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Phylogenomics of the tribe Leonureae reveals a new genus: *Paraleonurus* (Lamiaceae, Lamioideae)

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Abstract: With approximately 80 species across six genera (*Leonurus*, *Lagopsis*, *Panzerina*, *Lagochilus*, *Chaiturus*, and *Loxocalyx*), the tribe Leonureae represents one of the most morphologically diverse tribes within the subfamily Lamioideae of Lamiaceae. Previous studies indicated that *Leonurus* and *Lagopsis* are not monophyletic, and relationships among and within these genera remain unclear due to limited sampling and DNA markers. In this study, we present the most comprehensive phylogenomic analyses of Leonureae based on complete plastome sequences and 127 nuclear loci retrieved from next-generation sequencing data. Our results provide strong evidence for the monophyly of Leonureae, which can be further divided into six major clades. Consistent with previous studies, our analyses show that the traditionally defined *Leonurus* and *Lagopsis* are not monophyletic and should be reclassified. Consequently, we propose a new genus, *Paraleonurus*, along with six new combinations, based on molecular phylogenetic results and morphological evidence. The new genus differs from other genera of Leonureae by having palmately 3-lobed leaves, long spike-like verticillasters, inconspicuously 2-lipped calyces, and an emarginate middle lobe of the lower corolla lip.

Key words: *Lagochilus*, *Lagopsis*, *Leonurus*, next-generation sequencing, taxonomy

1. Introduction

As the sixth largest family of flowering plants, Lamiaceae have played a significant role in many ways. Multiple species of Lamiaceae have extensive applications in cosmetics, cuisine, and horticulture. They are also an important source of medicinal herbs (Zhao et al., 2021a; Selvi et al., 2022). Recent molecular phylogenetic studies have largely clarified relationships among this family (Harley et al., 2004; Scheen et al., 2010; Li et al., 2016; Zhao et al., 2021a, 2021b). Among the 12 subfamilies of Lamiaceae, Lamioideae is the second largest, containing 12 tribes and ca. 1260 species across 59 genera (Scheen et al., 2010; Bendiksby et al., 2011; Xiang et al., 2013; Roy and Lindqvist, 2015; Li et al., 2016; Siadati et al., 2018; Zhao et al., 2021a, 2021b; Dirmenci et al., 2023; Zhao et al., 2023a, 2023b, 2024; Yuan et al., 2024). The tribe Leonureae, although initially established in 1827, has only recently been circumscribed in its current form in Lamioideae. As currently defined, Leonureae consists of six genera and approximately 80 species widely distributed in Eurasia (Harley et al., 2004; Zhao et al., 2021a).

When Dumortier (1827) first established Leonureae, it included four genera: *Ballota* L., *Leonurus* L., *Marrubium* L., and *Phlomis* L. Later taxonomic treatments by Bentham (1832–1836, 1876) reassigned *Ballota*, *Leonurus*, and *Phlomis* to the tribe Lamieae and *Marrubium* to the tribe

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Marrubieae, a classification further endorsed by Briquet (1895–1897). Wunderlich (1967) also supported the placement of *Marrubium* in Marrubieae, but reassigned the other three genera to the subtribe Lamiinae within Stachydeae. However, recent molecular phylogenetic analyses (Scheen et al., 2010; Bendiksby et al., 2011; Roy and Lindqvist, 2015; Li et al., 2016; Zhao et al., 2021a) have revealed that these four genera belong to four different tribes of Lamioideae. In their genus-level phylogenetic analyses of Lamioideae, Scheen et al. (2010) expanded the circumscription of Leonureae to include five genera, i.e. *Chaiturus* Willd., *Lagochilus* Bunge ex Benth., *Lagopsis* (Bunge ex Benth.) Bunge, *Leonurus*, and *Panzerina* Soják. Subsequently, Bendiksby et al. (2011) refined the phylogeny and classification of Lamioideae and added *Loxocalyx* Hemsl. to Leonureae.

The type genus, *Leonurus*, comprises 24 species with a broad distribution across Eurasia. Kupriyanova (1954) proposed the first classification for *Leonurus*, dividing the genus into two subgenera: *Leonurus* subg. *Cardiochilium* Krecz. & Kuprian. and *Leonurus* subg. *Leonurus.* Later, Wu and Li (1965) established *Leonurus* subg. *Chaituroides* C.Y.Wu & H.W.Li to accommodate *Leonurus chaituroides* C.Y.Wu & H.W.Li, a species endemic to China. Krestovskaja (1989, 1990) synthesized these treatments into an updated and comprehensive classification, dividing *Leonurus* into three sections, i.e. *Leonurus* sect. *Cardiochilium* (V.I.Krecz. & Kuprian.) Krestovsk., *Leonurus* sect. *Chaituroides* (C.Y.Wu & H.W.Li) Krestovsk., and *Leonurus* sect. *Leonurus*, based on the morphology of leaves, calyces, and corollas. Five subsections and five series were further recognized within these sections (Krestovskaja, 1989, 1990). More recently, Lazkov and Sennikov (2015) elevated *Leonurus* subsect. *Panzerioidei* Krestovsk. to the rank of section, i.e. *Leonurus* sect. *Panzerioidei* (Krestovsk.) Lazkov, due to its lanate, abbreviated inflorescences with soft filiform bracts.

Lagochilus is the largest genus within Leonureae, encompassing 45 species distributed across Central Asia (Mamadalieva et al., 2021). Based on the presence or absence of spinous bracteoles at the leaf axils of sterile branches, Fischer and Meyer (1841) recognized two sections within *Lagochilus*: *Lagochilus* sect. *Spinosi* Fisch. & Mey. and *Lagochilus* sect. *Inermes* Fisch. & Mey. Knorring (1954a) further divided these two sections into ten series based on characteristics of calyces, leaves, and trichomes. Zuckerwanik (1985) synonymized *Lagochilus* sect. *Spinosi* with *Lagochilus* sect. *Lagochilus* and established a new section, *Lagochilus* sect. *Australes* Kamelin & Zuckerw., including the only species, *Lagochilus cuneatus* Benth., due to its spinous bracteoles on the sterile stem. Lazkov and Sennikov (2015) further separated the *Lagochilus* subsect. *Chlainanthus* (Briq) Zuckerw. with largely connate calyx teeth from *Lagochilus* sect. *Lagochilus* and elevated it to section level as *Lagochilus* sect. *Chlainanthus* (Briq.) Lazkov.

Lagopsis, despite consisting of only five species distributed across Siberia and Mongolia to Kazakhstan, southern China, and Korea, has a complex taxonomic history. It was originally placed within *Marrubium* as a section, i.e. *M.* sect. *Lagopsis* Bunge ex Benth. (Bentham, 1832–1836, 1876; Briquet, 1895–1897). Later, Ikonnikov-Galitzky (1937) raised the section to an independent genus and the treatment was accepted by Knorring (1954b). Pyak (2006) divided *Lagopsis* into two sections: *Lagopsis* sect. *Lagopsis* includes four species characterized by compact, spike-like inflorescences and sooty or yellow corollas with rounder upper lips and subequal lower lips; *Lagopsis* sect. *Lagopsioides* Pjak comprises the sole species, *Lagopsis supina* (Stephan ex Willd.) Ikonn.-Gal., which is characterized by having axillary whorled inflorescences and white or rose corollas with much longer upper lips. *Loxocalyx* is a small genus consisting of two species endemic to China (Li and Hedge, 1994), while *Panzerina* includes four species distributed from Siberia to China. *Chaiturus* is a monotypic genus containing only a single species, *C. marrubiastrum* (L.) Ehrh. ex Rchb., which was originally placed in *Leonurus* as *Leonurus marrubiastrum* L., but was later separated by Willdenow (1787).

Although previous studies strongly supported the monophyly of the tribe Leonureae within Lamioideae (Scheen et al., 2010; Bendiksby et al., 2011; Zhao et al., 2021a), the phylogenetic relationships within the tribe have not been satisfactorily resolved due to insufficient sampling and/or the limited DNA markers employed. Scheen et al. (2010) reported the nonmonophyly of *Leonurus*, although only two species were sampled. Bendiksby et al. (2011) further demonstrated the nonmonophyly of both *Lagopsis* and *Leonurus*. Specifically, eight species of *Leonurus* were separated into two distinct clades, with one species of *Lagopsis* recovered as sister to one of these clades, while another sister to *Panzerina* (Bendiksby et al., 2011). However, due to the lack of distinguishing synapomorphies and the low resolution of their phylogenetic trees, Bendiksby et al. (2011) refrained from making any taxonomic revisions for *Lagopsis* and *Leonurus*.

Based on a more comprehensive sampling than in all previous studies, in the present study the aim was to reconstruct a robust backbone phylogeny of Leonureae using plastomes and nuclear sequences obtained from genome skimming data. The specific objectives were to: (1) test the monophyly of *Leonurus* and *Lagopsis*; (2) clarify the boundaries between *Leonurus* and its allied genera; (3) assess the relationships among genera within Leonureae; and (4) provide new insights into the taxonomy and phylogeny of Lamioideae.

2. Materials and methods

2.1. Taxon sampling

A total of 40 accessions representing 20 Leonureae species were newly sequenced here, and 160 sequences of 32 species were downloaded from GenBank, including both complete plastome sequences and plastid DNA regions. In total, the in-group sampling of all datasets consists of 48 species (75 accessions) representing all six genera of Leonureae and including one species (two accessions) of *Chaiturus*, 30 species (37 accessions) of *Lagochilus*, four species (11 accessions) of *Lagopsis*, ten species (16 accessions) of *Leonurus*, one species (four accessions) of *Loxocalyx*, and two species (five accessions) of *Panzerina*. Four genera from four tribes were selected as outgroups based on a previous study (Zhao et al., 2021a), including one species each of *Paraphlomis* (Prain) Prain (Paraphlomideae), *Lamium* L. (Lamieae), *Marrubium* L. (Marrubieae), and *Leucas* R. Br (Leucadeae). Voucher information and GenBank accession numbers are provided in Supplementary Tables S1 and S2.

2.2. DNA extraction, library construction, and sequencing

Total genomic DNA was extracted from silica gel-dried leaves using the CTAB protocol (Doyle and Doyle, 1987) or from herbarium materials using the TIANGEN DNAsecure Plant Kit (DP320), and was quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Genomic DNA was then sheared into ca. 300-bp fragments with a Covaris M220 Focused-ultrasonicator, which were used to construct libraries following the standard protocol of manufacture (NEBNext Ultra II DNA Library Prep Kit for Illumina). The fragments were sequenced from both ends for 150 bp on the Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA) at BGI Genomics (BGO-Shenzhen, China), resulting in 2–10 GB of clean data for each sample.

2.3. Assembly of plastid and nuclear datasets

For the plastome datasets, 49 accessions representing 25 species were included, with plastomes of eight species downloaded from GenBank, while the remaining plastomes were newly sequenced.

Raw reads were filtered using Fastp v.0.20.1 (Chen et al., 2018) prior to downstream assembling using GetOrganelle v.1.7.5 (Jin et al., 2020). All plastomes were annotated by Plastid Genome Annotator (Qu et al., 2019), using the plastome of *Leonurus japonicus* Houtt. (Wang et al., 2023) as the reference. Annotated plastomes were manually adjusted in Geneious v.11.0.3 (Kearse et al., 2012). Maps of all plastomes were visualized and drawn with the online tool Chloroplot (https://irscope.shinyapps.io/Chloroplot/; Zheng et al., 2020). A Python script, "get_annotated_regions_from_gb.py" (Jin et al., 2020), was used to extract protein-coding genes and noncoding regions of plastomes, with parameter "-t CDS" specified.

Except for the plastome data, we also employed six plastid DNA regions (*matK*, *psbA*, *psbB-psbH*, *psbK-psbI*, *rps16*, and *trnL-trnF*; dataset CP6) to reconstruct the phylogeny of Leonureae. These regions have been widely used in phylogenetic studies of Lamioideae (Scheen et al., 2008, 2010; Scheen and Albert, 2009; Roy et al., 2013; Xiang et al., 2013; Siadati et al., 2018; Chen et al., 2021). For newly sequenced accessions, these regions were directly extracted from plastomes using Geneious v.11.0.3 (Kearse et al., 2012). In addition, 152 sequences were downloaded from GenBank as listed in Supplementary Table S3.

Nuclear sequences were acquired using the pipeline of REFMAKER (Pouchon and Boluda, 2023), a method for targeting nuclear loci from low-coverage genome skimming data. The parameter "MIN_SEQ=33" was set to keep a minimum coverage of samples ≥70%.

2.4. Sequence alignment and dataset construction

All sequences were aligned by MAFFT v.7.520 (Katoh and Standley, 2013) with the G-INS-I algorithm. TrimAL v.1.4.1 (Capella-Gutéirrez et al., 2009) was used to exclude regions that were obscurely aligned, followed by manual adjustment if necessary.

In order to include as many species of the tribe Leonureae as possible, we created five complementary datasets: (1) complete plastome dataset (CP, 25 species), (2) dataset of combined coding regions of plastomes (CR, 25 species), (3) dataset of combined noncoding regions of plastomes (NCR, 25 species); (4) dataset of combined six plastid DNA regions (*trnL-trnF*, *matK*, *psbA*, *psbB-psbH*, *psbK-psbI*, and *rps16*) (CP6, 52 species), and (5) dataset of combined 127 nuclear loci (NR127, 24 species). For each dataset, a concatenated matrix was generated using PhyloSuite v.1.2.3 (Zhang et al., 2020).

2.5. Phylogenetic reconstruction

For the four plastid datasets (CP, CR, NCR, and CP6), maximum likelihood (ML) and Bayesian inference (BI) methods were used to infer phylogenetic relationships. ML and BI analyses were conducted on the Cyberinfrastructure for Phylogenetic Research Science (CIPRES) Science Gateway v.3.3 (Miller et al., 2010), using RAxML v.8.2.9 (Stamatakis, 2014) and MrBayes (Ronquist et al., 2012), respectively. For ML analysis, all datasets were analyzed using a GTRGAMMA model, with bootstrap (BS) values calculated from 1000 replicates. For BI analyses, four Markov chain Monte Carlo (MCMC) chains were run for 20 million generations, sampling every 1000 generations. The best-fit model was determined by jModeltest v.2.1.4 (Darriba et al., 2012) based on the Akaike information criterion (AIC) score. Convergence of runs was accepted when the average standard deviation of split frequencies (ASDSF) dropped below 0.01. Tracer v.1.7.1 (Rambaut et al., 2018) was used to inspect the convergence of model

parameters and check whether the values of effective sample size (ESS) were >200. The first 25% of the sampled generations were discarded as burn-in and a majority-rule consensus tree was summarized from the remaining trees.

For the nuclear dataset NR127, both concatenation and coalescent methods were utilized. For the concatenation method, the ML tree was inferred using IQ-TREE v.2.2.6 (Minh et al., 2020), with the "-q" option selected for ModelFinder (Kalyaanamoorthy et al., 2017) to estimate the best substitution models for each partition. For the coalescent method, single gene trees were inferred using RAxML v.8.2.12 (Stamatakis, 2014) with the GTRGAMMA model and 100 replicates. Branches with low support values (BS ≤ 10%) in the gene trees were collapsed by Newick Utilities v.1.6.0 (Junier and Zdobnov, 2010) to enhance accuracy. These gene trees were then used to infer a coalescent-based species tree with ASTRAL-III v.5.7.8 (Zhang et al., 2018).

In addition, the MIKE (MinHash-based k-mer algorithm) method based on swift calculation of the Jaccard coefficient from raw sequencing reads (Wang et al., 2024) was used for phylogenetic construction. This distance-based method can improve the accuracy of Jaccard coefficient estimation. Therefore, we utilized MIKE to reconstruct phylogeny and compare its topology with those obtained from BI and ML analyses.

3. Results

3.1. Plastome features and gene content

Plastomes of all 44 newly sequenced samples (including four out-groups) were successfully assembled. All plastomes exhibited the typical quadripartite structure with a large single copy (LSC) region and a small single copy (SSC) region separated by a pair of inverted repeats (IRa and IRb) (Supplementary Figure S1). All plastomes contained 114 unique genes, including 80 protein-coding genes, 30 tRNAs, and four rRNAs. The sequence lengths of complete plastomes ranged from 150,650 bp (*Lamium amplexicaule* L.) to 152,197 bp (*Paraphlomis hispida* C.Y.Wu) with GC contents ranging from 38.38% (*Chaiturus marrubiastrum* and *Leonurus panzerioides* Popov) to 38.67% (*Leucas neuflizeana* Courbon). The length of LSC regions ranged from 82,152 bp (*Lagochilus ilicifolius* Bunge ex Benth.) to 83,294 bp (*Paraphlomis hispida*), the SSC regions varied between 17,439 bp (*Lagopsis eriostachya* (Benth.) Ikonn.-Gal.) and 17,844 bp (*Leonurus japonicus*.), and the IR regions ranged from 25,180 bp (*Lamium amplexicaule*) to 25,693 bp (*Loxocalyx quinquenervius* Hand.-Mazz.). Detailed information about GenBank accession number, length of four regions, GC content, and number of genes for all plastomes is listed in Supplementary Table S2.

3.2. Dataset characterization

The aligned lengths of the four plastid datasets (CP, CR, NCR, and CP6) were 68,408 bp, 68,875 bp, 137,283 bp, and 4185 bp, respectively. The characteristics of all four plastid datasets are summarized in Table.

In total, 127 nuclear loci were acquired from the REFMAKER analysis. The aligned length of the concatenated matrix (NR127) was 1,088,731 bp, including 16,430 informative sites. The median number of informative sites per locus was 49, with a median locus size of 807 bp. The percentage of missing data across all loci ranged from 3.24% to 40.9%, with an average of 27.21%. The sequence characteristics of the concatenated matrix and 127 nuclear loci are listed in Table and Supplementary Table S4, respectively.

3.3. Phylogenetic relationships

For two plastome datasets (CP and CR), phylogenetic reconstructions based on ML and BI analyses yielded largely congruent relationships, while trees generated from the NCR dataset showed slight differences in relationships among clades. Since the phylogenetic trees inferred from the CR dataset exhibited the highest resolution, only the BI tree of the CR dataset is shown here for further discussion, with support values from the ML tree presented (Figure 1).

The monophyly of Leonureae was strongly supported based on all plastome datasets (Figure 1, BI-PP = 1.00/ML-BS = 100%, all support values follow this order hereafter; Supplementary Figures S2–S5, 1.00/100%). Within the tribe, six strongly supported clades can be further recognized. The *Panzerina-Lagopsis* clade consists of the genus *Panzerina* and two species of *Lagopsis* (Figure 1, 1.00/100%). Within this clade, one accession each of

Table. Characteristics of datasets constructed in the present study.

Dataset	No. of sequences	No. of taxa	Aligned length (bp)	Variable sites (bp)	Percentage of variable sites	Parsimony-informative sites (bp)	Percentage of parsimony-informative sites
CR	52	25	68,408	4031	5.90%	1509	2.20%
NCR	52	25	68,875	5567	8.10%	2074	3.00%
CP	52	25	137,283	9598	7.00%	3583	2.60%
CP6	79	52	4185	530	12.70%	203	4.90%
NR127	44	24	1,088,731	24,448	9.90%	14,093	5.70%

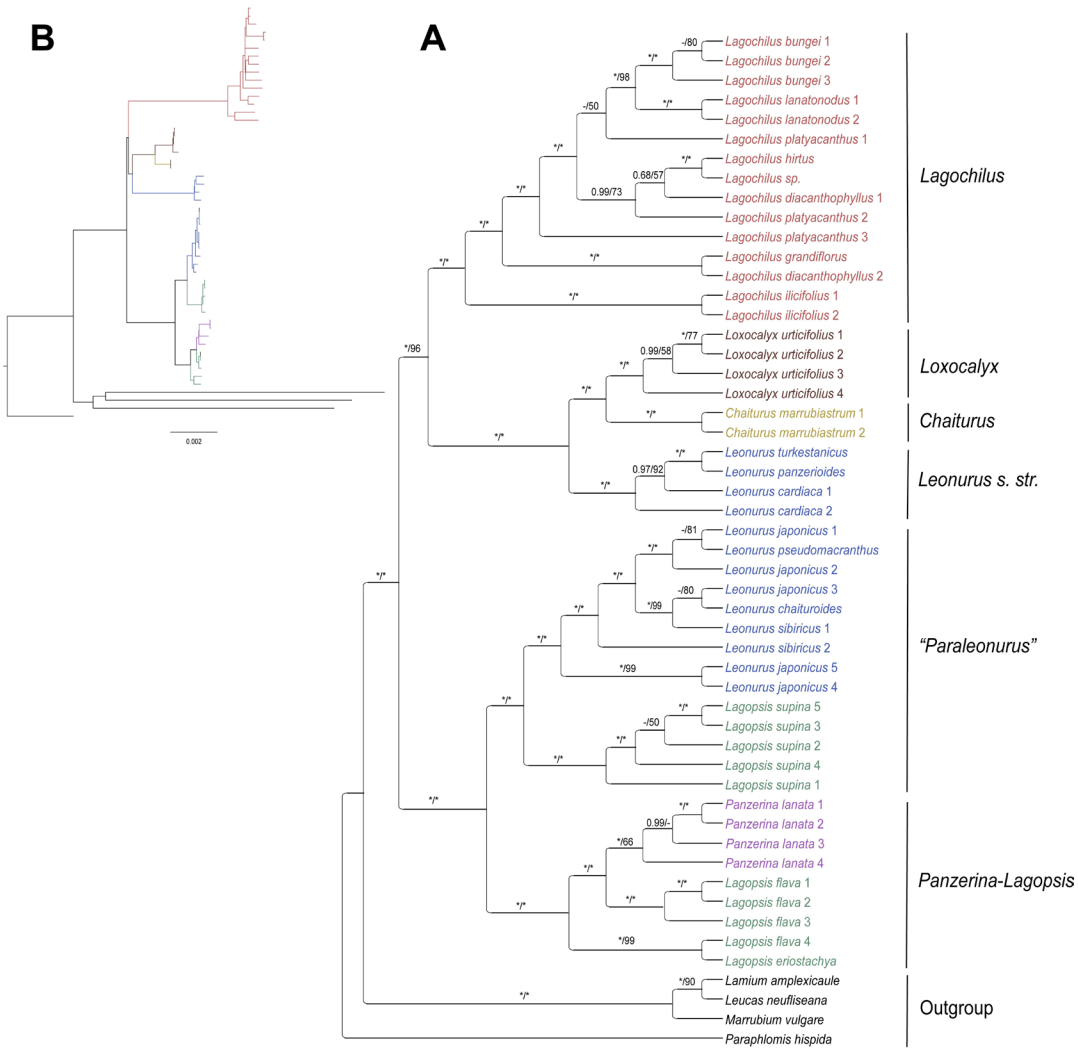

Figure 1. Cladogram (A) and phylogram (B) of the Bayesian 50% majority-rule consensus tree of Leonureae inferred from the CR dataset. Support values displayed on the branches follow the order ML-BS/BI-PP (a "-" indicates PP < 0.5 or BS < 50% and an "*" indicates BS = 100% or PP = 1.00). Multiple accessions of the same species are numbered according to Table S1.

Lagopsis flava Kar. & Kir. and *Lagopsis eriostachya* formed a highly supported subclade (Figure 1, 1.00/99%) sister to another subclade consisting of three accessions of *Lagopsis flava* and four accessions of *P. lanata* (L.) Soják (Figure 1, 1.00/100%). Recovered as sister to the *Panzerina-Lagopsis* clade, the "*Paraleonurus*" clade can also be divided into two subclades; the first subclade contained three species from *Leonurus* sect. *Cardiochilium* (*Leonurus japonicus*, *Leonurus sibiricus* L., and *Leonurus pseudomacranthus* Kitag.) and one species from *Leonurus* sect. *Chaituroides* (*Leonurus chaiturioides*) (Figure 1, 1.00/100%), and the second subclade included five accessions of *Lagopsis supina* (Figure 1, 1.00/100%). The two clades were further sister to the remaining clades of Leonureae.

The *Chaiturus* clade, represented by two accessions of the type species of *Chaiturus*, was robustly supported as sister to the *Loxocalyx* clade (Figure 1, 1.00/100%), which comprised four accessions of the type species of *Loxocalyx*. The *Chaiturus-Loxocalyx* clade was found to be sister to a clade consisting of three species from *Leonurus* sect. *Panzerioidei* and *Leonurus* sect. *Leonurus*, which we have designated as the *Leonurus s. str.* clade (Figure 1, 1.00/100%). As the sister group to the *Chaiturus-Loxocalyx-Leonurus s. str.* clade, the *Lagochilus* clade encompassed eight species representing two sections (*Lagochilus* sect. *Lagochilus* and *Lagochilus* sect. *Inermes*) of *Lagochilus*.

The dataset combining six plastid DNA regions (CR6) included the largest number of species (48 species of

Leonureae). Phylogenetic trees inferred from this dataset (Supplementary Figures S6 and S7) identified the same six clades as previously listed. However, the topology differed somewhat from those generated using the CP and CR datasets of 21 species of Leonureae, as the *Leonurus s. str.* clade was resolved as sister to the *Panzerina-Lagopsis-Paraleonurus* clade (Supplementary Figures S6 and S7, 0.868/96%) rather than to the *Chaiturus-Loxocalyx* clade (Figure 1; Supplementary Figures S2 and S3).

The monophyly of Leonureae and the six clades identified in the plastid trees was also strongly supported by the coalescent tree (Figure 2) and the ML tree (Supplementary Figure S8) estimated from 127 nuclear loci. The topological relationships among the six clades in the nuclear coalescent tree (Figure 2) were identical to those in the plastid trees inferred from the CP and CR datasets (Figure 1; Supplementary Figures S2 and S3), despite the fact that the sister relationship between the *Lagochilus* clade and the *Chaiturus-Loxocalyx-Leonurus s. str.* clade was poorly supported (Figure 2, local posterior probability (LPP) = 0.52). Notably, within the *Panzerina-Lagopsis* clade, all accessions of *Lagopsis* (except for *Lagopsis supina*) grouped together, forming a robustly supported clade (LPP = 1.00). Species relationships within

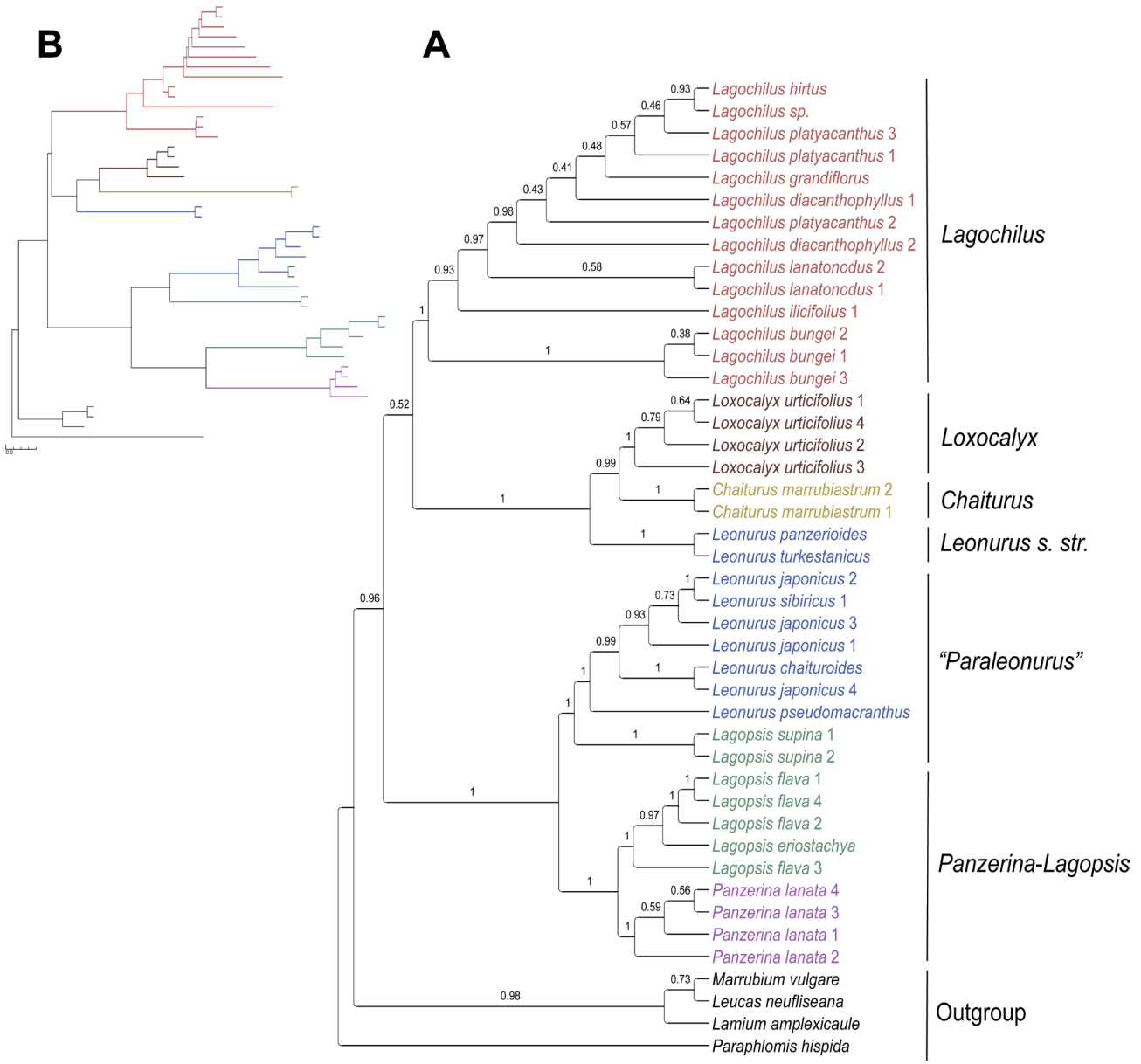

Figure 2. Cladogram (A) and phylogram (B) of the multiple species coalescent tree of Leonureae based on 127 nuclear loci produced with REFMAKER. Numbers on the branches are local posterior probability (LPP).

the *Lagochilus* clade and the "*Paraleonurus*" clade also showed some incongruities between the coalescent tree (Figure 2) and the plastid trees (Figure 1; Supplementary Figures S2–S5). In the plastome trees (Figure 1; Supplementary Figures S2–S5), two species of *Lagochilus* sect. *Inermes*, *Lagochilus hirtus* and *Lagochilus bungei*, were grouped with species from *Lagochilus* sect. *Lagochilus*, but in the nuclear trees (Figure 2; Supplementary Figure S8), accessions of *Lagochilus bungei* formed a monophyletic group, sister to a large clade that included the two species of *Lagochilus* sect. *Inermes* (*Lagochilus hirtus* and *Lagochilus ilicifolius*) and species of *Lagochilus* sect. *Lagochilus*. The major topological difference between the nuclear ML tree (Supplementary Figure S8) and the coalescent tree (Figure 2) was the placement of the *Lagochilus* clade. In the ML tree, the *Lagochilus* clade was shown to be sister to the *Panzerina-Lagopsis*-"*Paraleonurus*" clade with strong support (Supplementary Figure S8, ultrafast bootstrap (UFBS) = 100%).

The tree generated from the MIKE analysis (Supplementary Figure S9) exhibited a topology similar to that of the NCR dataset (Supplementary Figures S4 and S5). Both trees divided Leonureae into the same six clades, with the major difference from other plastid and nuclear trees (Figures 1 and 2; Supplementary Figures S2, S3, S6, and S7) being that the *Lagochilus* clade was resolved as sister to all other clades. Despite this, the "*Paraleonurus*" clade was consistently recovered and the sister relationship between the "*Paraleonurus*" clade and the *Panzerina-Lagopsis* clade was again supported (Supplementary Figures S4, S5, and S9).

4. Discussion

Our study involved the most comprehensive molecular phylogenetic analysis of Leonureae to date. A total of 52 species (79 individuals) were included across five datasets for phylogenetic reconstruction. In all analyses, Leonureae was strongly supported as a monophyletic group, consistent with previous studies (Scheen et al., 2010; Bendiksby et al., 2011; Roy and Lindqvist, 2015). For the first time, we identified six distinct clades within Leonureae, with the monophyly of *Chaiturus*, *Lagochilus*, *Loxocalyx*, and *Panzerina* being highly supported. In contrast, the traditionally defined *Leonurus* and *Lagopsis* were found to be nonmonophyletic, indicating that their boundaries require recircumscription.

4.1. Overall phylogenetic relationships within Leonureae

The present study revealed that Leonureae can be divided into six major clades in all analyses, although the relationships among these clades varied somewhat depending on the dataset used. Most phylogenetic trees (Figures 1 and 2; Supplementary Figures S2 and S3) supported a sister relationship between a large clade composed of *Lagochilus*, *Loxocalyx*, *Chaiturus*, and *Leonurus s. str.* and another large clade comprising the "*Paraleonurus*" and *Panzerina-Lagopsis* clades. However, trees generated from the CR6 dataset (Supplementary Figures S6 and S7) instead identified *Leonurus s. str.* as sister to the "*Paraleonurus*"-*Panzerina-Lagopsis* clade. This inconsistency is likely due to the CR6 dataset having fewer informative sites compared to the other three plastome datasets. In addition, in the nuclear concatenated tree (Supplementary Figure S8), the *Lagochilus* clade was recovered as sister to the "*Paraleonurus*"-*Panzerina-Lagopsis* clade, while the sister relationships among *Loxocalyx*, *Chaiturus*, and *Leonurus s. str.* were still supported. In the trees constructed from the MIKE analysis and NCR dataset (Supplementary Figures S4, S5, and S9), *Loxocalyx*, *Chaiturus*, and *Leonurus s. str.* formed a large clade that was sister to the "*Paraleonurus*"-*Panzerina-Lagopsis* clade, with the *Lagochilus* clade as sister to the clade comprising all five other clades.

The "*Paraleonurus*" clade was consistently sister to the *Panzerina-Lagopsis* clade in all trees. Both clades share palmately lobed leaves, subequal calyx teeth, and a cordate, emarginate middle lobe of the lower corolla lip (Li and Hedge, 1994; Harley et al., 2004). However, the "*Paraleonurus*" clade can be readily distinguished from the *Panzerina-Lagopsis* clade by having loose and elongated inflorescences with appressed minutely pubescent indumentum in contrast to the compact inflorescences with densely hairy calyces and corollas in *Panzerina* (tomentose) and *Lagopsis* (lanate) (Li and Hedge, 1994; Harley et al., 2004). Geographically, species of "*Paraleonurus*" are mainly distributed in East Asia, while species of *Panzerina* and *Lagopsis* are typically found in the arid and semiarid regions of Central Asia and Siberia (Krestovskaja, 1992; Li and Hedge, 1994).

Chaiturus was supported as sister to *Loxocalyx* by all trees (Figures 1 and 2; Supplementary Figures S1–S9). Both genera have ovate leaves and nutlets truncate at the apex. However, *Chaiturus* differs from *Loxocalyx* by having equal calyx teeth (vs. anterior teeth much longer than posterior ones), a short corolla tube hardly exserted from the calyx (vs. obviously exserted), subequal stamens included in the corolla (vs. exserted stamens with longer anterior pair), and nutlets puberulent at the apex (vs. glandular at the apex). The *Leonurus s. str.* clade was recovered as sister to the *Chaiturus-Loxocalyx* clade in all trees, except for those generated from the CR6 dataset (Supplementary Figure S6). All these three clades are characterized by nutlets, which are truncate at the apex. Trees inferred from the CR6 dataset indicated a sister relationship between the *Leonurus s. str.* clade and a large clade comprising the "*Paraleonurus*" and *Panzerina-Lagopsis* clades, but morphological differences in the length of calyx teeth and

the shape of the middle lobe of the lower corolla lip do not support this phylogenetic relationship.

Morphologically, *Lagochilus* shares similarities with "*Paraleonurus*", *Panzerina*, and *Lagopsis* as all these genera possess palmately lobed leaves and a cordate middle lobe of the lower corolla lip (Li and Hedge, 1994; Harley et al., 2004). However, close phylogenetic relationships among these genera were only supported by the nuclear concatenated tree (Supplementary Figure S8). Despite most analyses indicating a close relationship between *Lagochilus* and the *Chaiturus* + *Loxocalyx* + *Leonurus s. str.* clade (Figures 1 and 2; Supplementary Figures S2–S3), no synapomorphies have yet been identified for these genera. Further morphological studies are necessary for all genera within Leonureae to clarify these relationships.

4.2. Systematic placement of *Lagopsis supina* and circumscription of *Lagopsis*

In the present study, we included four species of *Lagopsis* for phylogenetic reconstruction. Across all analyses, all accessions of *Lagopsis supina* were consistently grouped within the "*Paraleonurus*" clade with high supported values (Figures 1 and 2; Supplementary Figures S2–S9). This finding aligned with the previous study by Bendiksby et al. (2011), which was based on four plastid DNA regions (*matK*, *rps16*, *trnL* intron, and *trnL-trnF* spacer). *Lagopsis supina* was originally described as *Leonurus supinus* Stephan ex Willd. by Willdenow (1800). However, Bentham (1832–1836) later transferred this species to *Marrubium* based on several characteristics: a corolla tube shorter than the calyx, stamens and style included in the corolla tube, and nutlets rounded at the apex. Subsequently, Ikonnikov-Galitzky (1937) separated *Lagopsis supina* from *Marrubium* due to distinct morphological traits, including the presence of five calyx teeth (vs. 5–10 teeth in *Marrubium*), the absence of a hairy annulus at the base of the corolla tube (vs. presence of a hairy annulus), an entire upper lip of the corolla (vs. notched or 2-lobed), and rounded, palmately lobed leaves (vs. ovate, toothed leaves). The separation of *Lagopsis supina* from the other four species of *Lagopsis* is also supported by morphological differences. *Lagopsis supina* is an annual or perennial herb with rigidly curved and short hairy, axillary whorled inflorescences; a white or rose corolla; and a much longer upper lip (Li and Hedge, 1994; Pyak, 2006). In contrast, the other four species of *Lagopsis* are perennial herbs having multicellular soft hairy, compact, spike-shaped inflorescences; sooty or yellow corollas; a rounder upper lip; and a subequal lower lip (Li and Hedge, 1994; Pyak, 2006). Geographically, *Lagopsis supina* is widely distributed in East Asia and southern Siberia, whereas the other four species are endemic to Central Asia and southern Mongolia and Siberia (Li and Hedge, 1994). As demonstrated by the present study and the previous study by Bendiksby et al. (2011), *Lagopsis supina* should be excluded from the genus *Lagopsis*.

After the exclusion of *Lagopsis supina*, the genus *Lagopsis* currently consists of at least four species: *Lagopsis darwiniana* A.I.Pjak, *Lagopsis eristachya*, *Lagopsis flava*, and *Lagopsis marrubiastrum* (Stephan) Ikonn.-Gal. (the type species of the genus). In the present study, we included three species (*Lagopsis flava*, *Lagopsis eriostachya*, and *Lagopsis marrubiastrum*) in the phylogenetic analyses. However, the monophyly of *Lagopsis* and its relationship with *Panzerina* remain unresolved due to topological incongruities between the nuclear and plastid trees (Figures 1 and 2; Supplementary Figures S2–S8). In the plastid trees (Figure 1; Supplementary Figures S2–S7), species of *Lagopsis* did not form a monophyletic group; three accessions of *Lagopsis flava* formed a subclade with *Panzerina*, which was sister to another subclade composed of the remaining accessions of *Lagopsis flava* and *Lagopsis eriostachya* (the subclade also included an accession of *Lagopsis marrubiastrum* in the CR6 trees). Conversely, in the nuclear trees (Figure 2; Supplementary Figure S8), both *Lagopsis* and *Panzerina* were monophyletic. Morphologically, *Panzerina* and these four *Lagopsis* species share several characteristics: perennial herb, palmately lobed leaves, densely hairy leaves, calyx and corolla (tomentose in *Panzerina*, lanate in *Lagopsis*), corolla tube and calyx tube equal or subequal in length, and nutlets that are rounded at the apex (Li and Hedge, 1994). However, there are also notable morphological differences between the two genera. *Panzerina* has a white to yellow-white corolla (vs. the yellow or sooty corolla in *Lagopsis*), slightly longer anterior calyx teeth than posterior ones (vs. subequal calyx teeth), stamens that are exserted from the corolla tube (vs. included), and a larger corolla with a length of 3.3–4.0 cm (vs. 0.7 cm) (Krestovskaja, 1991, 1992; Li and Hedge, 1994; Pyak, 2006). Considering both the systematic conflicts and morphological evidence, we refrained from making any taxonomic changes to the two genera in the present study. More comprehensive sampling and analyses are needed to further clarify the relationships between the two genera.

4.3. Nonmonophyly of *Leonurus* and the establishment of a new genus

Our analyses showed that species of the traditionally defined *Leonurus* grouped into two distinct clades, indicating the nonmonophyly of this genus. The first clade, referred to as the *Leonurus s. str.* clade, comprises two species (*Leonurus panzerioides* Popov and *Leonurus turkestanicus* V.I.Krecz. & Kuprian.) in the nuclear tree (Figure 2; Supplementary Figure S8), three species (*Leonurus panzerioides*, *Leonurus turkestanicus*, and *Leonurus cardiaca* L., the type species of the genus) in plastome trees (Figure 1; Supplementary Figures S2–S5), and five species (*Leonurus panzerioides*, *Leonurus turkestanicus*, *Leonurus cardiaca*, *Leonurus*

glaucescens, and *Leonurus quinquelobatus* Gilib.) in the plastid tree generated from the CR6 dataset (Supplementary Figure S6). *Leonurus panzerioides* belongs to *Leonurus* sect. *Panzerioidei* and the other four species are part of *Leonurus* sect. *Leonurus*. The second clade, defined as the "*Paraleonurus*" clade, includes *Lagopsis supina* and five species of *Leonurus* (*Leonurus japonicus*, *Leonurus chaiturоides*, *Leonurus macranthus*, *Leonurus sibiricus*, and *Leonurus pseudomacranthus*) from *Leonurus* sect. *Cardiochilium* and *Leonurus* sect. *Chaituroides*. Notably, accessions of *Leonurus japonicus* were nonmonophyletic in all trees (Figures 1 and 2; Supplementary Figures S1–S9). The "*Paraleonurus*" clade is also strongly supported by distinct morphological features, such as 3-lobed leaves (Figure 3G), an indistinctly 2-lipped calyx (Figures 3K and 3L), the presence or absence of a horizontal hairy annulus within the corolla tube without dilation above (Figure 3I), and an erect lower lip of the corolla with an emarginate middle lobe (Figures 3H and 3I; Krestovskaja, 1989, 1990, 1992; Harley et al., 2004). In contrast, species

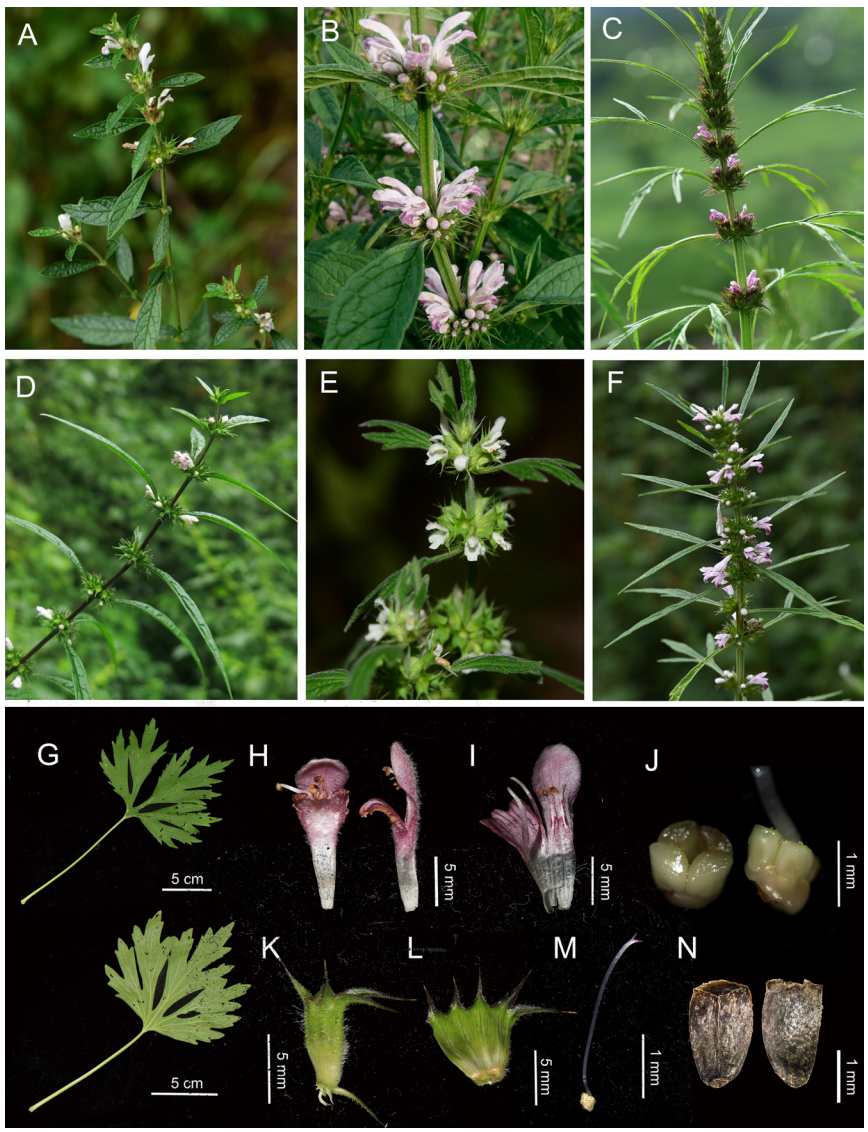

Figure 3. Morphological characters of six species of *Paraleonurus*. A. *P. pseudomacranthus*; B. *P. macranthus*; C. *P. sibiricus*; D. *P. chaituroides*; E. *P. supinus*; F. *P. japonicus*; G. Leaves of *P. japonicus*; H. Corollas of *P. sibiricus*; I. Dissected corolla of *P. sibiricus*; J. Ovaries of *P. japonicus*; K. Calyx of *P. sibiricus*; L. Dissected calyx of *P. sibiricus*; M. Pistil of *P. japonicus*; N. Nutlets of *P. japonicus*. A, photographed by X.-X. Zhu; B, photographed by B.-Y. Zhang; C, E–G, J, M–N, photographed by Y.-P. Chen; D, photographed by H.-J. Dong; H–I, K–L, photographed by Y. Zhao

in the *Leonurus s. str.* clade have 5- to 7-lobed leaves, a distinctly 2-lipped calyx, an obliquely hairy annulus with dilation above, and a subhorizontally reflexed lower lip of corolla with an oblong-ovate middle lobe (Krestovskaja, 1989, 1990, 1992; Harley et al., 2004).

Geographically, species within the "*Paraleonurus*" clade are predominantly distributed in eastern China, with some extending into southern Mongolia and Siberia, whereas species of the *Leonurus s. str.* clade are distributed in Central Asia and Siberia (Krestovskaja, 1992; Li and Hedge, 1994). Additionally, the inclusion of *Lagopsis supina* within the "*Paraleonurus*" clade is supported by morphological traits like herbaceous habit, palmately 3-lobed leaves, elongated and nonlanate verticillasters, an indistinctly 2-lipped calyx, and an emarginate middle lobe of the lower corolla lip (Figure 3E; Li and Hedge, 1994; Harley et al., 2004). The geographic distribution of these species also largely overlaps, as they are all found in East Asia and southern Siberia (Li and Hedge, 1994). Therefore, to maintain the monophyly of all genera within Leonureae, we propose naming the "*Paraleonurus*" clade as a new genus based on phylogenetic data, morphological characteristics, and geographical distribution. The new genus comprises species of *Leonurus* sect. *Cardiochilium*, *Leonurus* sect. *Chaituroides*, and *Lagopsis supina* (see taxonomic treatment).

4.4. Taxonomic treatment

***Paraleonurus* M.L.Qian, C.L.Xiang & Bo Li, gen. nov.**

Type: *Paraleonurus sibiricus* (L.) M.L.Qian, C.L.Xiang & Bo Li ≡ *Leonurus sibiricus* L., in: *Sp. Pl.* 2: 584. 1753.

Diagnosis: Phylogenetically, *Paraleonurus* is closely related to *Panzerina* and *Lagopsis*, but can be distinguished by its inflorescence structure (loose and long inflorescences vs. compact inflorescences); indumentum on leaves, calyces, and corolla [puberulent vs. densely tomentose (*Panzerina*) or lanate (*Lagopsis*)]; and color of corolla (white to purplish in *Paraleonurus* vs. white to yellow-white in *Panzerina* or yellow or sooty in *Lagopsis*). Morphologically, *Paraleonurus* is most similar to *Leonurus*, but can be readily distinguished by having an indistinctly 2-lipped calyx (vs. distinctly 2-lipped), a corolla tube with a horizontal hairy annulus present or not and not dilated above (vs. obliquely hairy annulus with above dilated), and an erect lower corolla lip (vs. subhorizontally reflexed) with a conspicuously emarginate middle lobe (vs. oblong-ovate), and 3-lobed leaves (vs. 5- to 7-lobed leaves).

Description: Herbs annual, biennial, or perennial, erect. Leaves palmately 3-lobed, with or without rounded or oblong teeth. Verticillasters densely or sparsely flowered, forming a long spike-like inflorescence. Bracts and bracteoles present, subulate or spinescent. Calyx obconical or tubular-campanulate, inconspicuously 2-lipped, 5-lobed, lobes subequal, upper teeth 3, lower teeth 2. Corolla white, pink, or purple; tube with or without interior horizontal hairy annulus, not dilated above annulus; corolla slightly or obviously exserted from calyx, and strongly 2-lipped, posterior lip obovate, hooded, margin entire, pubescent above; anterior lip erect, spreading, 3-lobed, lateral lobes short, ovate, middle lobe shallowly cordate, emarginate. Stamens 4, exserted or included, anterior 2 longer, parallel, thecae 2, parallel. Style 2-lobed, lobes subequal. Nutlets acutely triquetrous or oblong-ovoid, apex truncate or rounded.

Etymology: The generic name indicated that the new genus is separated from the traditionally circumscribed *Leonurus* and morphologically similar to *Leonurus*.

Common name (assigned here): Jia Yi Mu Cao Shu (假益母草属; Chinese name).

Taxonomic notes: The new genus is characterized by having palmately 3-lobed leaves, verticillasters forming elongated spike-like inflorescences, an indistinctly 2-lipped calyx, and an emarginate middle lobe of the lower corolla lip. Originally, *Leonurus* sect. *Cardiochilium*, established by Krestovskaja (1989, 1990), included six species, but our analyses included only four of them. Based on morphological characteristics, *Leonurus villosissimus* C.Y.Wu & H.W.Li and *Leonurus tibeticus* Krestovsk. should be incorporated into *Paraleonurus*. Nonetheless, due to the absence of corresponding samples, we refrain from proposing these combinations at this time. Additionally, our findings highlight the need for further morphological and molecular phylogenetic research to establish a robust infrageneric classification of *Paraleonurus*. With the description of this new genus, the number of species in *Leonurus* is reduced to 15, distributed across *Leonurus* sect. *Leonurus* and *Leonurus* sect. *Panzerioidei*. Further studies are also necessary to refine the infrageneric classification of *Leonurus*.

Distribution: The genus is widely distributed from East Asia to southern Siberia and Mongolia (Figure 4). It can be found in stony or sandy grasslands, in thickets, on slopes, or at roadsides at elevations of 0–3400 m.

New combinations:

Paraleonurus sibiricus (L.) M.L.Qian, C.L.Xiang & Bo Li, **comb. nov.**

≡ *Leonurus sibiricus* L. in Sp. Pl. 2: 584. 1753.

Paraleonurus chaituroides (C.Y.Wu & H.W.Li) M.L.Qian, Celep & C.L.Xiang, **comb. nov.**

≡ *Leonurus chaituroides* C.Y.Wu & H.W.Li in Acta Phytotax. Sin. 10: 161. 1965.

Paraleonurus pseudomacranthus (Kitag.) M.L.Qian, Dirmenci & C.L.Xiang, **comb. nov.**

≡ *Leonurus pseudomacranthus* Kitag. in Bot. Mag. (Tokyo) 48: 109. 1934.

Paraleonurus macranthus (Maxim.) M.L.Qian, Y.P.Chen & C.L.Xiang, **comb. nov.**

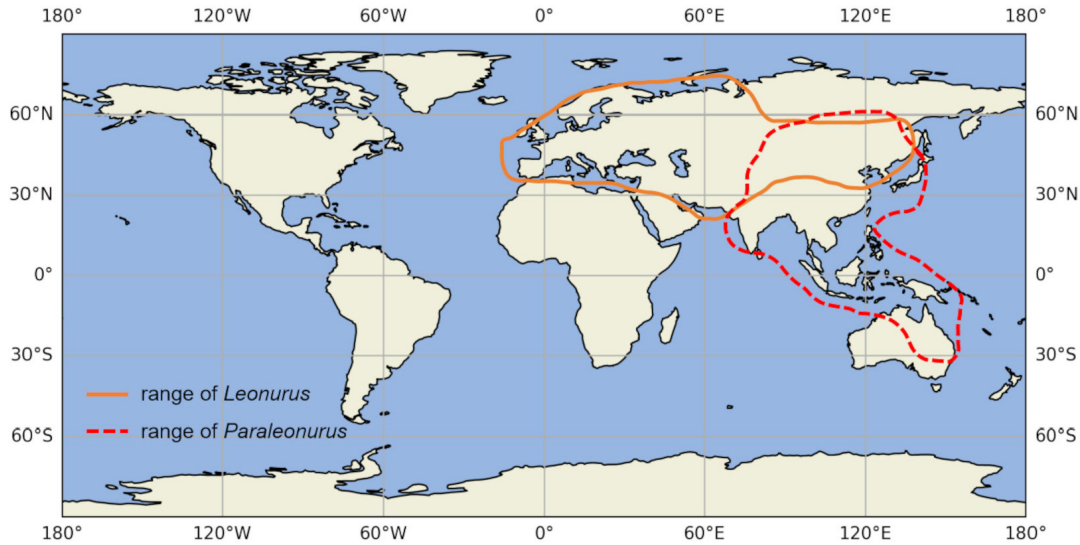

Figure 4. Geographical distribution of *Paraleonurus* and *Leonurus*.

≡ *Leonurus macranthus* Maxim. in Mém. Acad. Imp. Sci. St.-Pétersbourg Divers Savans 9: 476. 1859.

Paraleonurus japonicus (Houtt.) M.L.Qian, Y.P.Chen & C.L.Xiang, **comb. nov.**

≡ *Leonurus japonicus* Houtt. in Nat. Hist. 2: 366. 1778.

Paraleonurus supinus (Stephan ex Willd.) M.L.Qian, Y.Zhao & C.L.Xiang, **comb. nov.**

≡ *Leonurus supinus* Stephan ex Willd. in Sp. Pl. 3: 116. 1800 ≡ *Lagopsis supina* (Stephan ex Willd.) Ikonn.-Gal. in Bot. Mater. Gerb. Bot. Inst. Komarova Akad. Nauk S.S.S.R. 7: 45. 1937.

5. Conclusion

The present study involves the most comprehensive molecular phylogenetic analyses of the tribe Leonureae to date, utilizing six plastid regions, complete plastome sequences, and 127 nuclear loci. Our results confirm the monophyly of Leonureae and identify six major clades within the tribe. The monophyly of *Lagochilus*, *Loxocalyx*, and *Chaiturus* is well supported. However, the monophyly of *Lagopsis* and *Panzerina* and their relationships remain unresolved and require further investigation with more extensive sampling. Notably, species of the traditionally defined *Leonurus* are divided into two distinct clades. To keep the monophyly of each clade, we propose the establishment of a new genus, *Paraleonurus*, to accommodate five species of *Leonurus* and *Lagopsis supina*, resulting in six new combinations. Future research should prioritize: (1) resolving species relationships within *Lagochilus* and *Paraleonurus* and establishing a robust infrageneric classification for both genera; (2) clarifying the circumscription of *Lagopsis* and *Panzerina*; and (3) exploring the causes of cytonuclear discordance within the tribe using more molecular data and comprehensive samplings.

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Supplementary material

Supplementary figures and tables for this manuscript are available at: https://aperta.ulakbim.gov.tr/record/274016

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