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Phylogenetic Position of *Lagotis kunawurensis* (Plantaginaceae) in Himalaya, a Species of Boreal *L. glauca* Aggregate

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Abstract Phylogenetic position of a Himalayan species *Lagotis kunawurensis* is elucidated. This species is recognized as a member of *L. glauca* aggregate. The idea of close relationship between *L. kunawurensis* in Himalaya and *L. glauca* in the north pacific rim, however, contradicts with the scenario of the migration of the genus from Himalaya to the boreal region indirectly via Central Asian highlands. In our phylogenetic analysis of the genus based on chloroplast DNA sequence data, *L. kunawurensis* and *L. glauca* were placed together in a clade of ser. *Lagotis*. Within the clade, *L. kunawurensis* formed a subclade with other six Himalayan species, among which *L. clarkei* was its sister species. Phylogenetic affinity of *L. kunawurensis* to *L. glauca* is not supported, disagreeing with the view that *L. kunawurensis* is a member of *L. glauca* aggregate and being compatible with the migratory scenario.

Keywords: alpine plant, Bhutan, Himalaya, *Lagotis*, molecular phylogeny, series assignment.

Introduction

Lagotis J.Gaertn. (Plantaginaceae) comprises ca. 30 species of perennial herbs (Hong *et al.*, 1998) distributed in Himalaya, Central Asia, boreal Asia, and northwest America (Lu, 1992). In the genus, two sections (*Lagotis* and *Acaules* Maxim.) and three series (*Lagotis*, *Pharicae* X.F.Lu, and *Ramalanae* X.F.Lu) in sect. *Lagotis* are recognized (Lu, 1992). Molecular phylogeny of *Lagotis* was published including ca. 80% of the described species (Li *et al.*, 2014), and sect. *Acaules* and the three series were reported to be respectively monophyletic. Besides, the molecular analysis well covered the geographic range of the genus and inferred the migratory history (Li *et al.*, 2014); *Lagotis* have likely originated in the Tibetan Plateau in the Miocene and diversified there from the Miocene to Pleistocene, and spread to the boreal region via Central Asian highlands.

Among the unanalyzed species in the molecular study (Li *et al.*, 2014) is *L. kunawurensis* (Royle ex Benth.) Rupr. (Fig. 1A). This species is broadly distributed in Himalaya (Bhutan, India, Nepal, and Pakistan) at around 2,700–5,600 m asl. and grows on wet semi-stable scree and marshland (Polunin *et al.*, 1987; Grierson

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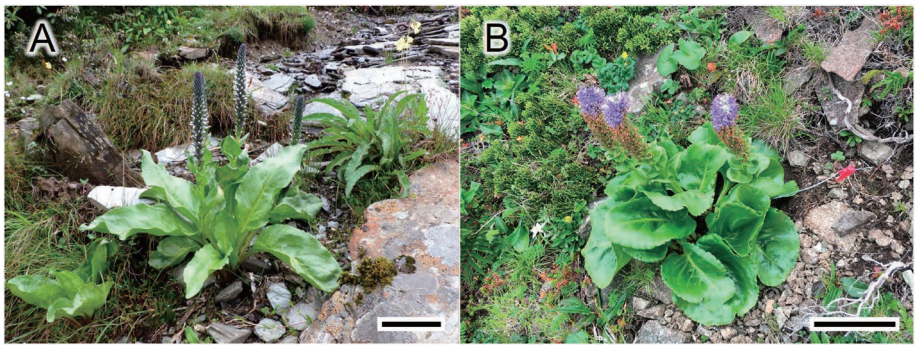

Fig. 1. Habit of *Lagotis kunawurensis* (A; Mt. Jomolhari range, western Bhutan) and *L. glauca* (B; Rebun Island, northern Japan). Scale bars indicate 5 cm.

and Long, 2001; GBIF.org, 2021). *Lagotis kunawurensis* is recognized as a member of *L. glauca* aggregate, a group of species with highly variable and overlapping morphology (Grierson and Long, 2001) and was previously named as *L. glauca* Gaertn. var. *sikkimensis* Hook.f., which name is now treated as its synonym (Grierson and Long, 2001). *Lagotis glauca* is a boreal species and widely distributed in Japan (Rebun Island in northernmost Japan and alpine ranges of central Honshu), the Kuriles, Kamchatka, the Aleutian, and Alaska (Yamazaki, 1993; Takahashi, 2015) (Fig. 1B). The idea of close relationship between *L. kunawurensis* in Himalaya and *L. glauca* in the north pacific rim, however, contradicts with the above-mentioned "Central Asiatic Highland Corridor" scenario for migration from Himalaya to the boreal region (Li *et al.*, 2014). The species relationship should be clarified with molecular analysis. This study aims to elucidate the phylogenetic position of *L. kunawurensis* and to discuss its series assignment and the relationship with *L. glauca*.

Materials and Methods

In our collaborative field research in Mt. Jomolhari range, Paro, Bhutan, a sample of *L. kunawurensis* was collected. The voucher specimen (voucher number: THIM16153) was deposited in the National Herbarium (THIM) of National Biodiversity Centre, Bhutan. DNA sequence data obtained from the sample were analyzed together with GenBank data used in Li *et al.* (2014), that included 22 species from China, northeastern Russia, Kazakhstan, Armenia, Alaska, and India (Table 1). For monophyletic species in Li *et al.* (2014), one sample each was randomly selected and used (in *L. integrifolia*, *L. integra*, *L. alutacea*, *L. brevituba*, *L. angustibracteata*, *L. decumbens*, *L. globosa*, and *L. brachystachya*). *Wulfenia carinthiaca* Jacq. was selected as an outgroup following Li *et al.* (2014).

Genomic DNA was extracted from the silica gel-dried leaf sample of *L. kunawurensis* using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990). Four chloroplast DNA regions (*mat*K, *rps*16, *trn*G–*trn*S, and *trn*L–F) used in Li *et al.* (2014) were PCR amplified using common primers (Taberlet *et al.*, 1991; Johnson and Soltis, 1994; Steele and Vilgays, 1994; Oxelman *et al.*, 1997; Hamilton, 1999). PCR amplification was performed in 20 µl total volume: 10 µl of Taq DNA polymerase 2 × master mix (Amplicon, Rødovre, Denmark), 0.8 µl of each forward and reverse primers (10 pmol/µl), 0.4 µl of dimethyl sulfoxide (DMSO), 0.8 µl of total DNA (ca. 20 ng/µl) and 7.2 µl of distilled water. The PCR cycle conditions were as follows: initial template denaturation for 4 min at 95°C; 25 cycles of 50 s at 94°C, 40 s at 50°C, and 40 s at 72°C; a final extension for 10 min at 72°C. The purified PCR products were amplified using

Table 1. Section/series assignment, sample locality, and GenBank accession numbers of *Lagotis* species in the phylogenetic analysis. A dash in GenBank accession indicates missing data

Taxa	Section/series	Sample locality	*mat*K	*rps*16	*trn*G–*trn*S	*trn*L–F
L. alutacea W.W.Sm.	*Lagotis*/*Lagotis*	Yunnan, China	KC413450	KC413529	KC413608	KC413568
L. angustibracteata Tsoong & H.P.Yang	*Lagotis*/*Lagotis*	Qinghai, China	KC413452	KC413531	KC413610	KC413570
L. brachystachya Maxim.	*Acaules*/—	Qinghai, China	KC413454	KC413533	KC413612	KC413572
L. brevituba Maxim.	*Lagotis*/*Lagotis*	Qinghai, China	KC413459	KC413538	KC413617	KC413577
L. cashmeriana Rupr.	*Lagotis*/*Lagotis*	Rohtang, India	KC413461	—	—	KC413579
L. clarkei Hook.f.	*Lagotis*/*Lagotis*	Tibet, China	KC413462	KC413540	KC413619	KC413580
L. crassifolia Prain	*Lagotis*/*Lagotis*	Tibet, China	KC413463	KC413541	KC413620	KC413581
L. decumbens Rupr.	*Lagotis*/*Lagotis*	Xinjiang, China	KC413474	KC413552	KC413632	KC413592
L. glauca Gaertn.	*Lagotis*/*Lagotis*	Commander Island, Russia	KC413465	KC413543	KC413622	KC413583
L. globosa Hook.f.	*Lagotis*/*Pharicae*	Xinjiang, China	KC413467	KC413545	KC413624	KC413585
L. humilis Tsoong & H.P.Yang	*Lagotis*/*Lagotis*	Tibet, China	KC413469	KC413547	KC413626	KC413587
L. integra W.W.Sm.	*Lagotis*/*Lagotis*	Qinghai, China	KC413470	KC413548	KC413628	KC413588
L. integrifolia (Willd.) Schischk.	*Lagotis*/*Lagotis*	Kazakhstan	KC413478	KC413556	KC413634	KC413596
L. kongboensis T. Yamaz.	*Lagotis*/*Lagotis*	Tibet, China	KC413479	KC413557	KC413635	KC413597
L. korolkowii Maxim.	*Acaules*/—	Armenia	KC413480	KC413558	KC413636	KC413598
L. kunawurensis (Royle ex Benth.) Rupr.	*Lagotis*/*Lagotis*	Paro, Bhutan	LC314879	LC314814	LC314909	LC314849
L. macrosiphon Tsoong & H.P.Yang	*Lagotis*/*Lagotis*	Tibet, China	KC413481	KC413559	KC413637	KC413599
L. minor (Willd.) Standl.	*Lagotis*/*Lagotis*	Alaska, USA	KC413466	KC413544	KC413623	KC413584
L. pharica Prain	*Lagotis*/*Pharicae*	Sichuan, China	KC413482	KC413560	KC413638	KC413600
L. praecox W.W.Sm.	*Lagotis*/*Rhamalanae*	Sichuan, China	KC413484	KC413562	KC413640	KC413602
L. ramalana Batalin	*Lagotis*/*Rhamalanae*	Qinghai, China	KC413485	KC413563	KC413641	KC413603
L. stolonifera Maxim.	*Acaules*/—	Kazakhstan	—	KC413564	KC413642	KC413604
L. yunnanensis W.W.Sm.	*Lagotis*/*Lagotis*	Yunnan, China	KC413486	KC413565	KC413643	KC413605
Wulfenia carinthiaca Jacq.	—	—	AY492169	AY218804	—	AF486409

ABI PRISM Big Dye Terminator v.3.1 (Applied Biosystems, Foster, CA, USA) with the same primers as those used for the PCR. DNA sequencing was performed on an ABI Prism 3730 DNA analyzer (Applied Biosystems). Automatic base-calling was checked manually using Chromas 2.6.2 (http://technelysium.com.au/wp/chromas/). Sequence alignment was conducted using ClustalX v.2.1 (Larkin *et al.*, 2007) and then edited manually using SeaView v. 4.6.1 (Gouy *et al.*, 2010).

Phylogenetic analyses were conducted using Bayesian (BI) and maximum parsimony (MP) inferences. To perform BI analyses, the best model of nucleotide substitution was estimated as SYM + G with MrModeltest (Nylander, 2004) using Akaike information criterion (AIC; Akaike, 1974). BI analysis was conducted using MrBayes 3.2.6 (Ronquist *et al.*, 2012). Analyses were run for 10 million generations, sampling every 1,000 generations. Convergence and effective sample size ($>$ 200 after burn-in) of all parameters were checked with Tracer v.1.6 (Drummond and Rambaut, 2007) and then the first 2,500 trees were discarded as burn-in. The 50% majority rule consensus tree of all the post-burn-in trees and posterior probability (PP) was calculated. The MP analysis was performed with PAUP* v.4.0a (Swofford, 2002) using heuristic searches of 1,000 replicates with random taxon addition and tree bisection reconnection (TBR) branch swapping, with the MulTrees and steepest descent options on, and the MaxTrees option set to 10,000. Indels were treated as missing data because they were difficult to align among the wide range of taxa. Bootstrap support (BS) was estimated from 1,000 replicates with random taxon addition and TBR branch swapping, with the MulTrees option off and the MaxTrees option set to 200. The BI and MP trees were displayed using Figtree v.1.3.1 (Drummond and Rambaut, 2007).

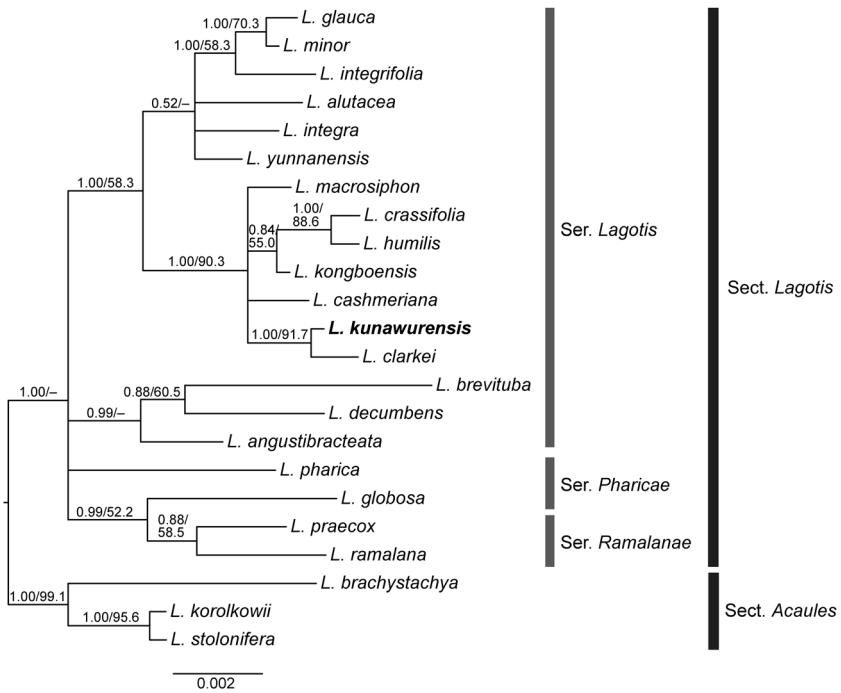

Fig. 2. Bayesian phylogenetic tree of 23 *Lagotis* species based on cpDNA. The outgroup *Wulfenia carinthiaca* is omitted from the figure for simplicity. Numerals on branches indicate Bayesian posterior probability (left) and bootstrap support (right) in the MP analysis. –means $<$ 50%. Scale bar indicates 0.002 substitutions per site.

Results and Discussion

The aligned length of cpDNA was 2,623 bp. In this study, clades with PP≥0.95 and/or BS≥70% were considered statistically supported. There was no topological incongruence between BI and MP trees (data not shown). Therefore, only BI tree is presented with BS. In the BI phylogenetic tree (Fig. 2), the two sections *Lagotis* (PP/BS = 1.00/—) and *Acaules* (1.00/99.1%) were reciprocally monophyletic. For each of the three series *Lagotis*, *Ramalanae*, and *Pharicae*, monophyly was not recovered. In the previous study (Li *et al.*, 2014), the three series were respectively monophyletic based on concatenated data of cpDNA and nuclear ribosomal DNA (nrITS). We tested the phylogenetic congruence between cpDNA and nrITS using the incongruence length difference test (Farris *et al.*, 1994) with PAUP and the test revealed significant heterogeneity between the cpDNA and nrITS data sets ($P < 0.05$). Thereby, the concatenation approach may be inappropriate and the monophyly of the three series needs further verification.

Series *Lagotis* was divided into two clades: one clade including *L. kunawurensis* and *L. glauca* plus other 11 species (1.00/58.3%) and the other clade comprising the other three species (0.99/—). Although the monophyly of ser. *Lagotis* was not indicated but *L. glauca* is the type species of the series and thereby the result supports the idea that *L. kunawurensis* is placed in ser. *Lagotis* (Lu, 1992). Within the former clade, one subclade including *L. kunawurensis* and other six species (1.00/90.3%) was recognized, where *L. glauca* was not included. These six are species of eastern Himalaya, i.e., Bhutan, Sikkim, Nepal, and Tibet (*L. clarkei*, *L. crassifolia*, *L. humilis*, *L. kongboensis*, and *L. macrosiphon*) and of western Himalaya and Pakistan (*L. cashmeriana*) (Hong *et al.*, 1998; Grierson and Long, 2001; Mill, 2015), and in this Himalayan clade, *L. kunawurensis* clustered with *L. clarkei* (1.00/91.7%). Phylogenetic affinity of *L. kunawurensis* to *L. glauca* is not supported, disagreeing with the view that *L. kunawurensis* is a member of *L. glauca* aggregate (Grierson and Long, 2001). The present result provides further support for Himalayan diversification of the genus and is compatible with the scenario of migration from Himalaya to the boreal region indirectly via Central Asian highlands (Li *et al.*, 2014).

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