Molecular phylogeny and biogeography of the arctic-alpine genus *Lagotis* (Plantaginaceae)

Guo-Dong Li,[1,2] Changkyun Kim,[1] Hong-Guang Zha,[1] Zhuo Zhou,[1] Ze-Long Nie[1] & Hang Sun[1]

1 *Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences, 132 Lanhei Road, Kunming, Yunnan 650204, P.R. China*

2 *Faculty of Traditional Chinese Pharmacy, Yunnan University of Traditional Chinese Medicine, 1076 Yuhua Road, Kunming, Yunnan 650500, P.R. China*

Author for correspondence: *Hang Sun, hsun@mail.kib.ac.cn*

ORCID: G.-D.L., 0000-0002-9108-5454

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Abstract It has been suggested that many plants now found in the arctic originated from ancestors that occurred at high altitudes in the southern mountains of the Northern Hemisphere during the Tertiary. However, this hypothesis has rarely been tested using a molecular phylogenetic approach. Here, we present a fossil-calibrated molecular phylogeny of *Lagotis*, an arctic-alpine genus with the greatest diversity in the Qinghai-Tibetan Plateau (QTP) and Central Asian mountains, based on five chloroplast (*matK*, *psbA-trnH*, *rps16*, *trnG-S*, *trnL-F*) and nuclear ribosomal ITS DNA markers. Within this framework, we infer the ancestral area and biogeographic history of the genus. Four major clades (A–D) within *Lagotis* were recovered with strong support, which largely correspond to the previous classification of the genus. Within clade A, *Lagotis* species from QTP were distributed among several subclades, and *L. integrifolia* from Central Asia was sister to *L. glauca* and *L. minor* from the arctic and subarctic region. The Bayesian molecular dating and the ancestral area reconstruction analyses suggested that *Lagotis* could have originated in the QTP in the Miocene (Tertiary), and that the genus radiated from the Miocene to Pleistocene. The diversification of *Lagotis* probably took place predominantly in the QTP and it subsequently spread to the Central Asian highlands, followed by northward migration into the arctic. Our results support the hypothesis that the "Central Asiatic Highland Corridor" as an important route for the migration of the flora between the arctic and QTP.

Keywords arctic-alpine; biogeography; Central Asiatic Highland Corridor; *Lagotis*; phylogeny

Supplementary Material Electronic Supplement (Fig. S1) and alignment are available in the Supplementary Data section of the online version of this article at http://www.ingentaconnect.com/content/iapt/tax

■ INTRODUCTION

The origins and migration patterns of Northern Hemisphere (NH) plants, which have their highest diversity in the highlands of Asia, have attracted the attention of numerous botanists for many years (e.g., Wulff, 1943; Wolfe, 1975; Ozenda, 1988; Donoghue & al., 2001; Donoghue & Smith, 2004; Jia & al., 2012). Molecular phylogenetic studies of genera containing species that are disjunctly and widely distributed across the NH have become common in recent decades (Xiang & al., 1998, 2004; Wen, 1999, 2001; Xiang & Soltis, 2001; Milne & Abbott, 2002; Donoghue & Smith, 2004; Milne, 2006; Zhang & al., 2009; Harris & al., 2013).

It has been proposed that the successive uplifts of the Qinghai-Tibetan Plateau (QTP) since the mid-Miocene have played an important role in triggering diversification in some large genera (with more than 100 species) endemic to the region, as well as in the formation of some small or monotypic endemic genera (see reviews by Liu & Tian, 2007; Wang & al., 2007; Qiu & al., 2011). With regard to biogeographic connections between the QTP and other NH regions, three different biogeographic patterns have emerged (Jia & al., 2012). First, most of such genera originated in the QTP and adjacent regions, and then migrated to other NH regions (e.g., Zhang & al., 2007, 2009; Xu & al., 2010). Second, the disjunct distribution of some genera in the NH represents local relics of a once continuous Arcto-Tertiary, Tethyan or boreal flora (e.g., Sun & al., 2001; Chen & al., 2005; Mao & al., 2010). Third, a few genera originated in other regions of the world and diversified greatly after reaching the QTP (e.g., Liu & al., 2002; Tu & al., 2010). However, few studies have focused on disjunctions involving genera with arctic-alpine distributions in the NH (Hoffmann & al., 2010; Winkler & al., 2012).

It has long been acknowledged that the arctic flora is very similar to that of the southern high mountains in both Asia and North America at the family, genus and even species level, and that this is the result of similar environmental conditions. This has led to the suggestion that many plants now found in

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the arctic originated from ancestors that occurred at high altitudes in the southern mountains of the NH during the Tertiary (Hultén, 1937; Hedberg, 1992). However, this hypothesis has rarely been tested using a molecular phylogenetic approach (Tkach & al., 2008). As summarized by Sun (2002a), two main pathways for the migration of the flora between the arctic regions and the QTP have been suggested, namely the "Himalayan-Hengduan Mountain-Qinling-NE China" route suggested by Wang (1992) and the "Central Asiatic Highland Corridor" proposed by Ohba (1988). In the present study, we investigated the diversification process and the historical biogeography of *Lagotis* J.Gaertn., a small genus of herbaceous plants containing approximately 28 species that are mainly found in the QTP, Himalayas, Pamir, Central Asian mountains, arctic and subarctic Asia (northwest Mongolia, northern Japan, Russia) and northwest America (Alaska and the Yukon; Lu, 1992). The centers of diversity of this genus are in two hotspots: the QTP (ca. 15 endemic species) and the Central Asian mountains (ca. 8 endemic species). Many *Lagotis* species are narrow endemics, but there are a few widespread taxa. Therefore, the genus is an ideal model to test the origins and migration pattern between the arctic regions and the QTP and Central Asian highlands.

The systematic position of *Lagotis* has been much debated since its description (Gärtner, 1770). It was variously classified as being either close to tribe Selagineae or tribe Veroniceae in Plantaginaceae (reviewed by Thieret, 1967). Based on the most recent evidence from phylogenetic analyses of *Veronica* and related genera, the position of *Lagotis* in Veroniceae is robustly supported (Albach & Chase, 2001; Albach & al., 2004a).

The infrageneric classification of *Lagotis* has been problematic (Li, 1954; Yang, 1979; Lu, 1992). Different classifications have been proposed based on various morphological characters. Maximowicz (1881) proposed two sections (sect. *Caulescentes* Maxim. and sect. *Acaules* Maxim.), and this was accepted by two other workers (Vikulova, 1955; Yamazaki, 1971) based on the presence/absence of an aerial stem. Yang (1979) also recognized two sections in *Lagotis*: sect. *Schizocalyx* Tsoong and sect. *Lagotis*, based on whether the calyx is split or not and on characteristics of the stem (stoloniferous or with conspicuous erect stem). This classification was modified by Lu (1992), who divided the genus into two sections but with different circumscriptions: sect. *Acaules* and sect. *Lagotis*, with the latter subdivided into three series: ser. *Pharicae* X.F.Lu, ser. *Ramalanae* X.F.Lu and ser. *Lagotis*. Molecular phylogenetic studies have not been conducted in this genus to evaluate these classifications.

In the present study, we used five chloroplast DNA (cpDNA) regions (*matK*, *psbA-trnH*, *rps16*, *trnG-S*, *trnL-F*) and the nuclear ribosomal internal transcribed spacer (nrITS) to reconstruct the phylogeny of *Lagotis*. The objectives of our study are: (1) to determine relationships among species of *Lagotis* and to evaluate previous classifications of the genus; (2) to estimate the time frame of species diversification; and (3) to reconstruct the biogeographic history of the genus and infer the migration route between the arctic and QTP regions.

■ MATERIALS AND METHODS

Taxon sampling. — Included in our nrITS and cpDNA sequence analyses were 37 accessions from China (Yunnan, Sichuan, Qinghai, Tibet, Xinjiang), northeastern Russia, Kazakhstan, Armenia, U.S.A. (Alaska), and India, representing 22 species of *Lagotis*. Among the 37 accessions, 23 were collected in the field and the others were herbarium specimens. Six species (*L. kunawurensis* (Royle ex Benth.) Rupr., *L. uralensis* Schischk., *L. ikonnikovii* Schischk., *L. spectabilis* Kurz, *L. takedana* Miyabe & Tatewaki, *L. blatteri* O.E.Schulz) were not included in this study, of which five species belong to sect. *Lagotis* and one to sect. *Acaules* sensu Lu (1992). However, our sample included species representing all sections and series recognized by Lu (1992) and the entire geographical range of the genus. Representative species of *Scrofella* Maxim. and *Picrorhiza* Kurroa were also included. All other sequences of Veroniceae (all genera of the tribe) and *Plantago coronopus* Phil. (Plantaginaceae) were downloaded from GenBank and selected as outgroups based on previous analyses of Veroniceae (Albach & Chase, 2004). The sampled species, voucher information, and GenBank accession numbers for the six datasets are listed in Appendix 1.

DNA extraction, amplification, and sequencing. — Total DNAs were extracted from silica-dried leaf samples or herbarium material using a Universal Genomic DNA Extraction Kit (TaKaRa, Dalian, China) following the manufacturer's protocol. One nuclear and five cpDNA markers (nrITS and *matK*, *psbA-trnH*, *rps16*, *trnG-S*, *trnL-F*) were employed in this study. The *matK* region was amplified and sequenced using primers matK-8F (Johnson & Soltis, 1994) and trnK-2R (Steele & Vilgays, 1994). The *psbA-trnH* region was amplified and sequenced using primers psbA and trnH (Sang & al., 1997). Sequences of the *rps16* intron were amplified and sequenced using primers rpsF and rpsR2 (Oxelman & al., 1997). The *trnG-S* region was amplified and sequenced using primers trnS and trnG (Hamilton, 1999). The *trnL-trnF* region was amplified and sequenced in two short fragments using primers "c" and "f" as described in Taberlet & al. (1991). The nrITS region was amplified and sequenced using primers ITS1 and ITS4 (White & al., 1990).

These DNA regions were amplified from total DNAs, using PCR on a PTC-200 thermal cycler (MJ Research, Waltham, Massachusetts, U.S.A.) programmed with a thermal profile of: 4 min at 95°C; 35 cycles of 50 s at 94°C (denaturation), 40 s at 50°C (annealing), and 40 s at 72°C (extension); with a final cycle of 10 min at 72°C. The amplified products were then purified with a QIAquick PCR Purification Kit (BioTeke, Beijing, China), and sequenced using an ABI 3730XL automated DNA sequencer (Applied Biosystems, Foster City, California, U.S.A.). Sequences were edited with Sequencher v.4.1.4 (GeneCodes Corporation, Ann Arbor, Michigan, U.S.A.) and then aligned using Clustal X v.1.83 (Thompson & al., 1997), followed by manual adjustments in BioEdit (Hall, 1999).

Phylogenetic analyses. — The nrITS and cpDNA datasets were assessed for supported topological differences (i.e., those subject to BS ≥70% following Oh & Potter, 2005; Zhou & al., 2009), in the absence of which the data were combined. Phylogenetic relationships were analyzed using maximum parsimony

(MP) and Bayesian inference (BI). The MP analyses were performed using heuristic searches of 1000 replicates with random stepwise taxon addition, tree bisection-reconnection (TBR) branch swapping, MulTrees on and Collapse option selected in PAUP* v.4.0b10 (Swofford, 2003). All characters were weighted equally and gaps were treated as missing data. Each individual dataset, the combined cpDNA data, and all regions combined were analysed. Bootstrap support (BS) was calculated from 1000 replicates using a heuristic search with simple taxon addition, TBR and MULPARS options implemented (Felsenstein, 1985).

The BI analysis (Rannala & Yang, 1996) was carried out for all data combined using MrBayes v.3.12 (Ronquist & Huelsenbeck, 2003). Applying the Akaike information criterion (AIC; Akaike, 1974), MrModeltest v.2.0 (Nylander, 2004) assigned the GTR+I+G model to the sequence data. The Markov chain Monte Carlo (MCMC) algorithm was run for 20 million generations with one cold and three heated chains, starting from random trees. Runs were repeated twice. The resulting log likelihood and number of generations were plotted to determine the point after which the log likelihoods had stabilized by using the ''sum parameters'' command. The 50% consensus tree was constructed from the last 18,001 of 20,001 trees sampled to obtain posterior probabilities of clades with the first 2000 trees were discarded as burn-in. Nodes with posterior probabilitiy (PP) values ≥95% were considered statistically significant.

Molecular dating analysis. — To estimate the age of *Lagotis* and its constituent clades, we constructed a broad dataset of the combined *rps16* and *trnL-F* sequences of Lamiales based on previous work (Nie & al., 2006). The matrix included all *Lagotis* species sampled (one representative per species) in this study and representative species of Veroniceae plus sequences of 40 species obtained from GenBank (Appendix 2). A few taxa were coded with missing data in the *rps16* intron region because fewer sequences of this region were available for Lamiales.

A likelihood ratio test provided no support for a molecular clock hypothesis ($P < 0.05$). Thus, we conducted the divergence time analysis using a Bayesian approach implemented in BEAST v.1.5.4 (Drummond & Rambaut, 2007) under a log-normal relaxed molecular clock (Drummond & al., 2006) and the Birth-Death prior, estimated base frequencies, gamma shape distribution (with four categories), and a proportion of invariant sites, with fossil calibration at four nodes described below. Based on the results from MrModeltest v.2.0 for the combined *rps16* and *trnL-F* dataset, we used the GTR+I+G model of nucleotide substitution. Posterior distributions of parameters were approximated using two independent MCMC analyses of 20 million generations with a 10% burn-in. Samples from the two runs, which yielded similar results, were combined and convergence of the chains was checked using the program Tracer v.1.4 (Drummond & Rambaut, 2007). The samples from the posterior distribution were summarized on the maximum clade credibility (MCC) tree, which has the maximum sum of posterior probabilities on its internal nodes (Drummond & al., 2007), using TreeAnnotator v.1.5.4 (Drummond & Rambaut, 2007) with the posterior probability limit set to 0.5 and mean node heights summarized. The MCC tree was visualized using FigTree v.1.3.1, from which the means and 95% higher posterior densities (HPD) could be obtained. The 95% HPD represent the shortest interval that contains 95% of the sampled values from the posterior (Drummond & al., 2007).

The fossil record for Plantaginaceae is poor. Pollen referred to *Plantago* L. extends to the upper Miocene (5–11 million years ago [Ma]; Muller, 1981). Rønsted & al. (2002) calculated the time of the split between *Plantago* and *Aragoa* Kunth to 7.1 Ma, which is congruent with this fossil record. Thus, we constrained the *Plantago*-*Aragoa* node ("A" in Fig. 2) with the minimum age of 7.1 Ma. We applied a log-normal prior with offset of 7.1, and arbitrary mean and standard deviation of 1 to constrain this node (resulting in a prior distribution with upper 95% bound extending to ca. 11 Ma). Only a few other fossils have been reported for Lamiales (Manchester, 1999). Fossils of *Fraxinus* L. (Oleaceae) are known from the Eocene Claiborne Formation of southeastern North America (Call & Dilcher, 1992) and have been recorded from the Oligocene (Meyer & Manchester, 1997) and the Miocene of the Pacific Northwest (Chaney & Axelrod, 1959). The oldest reliable *Fraxinus* fossil is from the late Eocene of North America (Magallón-Puebla & al., 1999; Manchester, 1999). Seeds of *Catalpa* Scop. (Bignoniaceae) have been reported from Oregon from the early Oligocene (Meyer & Manchester, 1997; Manchester, 1999). The oldest reliable Bignoniaceae fossil is a fruit with seeds from the early Eocene (45–55 Ma) in Washington (Wehr & Hopkins, 1994; Pigg & Wehr, 2002; Wolfe & al., 2003). This fossil material was included in a previous study by Nie & al. (2006). In this paper, following Nie & al. (2006), the divergence between *Catalpa* and its relatives ("B" in Fig. 2) was constrained to 35 Ma. In addition, the Bignoniaceae node was constrained to 50 Ma ("C" in Fig. 2; see Wehr & Hopkins, 1994; Wolfe & al., 2003). The *Fraxinus*-*Osmanthus* node ("D" in Fig. 2) was constrained to 37 Ma. We follow the study of Nie & al. (2006) in setting the three constraints as normally distributed priors with a mean based on the age of the fossil record and an arbitrary standard deviation of 2. The soft-bounded constraints allow the posterior distribution to deviate from the prior expectations, which can represent uncertainty. The precise shape of the prior is however difficult to justify (Ho & Phillips, 2009) and it has been argued that uniform constraints with hard bounds are more appropriate to represent minimum constraints based on fossil evidence (Pirie & Doyle, 2012).

Biogeographic analyses. — The biogeographic history of *Lagotis* was investigated using RASP v.2.1 (Yu & al., 2012), which implements the S-DIVA (statistical dispersal-vicariance) method (Yu & al., 2010), and a maximum likelihood based method, LAGRANGE (Ree & al., 2005; Ree & Smith, 2008). To perform our analyses, we used the total combined data, and the matrix only included *Lagotis* and its close relative *Wulfenia*. Duplicate samples of *Lagotis* species were removed. Four areas were delimited based on the endemism and distribution of *Lagotis* and outgroup species: (A) the QTP (including adjacent regions), (B) Central Asia, (C) arctic and subarctic areas, and (D) Europe. Each terminal in the tree was coded according to the total distribution of the species (see Appendix 1). The outgroup of *Wulfenia* (here represented by *W. carinthiaca* Jacq.) was coded according to its overall distribution range.

In the S-DIVA analysis, we used the method described by Harris & Xiang (2009) to take into account phylogenetic uncertainty in the biogeographic reconstruction by utilizing the posterior distribution of trees resulting from the MrBayes analyses (the last 9000 of 10,000 trees, excluding burn-in). Results were summarized on the condensed tree that was computed from 9000 trees with "maxareas" constrained to three because no species currently occurs in more than three areas. In LAGRANGE, transitions between discrete states (ranges) along phylogenetic branches are modeled as a function of time, thus enabling maximum likelihood estimation of the ancestral states (range inheritance scenarios) at cladogenetic events. This program not only finds the most likely ancestral areas, but also calculates the probabilities of these most likely areas at each node (Ree & Smith, 2008). LAGRANGE was run using the MCC tree derived from the BEAST analyses.

■ RESULTS

The characteristics and statistics for the individual and combined nrITS and cpDNA regions based on the MP analyses are presented in Table 1. The alignments of the *matK*, *psbA-trnH*, *rps16*, *trnG-S*, and *trnL-F* datasets contained 480, 425, 837, 842, and 946 characters, respectively. The individual analyses of the five cpDNA datasets resulted in very low resolution among *Lagotis* species, with most branches unresolved in the five separate MP strict consensus trees (trees not shown). We combined all cpDNA datasets in our analysis because they exhibited low levels of sequence divergence and because incongruence is not expected from the uniparentally inherited, non-recombining plastid genome. The aligned matrix of the combined cpDNA data had 3530 positions including 291 (8.2%) parsimony informative sites. The alignment of the nrITS contained 612 characters, of which 167 (27.3%) were parsimony-informative.

The matrix of the combined cpDNA and nrITS data contained 4142 characters, including 458 (11.1%) parsimony-informative sites. The MP analysis of the total combined dataset yielded 1508 MPTs of 1729 steps, with a CI (excluding uninformative characters) of 0.603 and a RI of 0.775 (Table 1). The MP and BI analyses produced trees with similar topologies and only the BI phylogram is shown in Fig. 1 with parsimony bootstrap (BS) and Bayesian posterior probabilities (PP) indicated. The combined nrITS and cpDNA tree grouped sequences according to species, with the exception of *L. praecox* W.W.Sm. The BI phylogram (Fig. 1) shows that: (1) All species of *Lagotis* formed a monophyletic group with maximal support (BS = 100%, PP = 100%; Fig. 1); (2) *Lagotis* is sister to *Wulfenia* with moderate support (BS = 74%, PP = 96%); and (3) in *Lagotis*, four major clades (A–D) with high support values that correspond to the classification of Lu (1992) can be recognized. However, the monophyly of sect. *Lagotis* was not well supported and resolution of relationships among the four clades was low. Within clade A, three subclades (A-1, 2, 3) were recognized (Fig. 1). Subclade A-1, comprising *L. brevituba* Maxim., *L. angustibracteata* P.C.Tsoong & H.P.Yang, and *L. decumbens* Rupr., was sister to subclades A-2 plus A-3. *Lagotis decumbens*, whose position within *Lagotis* has been unclear, was found to be sister to *L. brevituba* and *L. angustibracteata* in subclade A-1 (Fig. 1). The six species endemic to the Central Himalayas formed subclade (A-2) and were sister to subclade (A-3). Three groups were recognized in subclade A-3: group I including *L. intergrifolia* (Willd.) Schischk., *L. minor* (Willd.) Standl., and *L. glauca* Gaertn. from Central Asian subarctic and arctic regions; group II comprising five accessions of *L. integra* W.W.Sm. from the QTP; and group III including *L. alutacea* W.W.Sm. and *L. yunnanensis* W.W.Sm. which are endemic to the Hengduan Mountains (HDM).

The molecular dating analysis suggested that *Lagotis* diverged from its sister group, *Wulfenia* in the early Miocene (Mean 19.3 Ma, 95% HPD: 11.3–29.6 Ma; node 1 in Fig. 2). The first radiation of *Lagotis*, which resulted in the formation of four major clades, was estimated to have occurred in the middle Miocene (Mean 12.7 Ma, 95% HPD: 7.3–19.5 Ma; node 2 in Fig. 2). The subarctic and arctic species, *L. minor*, and *L. glauca*, were estimated to have diverged from their closest relative, *L.integrifolia*, in the early Pleistocene (Mean 1.9 Ma, 95% HPD: 0.54–3.8 Ma; node 3 in Fig. 2).

The results from the LAGRANGE analysis (Fig. S1) were largely similar to those of the S-DIVA analysis (Fig. 3). Both reconstructions of ancestral areas indicated that the ancestral area of *Lagotis* is the QTP region, and that the genus subsequently migrated into the Central Asian highland. The results also suggested a single dispersal event into arctic and subarctic regions in the late Tertiary.

Table 1. Sequence characteristics of individual and combined datasets of *Lagotis*.

Parameters	nrITS	*matK*	*psbA-trnH*	*rps16*	*trnG-S*	*trnL-F*	Combined cpDNA	Combined nrITS + cpDNA
No. of sequences (ingroup/outgroup)	46 (37/9)	42 (35/7)	44 (36/8)	44 (35/9)	37 (35/2)	46 (37/9)	46 (37/9)	46 (37/9)
Aligned length [bp]	612	480	425	837	842	946	3530	4142
No. of parsimony-informative characters (%)	167 (27.3)	31 (6.5)	81 (19.1)	67 (8.0)	56 (6.7)	56 (5.9)	291 (8.2)	458 (11.1)
No. of trees (MP)	524	141	177	274	620	238	1729	1508
MP tree length [bp]	533	123	262	243	256	217	1166	1729
Consistency index (CI)[a]	0.604	0.698	0.705	0.706	0.710	0.710	0.631	0.603
Retention index (RI)	0.768	0.875	0.816	0.892	0.814	0.892	0.805	0.775

[a] The CI is calculated by excluding uninformative characters.

■ DISCUSSION

Monophyly and phylogenetic relationships of *Lagotis*. — Our molecular phylogenetic analyses using the combined nrITS and cpDNA dataset suggested that all sampled species of *Lagotis* form a monophyletic group with maximal support (Fig. 1). The monophyly of this genus is also supported by morphological and chromosomal evidence. *Lagotis* is characterized within Veroniceae by its reduced number of ovules (one per locule), reduced number of seeds (one or two), and schizocarpic fruit (Thieret, 1967). In addition, all chromosome numbers of *Lagotis* species were reported to be based on $x = 11$, which is unique in Veroniceae (Albach & al., 2008). All these characteristics make this genus a distinct group within Veroniceae.

Our phylogenetic results based on the combined dataset robustly support four clades within *Lagotis* (Fig. 1). Although the monophyly of sect. *Lagotis* was not supported due to the lack of phylogenetic resolution among four clades, our results largely correspond to the classification outlined by Lu (1992), who divided the genus into two sections: sect. *Acaules* and sect. *Lagotis*, with the latter subdivided into three series: ser. *Pharicae*, ser. *Ramalanae* and ser. *Lagotis*. Difficulty in resolving relationships among these clades may be attributed to low genetic divergence resulting from their rapid radiation (Baldwin & Sanderson, 1998; Richardson & al., 2001; Sun & al., 2012).

The two sections of Lu are morphologically distinct. Section *Acaules* is characterized by stoloniferous stems, root crowns enclosed by fibrous remnants of old leaves, and broadly linear to lanceolate leaf blades (Yang, 1979; Lu, 1992). In

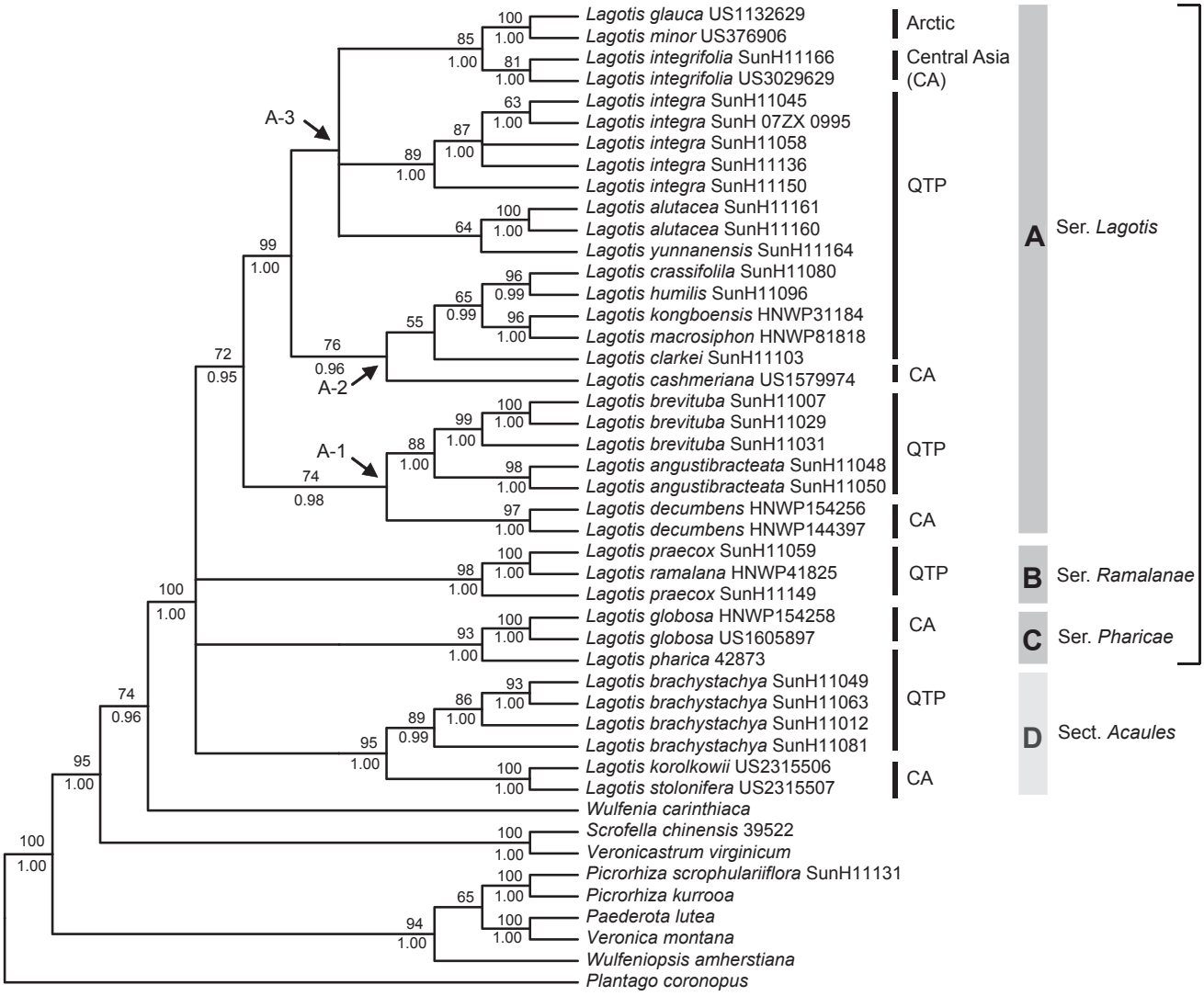

Fig. 1. Bayesian majority-rule consensus tree, inferred from the combined data of five chloroplast markers (*matK*, *psbA-trnH*, *rps16*, *trnG-S*, *trnL-F*) and nrITS sequences. Posterior probabilities (>0.70) are noted below branches, bootstrap support values (>50%) are indicated above branches. Four clades (A–D) and subclades (A1–A3) were recognized in *Lagotis*. Classification of *Lagotis* follows Lu (1992).

contrast, the species of sect. *Lagotis* share morphological characters such as conspicuous erect stems, leaves broad, rounded, or elliptic to ovate, and scapes usually as long as or longer than the radical leaves. Section *Acaules* contains only three species, which are disjunctly distributed between the QTP and Central Asia, and are found in more arid habitats than the species of sect. *Lagotis*. The species of sect. *Acaules* form a strongly supported clade (BS = 95%, PP = 100%; "D" in Fig. 1) in the phylogeny. In this clade, *L. brachystachya* Maxim., endemic to the QTP, is sister to the two species (*L. korolkowii* Maxim., *L. stolonifera* (C.Koch)Maxim.) from Central Asia. The close relationship between *L. korolkowii* and *L. stolonifera* is also supported by shared morphological characters including their glabrous habit and non-lobed ovary (Li, 1954).

Within sect. *Lagotis*, all three series of Lu (1992) were found to be monophyletic in our analyses (Fig. 1). However, our data did not resolve the monophyly of the section nor the relationships among the three series (ser. *Lagotis*, ser.

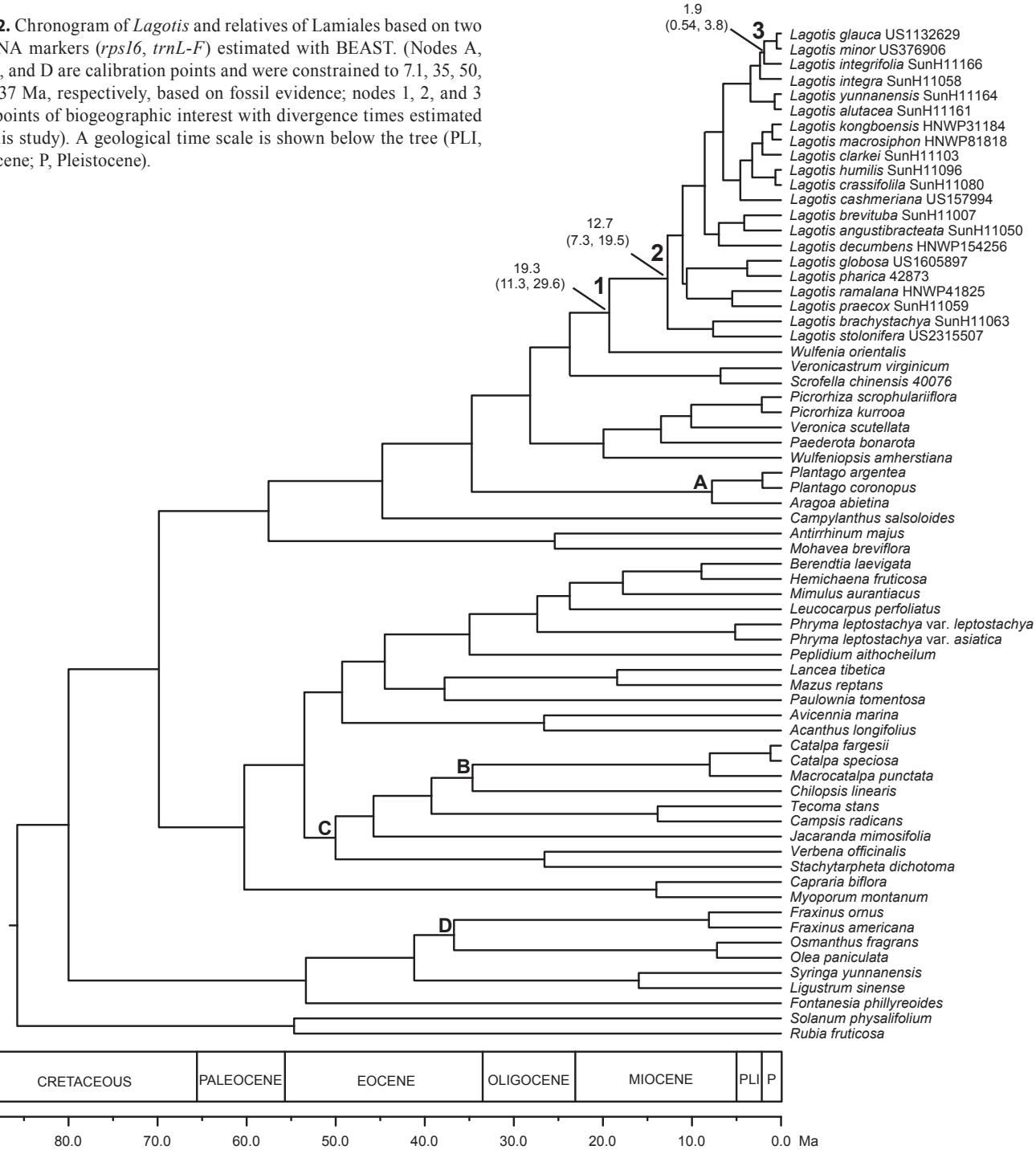

Fig. 2. Chronogram of *Lagotis* and relatives of Lamiales based on two cpDNA markers (*rps16*, *trnL-F*) estimated with BEAST. (Nodes A, B, C, and D are calibration points and were constrained to 7.1, 35, 50, and 37 Ma, respectively, based on fossil evidence; nodes 1, 2, and 3 are points of biogeographic interest with divergence times estimated in this study). A geological time scale is shown below the tree (PLI, Pliocene; P, Pleistocene).

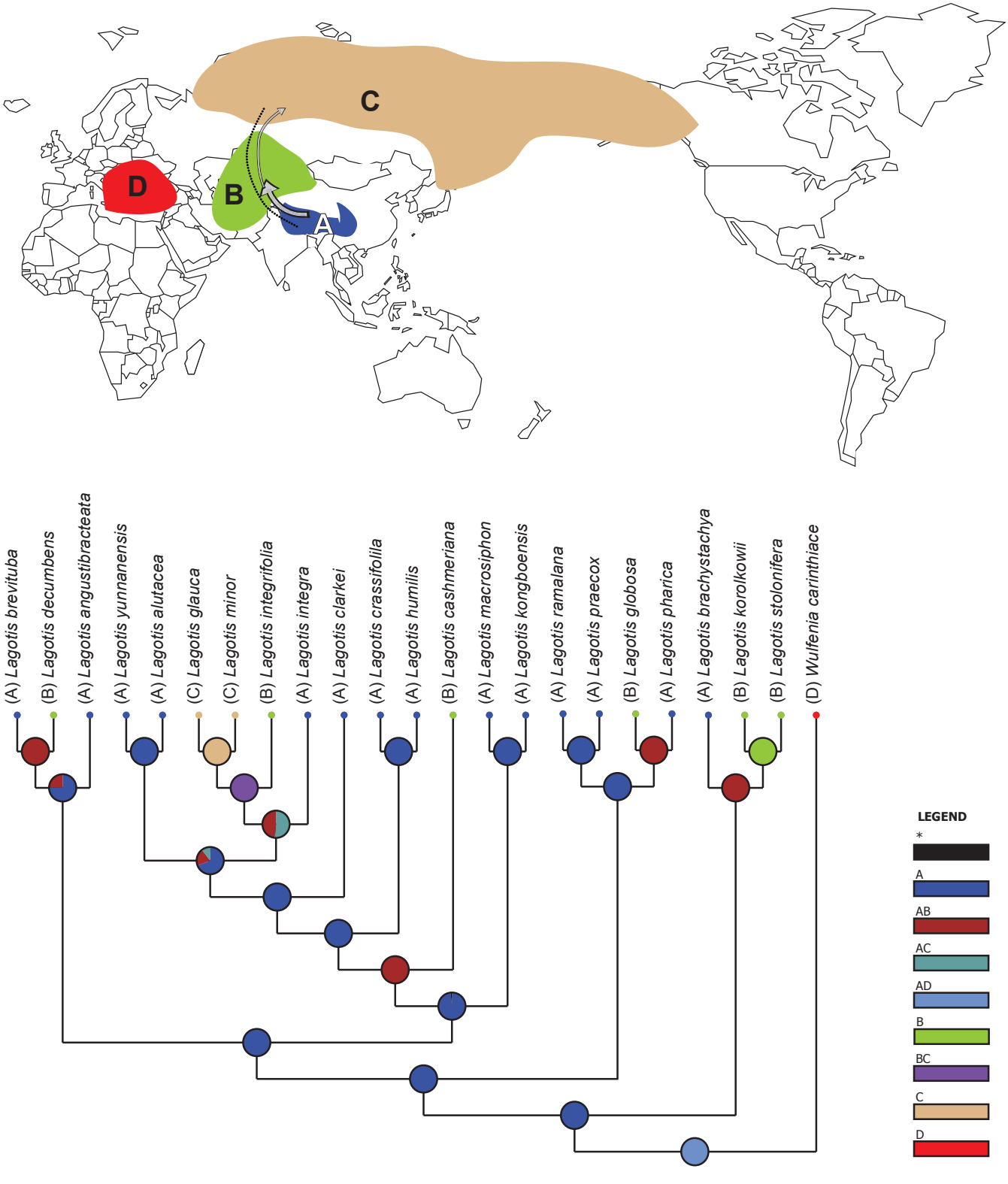

Fig. 3. Statistical dispersal-vicariance (S-DIVA) model of ancestral area reconstruction (AAR) in *Lagotis* based on a reduced BEAST combined-gene chronogram. The AARs are shown as colored circles at each node. Partitioning of the distribution area, based on centers of endemism of *Lagotis* and its closest relative (*Wulfenia carinthiaca*), is shown in the map: A, QTP (including adjacent regions); B, Central Asia; C, arctic and subarctic areas; and D, Europe. The dotted line represents the "Central Asiatic Highland Corridor" proposed by Ohba (1988). The curved arrows show the possible migrating route of *Lagotis*. A color key of ancestral ranges at different nodes is provided in the map.

Ramalanae, ser. *Praricae*). Series *Ramalanae* contains only two species endemic to the QTP and both were included in our analysis, represented by two individuals of *L. praecox* and one of *L. ramalana*. It has long been debated whether the two species should be treated as a single species. Li (1954) thought that *L. praecox* was undoubtedly identical with *L. ramalana* based on careful examination of the type specimens. However, Yang (1979) argued that many morphological characters of *L. praecox* differ from those of *L. ramalana* Batalin. For example, the petioles and abaxial leaf surfaces of *L. praecox* are purple-red, whereas those of *L. ramalana* are green; the bracts of *L. praecox* are subleathery but papery in *L. ramalana*. Our phylogenetic analyses appear to support Li (1954; clade B, Fig. 1), but more samples of these two taxa are needed to contribute to this debate. Series *Pharicae* is comprised of only two species: *L. pharica* Prain, a narrow endemic to the HDM, and *L. globosa* Hook.f. of the western Himalayas, and they formed a clade (clade C, Fig. 1). Morphologically, the species of ser. *Pharicae* are very similar to those of ser. *Ramalanae* (clade B), but the species of ser. *Pharicae* can be distinguished by having more deeply dissected leaves (Lu, 1992). According to our molecular phylogeny (Fig. 1), however, the close relationship between the two clades was not resolved.

Our samples of ser. *Lagotis* along with the unclassified species, *L. decumbens*, endemic to the Central Asian highlands, form a moderately supported clade in our analyses (clade A, Fig. 1). Morphologically, *L. decumbens* is characterized by petiolate leaves, bisected calyx, and short filaments, which suggest its close affinity to the species of ser. *Lagotis*. Thus, morphological data also support the placement of *L. decumbens* in ser. *Lagotis*.

Biogeographic history of *Lagotis*. — Appropriate calibration of molecular dating analyses is controversial, and the results should be treated with caution. However, when paleontological data are lacking, molecular estimates provide the only means of inferring the age of lineages (Li, 1997; Bromham & Penny, 2003). Multiple fossil-based calibration points and multiple molecular markers are important for the accuracy of divergence time estimates. In this paper, we used the combined cpDNA (*rps16*, *trnL-F*) dataset and the same fossil material as used in previous work (Nie & al., 2006). It was estimated that *Lagotis* diverged from its closest relative in the early Miocene (Mean 19.3 Ma, 95% HDP 11.3–29.6 Ma; node 1 in Fig. 2). *Wulfenia* is a small genus with three species which has a European–eastern Mediterranean distribution (Albach & al., 2004b). The distribution of *Lagotis* and its closest relative, *Wulfenia*, shows an east-west disjunction in Eurasia. Such a pattern may be circumstantial evidence for an earlier more continuous Tethyan distribution across Eurasia (Wu, 1988; Sun, 2002b). The uplift of the QTP in the Tertiary has been postulated as the major factor for the formation of this disjunct pattern, and this has been corroborated by a number of molecular phylogenetic studies of plants, including *Helleborus* L. (Ranunculaceae; Sun & al., 2001), *Cedrus* Trew (Pinaceae; Qiao & al., 2007), and Hyoscyameae and Mandragoreae (Solanaceae; Tu & al., 2010). Thus, the age of the split between *Lagotis* and *Wulfenia* coincides with the Tertiary vicariance hypothesis, and the current disjunction of the two genera supports the hypothesis of a disruption of the once continuous Tethyan distribution caused by the uplift of the Himalayas and the aridification of Central Asia.

At present, more than half of the species of *Lagotis* occur in the QTP, so that the uplift of the QTP may be one factor contributing to this high species diversity. On the basis of our results, we inferred that the QTP origin of *Lagotis* dates back to the Miocene, and that the species migrated out of this area through the Miocene and until the Pleistocene (Fig. 2). Our biogeographic analyses showed that the early diversification of the genus might have occurred predominantly in the QTP with subsequent multiple dispersals to the Central Asian highlands (Fig. 3).

Since 40 Ma (Chung & al., 1998), the QTP has persistently experienced tectonic uplift, playing an important role in the rapid differentiation and diversification of taxonomic groups. Geological evidence indicates that extensive uplifting of the QTP occurred during at least four different periods since the early Miocene, i.e., 25–17 Ma, 15–13 Ma, 8–7 Ma, and 3.5–1.6 Ma (Harrison & al., 1992; Li & al., 1995; Shi & al., 1998; Spicer & al., 2003). The origin of *Lagotis* (19.3 Ma, 95% HPD 11.3–19.6 Ma; node 1 in Fig. 2) might, therefore, be linked to the first period of uplift in the region of Tibet during the early Miocene 25–17 Ma; this has been called the Himalayan motion period and is one of the most significant events in the overall uplift of the QTP (Shi & al., 1999). Based on molecular phylogenetic and biogeographic analyses, many plant and animal taxa have been inferred to have evolved in response to the early Miocene QTP uplift (Guo & al., 2005; Liu & al., 2006). For example, both *Nannoglottis* Maxim. (Asteraceae; Liu & al., 2002) and Chinese sisorid catfishes (Guo & al., 2005) originated in the QTP at the Oligocene-Miocene boundary (24–19 Ma). The diversification events that resulted in four clades of *Lagotis* were estimated to have occurred in the middle Miocene (mean 12.7 Ma, 95% HPD 7.3–19.5 Ma; node 2 in Fig. 2). This timeframe of the early diversification is highly consistent with timeframes of diversification estimated for other genera of the QTP (e.g., *Pedicularis* L., Yang & al., 2003; *Saussurea* DC., Wang & al., 2009; the *Ligularia-Cremanthodium-Parasenecio* complex, Liu & al., 2006; *Caragana* Fabr., Zhang & Fritsch, 2010; *Rheum*, Sun & al., 2012), and the diversification has been considered to have been triggered by the earlier uplifts of the QTP.

The pattern found in *Lagotis* is partly consistent with that of *Gentiana* sect. *Cruciata* Gaudin (Gentianaceae; Zhang & al., 2009), *Androsace* L. (Primulaceae; Wang & al., 2004), *Aconitum* L. (Ranunculaceae; Luo & al., 2005), and *Rheum* L. (Polygonaceae; Sun & al., 2012). In these cases, the less diversified European clades are also closely related to those occurring in the QTP and in the adjacent Himalayan region. Their link seems to have been via the intervening Central Asian highland (Wang & al., 2004; Luo & al., 2005; Zhang & al., 2009; Sun & al., 2012). These studies support the long-standing hypothesis that modern alpine plants in Central Asia and Europe originated from the QTP and/or west China (Wulff, 1943; Wu, 1987, 1988; Ozenda, 1988; Axelrod & al., 1996; Wu & Wu, 1996). However, other biogeographic patterns have been proposed elsewhere (Sun & al., 2001; Milne, 2004; Tu & al., 2010). One

notable example is by Zhang & al. (2007), who investigated the genus *Epimedium* L. (Berberidaceae) and found that the European species are not closely related to those occurring on the QTP. Their link seems not to have been through the intervening mountain regions, but probably through a more northerly deciduous forest belt at some point in the past.

In this study, the arctic and subarctic species, *L. minor* and *L. glauca*, formed a subclade with *L. intergrifolia* of Central Asia, and these three species were deeply nested among species from the QTP and adjacent areas (Fig. 1), suggesting a single dispersal event into the arctic and subarctic regions in the late Tertiary. The sister-group relationship inferred between the arctic species and the Central Asian *L. integrifolia* supports the "Central Asiatic Highland Corridor" proposed by Ohba (1988) as a pathway for the migration of plants between the two regions. It has been suggested that high mountains could have served as an important source area for arctic plants because alpine taxa have a similar ecology to arctic taxa and could have migrated into the arctic along mountain ridges from areas further south (Murray, 1995; Abbott & Brochmann, 2003). Abbott & al. (2000) surveyed cpDNA variation across a large part of the range of the circumpolar arctic-alpine *Saxifraga oppositifolia* L. (Saxifragaceae). Their results indicated that *S. oppositifolia* might be derived from an ancestral stock located in the high mountains of Central Asia, which migrated to the Arctic in northern Siberia along mountain ranges that connect these two regions (Abbott & al., 2000; Abbott & Brochmann, 2003). The arctic flora is thought to have originated during the late Tertiary, approximately 3 Ma (Abbott & al., 2000). The present-day arctic flora replaced the Arcto-Tertiary forest at high latitudes when global temperatures dropped markedly towards the end of the Pliocene (Murray, 1995). Our divergence dates suggested that the subarctic and arctic species *L. minor* and *L. glauca* diverged from their closest relative, *L. integrifolia*, approximate in the early Pleistocene (Mean 1.9 Ma, 95% HPD 0.54–3.8 Ma; node 3 in Fig. 2).

The dispersal of subarctic and arctic species may also be related to Pleistocene cycles of glaciation. During the Quaternary (from 1.8 Ma) recurrent cycles of ice ages (glaciations) interspersed with shorter warmer periods (interglacials) caused arctic and alpine plants to experience frequent range fragmentations and expansions (Abbott, 2008).

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Appendix 1. Voucher information and GenBank accession numbers for specimens used in this study.

Taxon; GenBank accessions: nrITS; *trnL-F*; *psbA-trnH*; *rps16*; *matK*; *trnG-S* [a dash (–) indicates missing sequence]; voucher specimen (herbarium), collection locality; [scoring for biogeographic analysis].

INGROUPS: ***Lagotis alutacea*** W.W.Sm.; KC413410; KC413568; KC413489; KC413529; KC413450; KC413608; *H. Sun 11160* (KUN), China, Yunnan; [A]. ***L. alutacea***; KC413411; KC413569; KC413490; KC413530; KC413451; KC413609; *H. Sun 11161* (KUN), China, Yunnan. ***L. angustibracteata*** P.C.Tsoong & H.P.Yang; KC413412; KC413570; KC413491; KC413531; KC413452; KC413610; *H. Sun 11048* (KUN), China, Qinghai; [A]. ***L. angustibracteata***; KC413413; KC413571; KC413492; KC413532; KC413453; KC413611; *H. Sun 11050* (KUN), China, Qinghai. ***L. brachystachya*** Maxim.; KC413414; KC413572; KC413493; KC413533; KC413454; KC413612; *H. Sun 11012* (KUN), China, Qinghai; [A]. ***L. brachystachya***; KC413415; KC413573; KC413494; KC413534; KC413455; KC413613; *H. Sun 11049* (KUN), China, Tibet. ***L. brachystachya***; KC413416; KC413574; KC413495; KC413535; KC413456; KC413614; *H. Sun 11063* (KUN), China, Qinghai. ***L. brachystachya***; KC413417; KC413575; KC413496; KC413536; KC413457; KC413615; *H. Sun 11081* (KUN), China, Tibet. ***L. brevituba*** Maxim.; KC413418; KC413576; KC413497; KC413537; KC413458; KC413616; *H. Sun 11007* (KUN), China, Qinghai; [A]. ***L. brevituba***; KC413419; KC413577; KC413498; KC413538; KC413459; KC413617; *H. Sun 11029* (KUN), China, Qinghai. ***L. brevituba***; KC413420; KC413578; KC413499; KC413539; KC413460; KC413618; *H. Sun 11031* (KUN), China, Qinghai. ***L. cashmeriana*** Rupr.; KC413421; KC413579; KC413500; –; KC413461; –; *M. Nath s.n.* (US 1579974), India, Rohtang; [B]. ***L. clarkei*** Hook.f.; KC413422; KC413580; KC413501; KC413540; KC413462; KC413619; *H. Sun 11103* (KUN), China, Tibet; [A]. ***L. crassifolia*** Prain; KC413423; KC413581; KC413502; KC413541; KC413463; KC413620; *H. Sun 11080* (KUN), China, Tibet; [A]. ***L. decumbens*** Rupr.; KC413434; KC413592; KC413513; KC413552; KC413474; KC413632; *S.G. Wu, Y.H. Wu & Y. Fei 4904* (HNWP 154256), China, Xinjiang; [B]. ***L. decumbens***; KC413424; KC413582; KC413503; KC413542; KC413464; KC413621; *Y.H. Wu 870675* (HNWP 144397), China, Xinjiang. ***L. minor*** (Wild.) Standl.; KC413425; KC413583; KC413504; KC413543; KC413465; KC413622; *L. Stejneger 152* (US 1132629), U.S.A., Alaska; [C]. ***L. glauca*** Gaertn.; KC413426; KC413584; KC413505; KC413544; KC413466; KC413623; *L. Stejneger 180* (US 376906), Russia, The Commander Island; [C]. ***L. globosa*** Hook.f.; KC413427; KC413585; KC413506; KC413545; KC413467; KC413624; *S.G. Wu, Y.H. Wu & Y. Fei 4906* (HNWP 154258), China, Xinjiang; [B]. ***L. globosa***; KC413428; KC413586; KC413507; KC413546; KC413468; KC413625; *W. Koelz 2800a* (US 1605897), India, Kashmir. ***L. humilis*** Tsoong & Yang; KC413429; KC413587; KC413508; KC413547; KC413469; KC413626; *H. Sun 11096* (KUN), China, Tibet; [A]. ***L. integra*** W.W.Sm.; KC413430; KC413588; KC413509; KC413548; KC413470; KC413628; *H. Sun 11045* (KUN), China, Qinghai; [A]. ***L. integra***; KC413431; KC413589; KC413510; KC413549; KC413471; KC413629; *H. Sun 11058* (KUN), China, Tibet. ***L. integra***; KC413432; KC413590; KC413511; KC413550; KC413472; KC413630; *H. Sun 11136* (KUN), China, Tibet. ***L. integra***; KC413433; KC413591; KC413512; KC413551; KC413473; KC413631; *H. Sun 11150* (KUN), China, Tibet. ***L. integra***; KC413435; KC413593; KC413514; KC413553; KC413475; KC413627; *H. Sun 07ZX-0995* (KUN), China, Tibet. ***L. integrifolia*** (Wild.) Schischk.; KC413437; KC413595; KC413516; KC413555; KC413477; KC413633; *H. Sun 11166* (KUN), China, Xinjiang; [B]. ***L. integrifolia***; KC413438; KC413596; KC413517; KC413556; KC413478; KC413634; *T. Elias, S. Shetler & D. Murray* (US 3029629), Kazakhstan. ***L. kongboensis*** Yamazaki; KC413439; KC413597; KC413518; KC413557; KC413479; KC413635; *Y.H. Wu & al. 1816* (HNWP 31184), China, Tibet; [A]. ***L. korolkowii*** Maxim; KC413440; KC413598; KC413519; KC413558; KC413480; KC413636; *Maio 1850* (US 2315506), Armenia; [B]. ***L. macrosiphon*** Tsoong & Yan; KC413441; KC413599; KC413520; KC413559; KC413481; KC413637; *K.Y. Lang & al. 650* (HNWP 81818), China, Tibet; [A]. ***L. pharica*** Prain; KC413442; KC413600; KC413521; KC413560; KC413482; KC413638; *H. Sun HD42873* (KUN) China, Sichuan; [A]. ***L. praecox*** W.W.Sm.; KC413443; KC413601; KC413522; KC413561; KC413483; KC413639; *H. Sun 11059* (KUN), China, Tibet; [A]. ***L. praecox***; KC413444; KC413602; KC413523; KC413562; KC413484; KC413640; *H. Sun 11149* (KUN), China, Sichuan. ***L. ramalana*** Batalin; KC413445; KC413603; KC413524; KC413563; KC413485; KC413641; *Q. Feng & al. 288* (HNWP 41825), China, Qinghai; [A]. ***L. stolonifera*** (C.Koch) Maxim.; KC413446; KC413604; KC413525; KC413564; –; KC413642; *Maio 1853* (US 2315507), Kazakhstan; [B]. ***L. yunnanensis*** W.W.Sm.; KC413447; KC413605; KC413526; KC413565; KC413486; KC413643; *H. Sun 11164* (KUN), China, Yunnan; [A]. — **OUTGROUPS:** ***Paederota lutea*** L.f.; AF313024; AF486408; FJ848091; AY218807; –; –. ***Picrorhiza kurroa*** Royle; AF509813; AF486414; FJ848090; AY218806; AY492159; –. ***P. scrophulariiflora*** (Pennell) D.Y.Hong; KC413448; KC413606; KC413527; KC413566; KC413487; KC413644; *H. Sun 11131* (KUN), China, Tibet. ***Plantago coronopus*** L.; AF515217; AF486419; –; AY218801; AY492160; –. ***Scrofella chinensis*** Maxim.; KC413449; KC413607; KC413528; KC413567; KC413488; KC413645; *H. Sun HD39522* (KUN), China, Sichuan; KUN. ***Veronicastrum virginicum*** (L.) Farw.; AF313030; AF486412; FJ848087; AY218802; AY492168; –. ***Veronica montana*** L.; AF313014; AF486388; FJ848095; AY218824; AY492167; –. ***Wulfenia carinthiace*** Jacq.; AF313025; AF486409; FJ848088; AY218804; AY492169; –; [D]. ***Wulfeniopsis amherstiana*** (Benth.) D.Y.Hong; AF515216; AF486411; FJ848089; AY218808; –; –.

Appendix 2. Sequences of Lamiales downloaded from GenBank for divergence time analysis. A dash (–) indicates a missing sequence.

Taxon: GenBank accessions, *rps16*, *trnL-F*.

Acanthus longifolius Host: AJ431037, AJ430912; ***Antirrhinum majus*** L.: AY492195, AJ492270; ***Aragoa abietina*** Kunth: AY492194, HQ593823; ***Avicennia marina*** (Forssk.) Vierh.: AJ431038, AJ430913; ***Berendtia levigata*** Robinson & Greenm.: AJ609208, AJ608615; ***Campsis radicans*** Seem.: –, AY695865; ***Campylanthus salsoloides*** Webb: AY492199, AY492173; ***Capraria biflora*** L.: AJ609198, AJ608608; ***Catalpa fargesii*** f. ***duclouxii*** (Dode) Gilmour: DQ532491, DQ532488; ***Catalpa speciosa*** (Warder) Engelm.: AJ609197, AJ608599; ***Chilopsis linearis*** (Cav.) Sweet: DQ532492, DQ532489; ***Digitalis obscura*** L.: AY218799, AF486418; ***Fontanesia phillyreoides*** Labill.: AF225226, AF231818; ***Fraxinus americana*** L.: AF225233, AF231825; ***Fraxinus ornus*** L.: AF225240, AF231832; ***Hemichaena fruticosa*** Benth.: AJ609179, AY575501; ***Jacaranda mimosifolia*** D.Don: AJ431039, AJ430914; ***Lancea tibetica*** Hook.f. & Thomson: AJ609174, AF479003; ***Ligustrum sinense*** Lour.: AF225256, AF231847; ***Leucocarpus perfoliatus*** Benth.: –, AF478998; ***Macrocatalpa punctata*** (Griseb.) Britton: DQ532490, DQ532487; ***Mazus reptans*** N.E.Br.: –, F479004; ***Mimulus aurantiacus*** Curt.: AJ609163, AF478982; ***Mohavea breviflora*** Coville: AJ609223, AF479011; ***Myoporum montanum*** R.Br.: AJ431059, AJ430934; ***Olea paniculata*** Roxb.: AF225276, AF231867; ***Osmanthus fragrans*** Lour.: AF225278, AF231869; ***Paulownia tomentosa*** (Thunb.) Steud.: AJ609153, AF479005; ***Peplidium aithocheilum*** W.R.Barker: –, AF479002; ***Phryma leptostachya*** var. ***asiatica*** H.Hara: DQ532461, DQ532481; ***Phryma leptostachya*** var. ***Leptostachya*** L.: DQ532445, DQ532471; ***Plantago argentea*** Chaix: AJ431056, AJ430931; ***Plantago coronopus*** L.: AY218801, AY101937; ***Rubia fruticosa*** Ait. (outgroup): AF004078, AF102475; ***Solanum physalifolium*** Rusby (outgroup): AY727449, AY727207; ***Stachytarpheta dichotoma*** (Ruiz & Pavon) Vahl: AJ299259, AJ299260; ***Syringa yunnanensis*** Franch.: AF225293, AF231883; ***Tecoma stans*** (L.) Juss. ex Kunth: –, AY008826; ***Verbena officinalis*** L.: AF225295, AF231885; ***Veronica scutellata*** L.: AY218823, AF486393.