and cold) in the interior of Eurasia during the Pliocene (Fig. 3). In China, the distribution areas of *L. ilicifolius* were under pressure by mountains and deserts: the north–south Helan Mts. and the west-east Yinshan Mts.; at the north of the distribution areas are the Ulanbuhe and Kubuqi Deserts, and on the west and east are the Tengger Desert and Mu Us Sandy Land, respectively. These deserts can be considered as geographic barriers, but the Loess Plateau appears to have provided an ecological corridor for northward migration (Meng and Zhang, 2011). When all distribution areas of *L. ilicifolius* are taken into account, our results propose a “line” along the edges of the deserts, linking the Helan and Yinchuan Mts., as a biogeographic barrier across this region. That is to say, there is a genetic break between China and the rest of arid Eurasia. In particular, the Yinchuan Mts. might be the geographic barrier that resulted in the development of genetic isolation between China lineage and the Russia–Mongolia lineage. As a biogeographic boundary, Wallace’s line was proposed on the basis of numerous taxa, even some that are marine (Barber et al., 2000). Here, although we recommend the “line”, limitations in our ability to estimate geographic barriers more accurately confining the robustness of our inferences; information from additional taxa should reveal whether these findings apply to other desert plants.

4.3. Implications of conservation in Northern China

*L. ilicifolius* is mainly restricted to arid environments, and these regions could be defined as the exclusive biodiversity of a region. The area inhabited by *L. ilicifolius* in Northern China is considered as a diversification center contains many endemic genera (Wu et al., 2010; Zhao and Zhu, 2003). An area of endemism contains taxa found nowhere else and could be catalogued as irreplaceable and of high priority for conservation. However, there is still no evidence to prove that the area is a distinctive region. To the best of our knowledge, the spatial partitioning of genetic diversity in *L. ilicifolius* and integrated previous view points which offered information for conservation practices. Our results reveal that China clade is a distinctive evolutionary lineage (Figs. 2 and 3). Moreover, the area was simulated using distribution models, suggesting that the A1xa of Inner Mongolia is the most noticeable endemic area (Ma et al., 2012).

Arid areas of Northern China are vast, especially since the sand deserts are developing and expanding further by current anthropogenic processes (Zong and Qu, 2003). Conservation of the genetic resources of desert plants is crucial to combat desertification, to prevent further degradation of the fragile ecosystems in arid and semi-arid regions, and to sustain biodiversity in the deserts (Meng et al., 2014). According to the results, *L. ilicifolius* in China constitutes a separate evolutionary clade, and this area is confirmed as a diversification center (Meng and Zhang, 2011). So, strategies applicable to this area should be adopted for these populations for in situ and ex situ conservation of genetic diversity. Finally, the researched taxa are rare in this region, which limits conservation strategies and management decisions. Therefore, samples from different lineages in various habitats, such as from different mountain slopes and sandy deserts, should be taken into account for conservation, because desertification in these regions is increasing and the genetic diversity of desert plants is constantly diminishing.

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