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Reinstatement of *Pedicularis delavayi* (Orobanchaceae): a species separating from *P. siphonantha* revealed by phylogenetic analyses

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Background. Morphological identification of *Pedicularis* depends on floral characters because interspecific variations these characters is very large. However, some important characters may be lost during the process of pressing the specimen. *Pedicularis delavayi* was described from Lijiang, northwestern Yunnan, and it was widely adopted as a variety of *P. siphonantha*. Unfortunately, the name "*P. siphonantha* var. *delavayi*' incorrectly referred to *P. milliana*/*P. tenuituba* in some identified specimens and publications. Moreover, phylogenetic relationships between *P. delavayi* and *P. siphonantha* and its allies (*P. millana* and *P. tenuituba*) were not fully resolved. **Methods.** In total, we sampled 75 individuals representing 56 taxa. Of them, 10 taxa were from *P. siphonantha* group, and 11 individuals of *P. delavayi* represented 9 populations. One nuclear gene (nrITS) and four chloroplast genes/regions (*matK*, *rbcL*, *trnH-psbA*, and *trnL-F*) were sequenced for phylogenetic analyses. The nrITS and plastid datasets were combined to analyze. Bayesian Inference (BI) and Maximum Likelihood (ML) methods were used to reconstruct phylogenetic trees. Species identification, morphological comparison and line drawing were based on field observation, dried herbarium specimens and FFA-fixed samples. **Results.** Phylogenetic analyses showed that the *P. siphonantha* group was polyphyletic: monophyletic *P. delavayi*, sister to *P. obliquigaleata,* fell into clade A; and the remaining species of *P. siphonantha* group was in clade B as monophyletic (i.e. *P. siphonantha* lineage). In the *P. siphonantha* lineage, *P. milliana*, *P. siphonantha,* and *P. tenuituba* (= *P. siphonantha* var. *stictochila*) were well supported as monophyletic, and *P. dolichosiphon* (≡ *P. siphonantha* var. *dolichosiphon*) was sister to *P. leptosiphon*. Morphologically, *P. delavayi* differs from species of the *P. siphonantha* lineage in having a long petiole (~ 50 mm) and pedicel (~ 40 mm), a ridged corolla tube, and a folded lower-lip of the corolla.

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Discussion. Phylogenetic analyses showed evolution of *P. delavayi* was independent from the *P. siphonantha* lineage. The long-tubular corollas in *P. delavayi* and *P. siphonantha* lineage might be a convergent character. Both morphological and phylogenetic evidence strongly supported to reinstate *P. delavayi* as an independent species. In addition, *P. neolatituba* was proposed to reduce as a new synonymy of *P. delavayi*.

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Title Page

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ABSTRACT

Background. Morphological identification of *Pedicularis* depends on floral characters because interspecific variations these characters is very large. However, some important characters may be lost during the process of pressing the specimen. *Pedicularis delavayi* was described from Lijiang, northwestern Yunnan, and it was widely adopted as a variety of *P. siphonantha*. Unfortunately, the name "*P. siphonantha* var. *delavayi*' incorrectly referred to *P. milliana*/*P. tenuituba* in some identified specimens and publications. Moreover, phylogenetic relationships between *P. delavayi* and *P. siphonantha* and its allies (*P. millana* and *P. tenuituba*) were not fully resolved.

Methods. In total, we sampled 75 individuals representing 56 taxa. Of them, 10 taxa were from *P. siphonantha* group, and 11 individuals of *P. delavayi* represented 9 populations. One nuclear gene (nrITS) and four chloroplast genes/regions (*matK*, *rbcL*, *trnH-psbA*, and *trnL-F*) were sequenced for phylogenetic analyses. The nrITS and plastid datasets were combined to analyze. Bayesian Inference (BI) and Maximum Likelihood (ML) methods were used to reconstruct phylogenetic trees. Species identification, morphological comparison and line drawing were based on field observation, dried herbarium specimens and FFA-fixed samples.

Results. Phylogenetic analyses showed that the *P. siphonantha* group was polyphyletic: monophyletic *P. delavayi*, sister to *P. obliquigaleata,* fell into clade A; and the remaining species of *P. siphonantha* group was in clade B as monophyletic (i.e. *P. siphonantha* lineage). In the *P. siphonantha* lineage, *P. milliana*, *P. siphonantha,* and *P. tenuituba* (= *P. siphonantha* var. *stictochila*) were well supported as monophyletic, and *P. dolichosiphon* (≡ *P. siphonantha* var. *dolichosiphon*) was sister to *P. leptosiphon*. Morphologically, *P. delavayi* differs from species of the *P. siphonantha* lineage in having a long petiole (~ 50 mm) and pedicel (~ 40 mm), a ridged corolla tube, and a folded lower-lip of the corolla.

Discussion. Phylogenetic analyses showed evolution of *P. delavayi* was independent from the *P. siphonantha* lineage. The long-tubular corollas in *P. delavayi* and *P. siphonantha* lineage might be a convergent character. Both morphological and phylogenetic evidence strongly supported to reinstate *P. delavayi* as an independent species. In addition, *P. neolatituba* was proposed to reduce as a new synonymy of *P. delavayi*.

Keywords: *Pedicularis*, *Pedicularis delavayi*, *Pedicularis milliana*, *Pedicularis siphonantha*, species delimitation, the Hengduan Mountains

INTRODUCTION

Flowers of *Pedicularis* L. (Orobanchaceae) show striking interspecific variations (Li 1951; Yu et al. 2008), so morphological identification of these species depends much on floral characters (Liu, Yu & Wang 2013). Generally, one species is easily distinguished from another morphologically similar species using fresh flowers in the field. However, floral shape and structure may be changed during the process of pressing and drying the specimen and, in practice, herbarium specimens of closely related species are very difficult to discriminate. DNA barcodes have been widely applied to assist species identification (Hebert et al. 2003; Kress et al. 2005; China Plant BOL Group 2011), particularly when morphological identification is uncertain. In *Pedicularis*, the nuclear ribosomal internal transcribed spacer (nrITS) or nrITS+*rbcL* can discriminate at least 78% of species in the genus (Yu et al. 2011; Liu, Yu & Wang 2013). However, there is little consensus between the phylogenetic tree and traditional classification in *Pedicularis* (Ree 2005; Yang & Wang 2007; Tkach et al. 2014; Robart et al. 2015; Yu et al. 2015), and morphologically similar species may not be sister to each other in phylogenetic analyses. Therefore, DNA sequences are very useful to delimit species and to confirm phylogenetic relationship among species.

Pedicularis delavayi was firstly named by Franchet after J. M. Delavay, who collected the type material (Fig. S1) at Yulong Mountain in Lijiang, northwestern Yunnan, China in 1886, while it was validly published by Maximowicz (1888). This species was treated as an independent species (Prain 1890; Bonati 1910; Limpricht 1924; Li 1949) until Tsoong (1963), who downgraded it to a variety in *P. siphonantha* D. Don. According to illustrations in Chinese Floras (Tsoong 1963; Yang, Holmgren & Mill 1998), the lower lip of *P. siphonantha* var. *delavayi* (Franch. ex Maxim.) P. C. Tsoong should be similar to *P. siphonantha* D. Don var. *siphonantha* as spreading (see Fig. 1A). From illustrated publications, the name *"P. siphonantha* var. *delavayi* was used for a "long-tubed and purple-red species" (Fig. 1B), which is a common species in alpine meadow at altitudes from 3000 m to 4000 m a.s.l. (above sea level, hereafter) in northwestern Yunnan (e.g., Guan 1998; Yu, Zhang & Wang 2008; Xu, Li & Sun 2013). In addition, some publications used the name "*P. siphonantha*" for this "long-tubed and purple-red species" (e.g., Yang et al. 2002; Yang, Sun & Guo 2005; Huang & Fenster 2007; Yu et al. 2013a; Huang, Wang & Sun 2016).

During field expeditions for *Pedicularis* in the Hengduan Mountains region from 2006 to 2010, we collected an unknown long-tubed species with a purple-red corolla in Shangri-La, northwestern Yunnan, and Jiulong and Kangding, Sichuan, at altitudes around 4000 m (Figs. 1C-F). This species differs from infraspecfic taxa on *P. siphonantha* and other long-tubed and purple-red species in series *Longiflorae* Prain by having an incurved middle lobe of the lower lip and a ridged corolla tube (Figs. 1G, H). DNA barcoding showed that samples of this species were separated from *P. siphonantha* (Yu et al. 2011). We considered that this species may be new until we checked the type materials of *P. delavayi* conserved at the herbaria of the V. L. Komarov Botanical Institute in St. Petersburg (LE) (Fig. S1) and the Muséum National d'Histoire Naturelle in Paris (P). Based on morphological comparisons of specimens, we found that our specimens were very similar to the type of *P. delavayi*. In order to clarify the taxonomical confusion, we examined herbarium specimens of *P. siphonantha* collected from Yulong Mountain (type location of *P. delvayi*) conserved at the herbaria of CAS Kunming Institute of Botany (KUN) and CAS Institute of Botany (PE). We found that specimens labeled as "*P. siphonantha* var. *delavayi*" included two taxa: one is similar to the type of *P. delavayi*, and another is the "long-tubed and purple-red species" (Fig. 1B), a common species in northwestern Yunnan. Indeed, it is very difficult to discriminate the herbarium specimens as two taxa. Based on the field investigations, we found that *P. delavayi* differed from the "long-tubed and purple-red species" by having a long petiole and pedicel, inflated calyx tube in the middle upper parts, and folded lower lip of the corolla, as well as occurring at altitudes over 3600 m a.s.l. During recent field expeditions, specimens of *P. delavayi* were collected from the Yulong Mountain at over 4000 m a.s.l (Fig. 1F), and those of the "long-tubed and purple-red species" between 3600 m and 4000 m a.s.l. (Fig. 1B). Therefore, we confirmed that *P. delavayi* and the "long-tubed and purple-red species" were two separated species, and the "long-tubed and purple-red species" should be an undescribed species. To reduce descriptive confusion, we proposed a name "*P. millina* W. B. Yu & H. Wang" for this taxon in following statements and discussion.

Recently, a comprehensive phylogeny of Chinese *Pedicularis* shows that *P. siphonantha* is a polyphyletic group, var. *delavayi* (≡ *P. delavayi*) and other varieties of *P. siphonantha* falling into two subclades in clade 3 (Yu et al. 2015). *Pedicularis delavayi* was sister to *P. obliquigeleata* in subclude A. In the subclade 3B, var. *siphonantha* and var. *stictochila* H. Wang & W.B. Yu (= *P. tenuituba* H.L. Li), and five species from series *Longiflorae* with purple/red/pink corollas with twisted beaks formed a strongly supported lineage, i.e. the *P. siphonantha* lineage (that is molecular delimitation). In the present study, we made extensive sampling of *P. delavayi* from 9 populations (10 new samples), and two additional samples of *P. millina* from Yulong Mountains in Lijiang, one additional new samples of *P. tenuituba*, and all recognized taxa in clade 3 and the sister *P. axillaris* Franch. ex Maxim. (Yu et al. 2015). DNA sequences from nrITS and four chloroplast regions (*matK*, *rbcL*, *trnH-psbA* and *trnL-F*) were generated and analyzed. Our main goal was to evaluate the monophyly of *P. delavayi*, and its phylogenetic relationship to the *P. siphonantha* lineage, in particular to *P. milliana* and *P. tenuituba*. If monophyletic *P. delavayi* was excluded from the *P. siphonantha* lineage, *P. delavayi* should be reinstated as an independent species.

MATERIAL AND METHODS

Plant samplings. In total, we sampled 75 individuals representing 56 taxa, including all taxa were identified in clade 3 and its sister *P. axillaris* (Yu et al. 2015). *Pedicularis siphonantha* group, based on morphological delimitation, consisted of 11 species (Table S1). Of them, four species *P. delavayi*, *P. milliana* (new species), *P. siphonantha*, and *P. tenuituba* (= *P. siphonantha* var. *stictochila*) have a wide distribution range. The remaining seven species only collected once or few gatherings around the type locality: 1) *P. sigmoidea* Franch. ex Maxim. were found in around Eryuan and Lijiang, northwestern Yunnan; 2) *P. dolichosiphon*

(Hand.-Mazz.) H.L.Li) (≡ *P. siphonantha* var. *dolichosiphon* Hand.-Mazz.), *P. dolichantha* Bonati, *P. leptosiphon* H. L. Li, *P. variegata* H. L. Li and *P. humilis* Bonati were recollected from the type locality; and 3) *P. fastigiata* Franch. only had the type, which did not include in this study. In this study, we chose 11 samples of *P. delavayi* (three samples from the type locality Yulong Mountain, Lijiang), and three samples of *P. milliana* and *P. tenuituba* (Table S2). Natural population of *P. humilis* was just rediscovered in 2015 (Li et al. 2016). It is the first time to include this species for phylogenetic analyses.

Fresh leaf tissues were collected in the field and preserved in silica gel. All DNA samples and voucher specimens are stored at the Germplasm Bank of Wild Species and the herbarium of CAS Kunming Institute of Botany (KUN), respectively. There are 284 sequences from 64 individuals which have been published in other studies (China Plant BOL Group 2011; Yu et al. 2011; Liu & Yu 2015; Yu et al. 2015). In this study, we generated 62 new sequences from 23 individuals (with 11 new individuals). A conspectus of voucher information is presented in Table S2.

Specimen examination and identification. Fresh specimens were observed in the field. Fresh flowers were collected and fixed in FAA solution. Herbarium specimens from the herbaria CDBI, KUN, LE, and PE were examined and identified, and digital images of types from the herbaria E, K and P were accessed online. Flower and fruits characters in the line drawings of *P. delavayi* were based on field photos and FAA-preserved flowers.

Molecular methods. For the 11 new samples, total genomic DNA was extracted from silica gel-dried tissue using a modified 2× CTAB method. Five DNA loci, one nuclear gene (nrITS) and four chloroplast genes/regions (*matK*, *rbcL*, *trnH-psbA*, and *trnL-F*), were sequenced in this study. Primer information for the five loci were presented in previous studies (Yu et al. 2011; Yu et al. 2013b). Protocols for polymerase chain reaction (PCR) amplification and sequencing followed the study of (Yu et al. 2011).

Sequence assembly and alignment. The newly obtained raw sequences were assembled and edited using Geneious version 7.1 (Kearse et al. 2012). The nrITS is a multiple copy region. These copies showed evolutionary consistent in the sequenced 85 samples, only three samples had one ambiguous basecall (i.e. multiple superimposed peaks in chromatograms). Two samples, LID1199 and HW10244, belonging to *P. tenuituba*, and sample HW10163 is *P. milliana*. The ambiguous site was assigned using IUPAC ambiguity characters.

Preliminary alignments were automatically aligned using MAFFT version 7.2 (Katoh & Toh 2010), then adjusted manually in Geneious. The aligned matrix was concatenated to a combined matrix using SequenceMatrix version 1.73 (Vaidya, Lohman & Meier 2011). Sequence characteristics were calculated using MEGA version 6.0 (Tamura et al. 2013).

Phylogenetic analyses. Bayesian Inference (BI) and Maximum Likelihood (ML) methods were used to reconstruct phylogenetic trees. The nrITS and plastid datasets were combined to analyze. No nucleotide positions were excluded from analyses. Partitioned BI analyses were performed using MrBayes (Ronquist & Huelsenbeck 2003), with DNA substitution models selected for each gene partition by the Bayesian information criterion (BIC) using jModeltest (Guindon & Gascuel 2003; Darriba et al. 2012). Markov Chain Monte Carlo (MCMC) analyses were run in MrBayes for 10,000,000 generations for each dataset, with two simultaneous runs, and each run comprising four incrementally heated chains. The BI analyses were started with a random tree and sampled every 1000 generations. Number of generations for the three datasets were sufficient, because the average standard deviation of split frequencies for the datasets was lower than 0.005 (0.002900), and Potential Scale Reduction Factor of Convergence Diagnostic (Gelman & Rubin 1992) for the datasets was 1.00. The first 25% of the trees was discarded as burn-in, and the remaining trees were used to generate a majority-rule consensus tree. Posterior probability values (PP) ≥ 0.95 were considered as well supported (Alfaro, Zoller & Lutzoni 2003; Erixon et al. 2003; Kolaczkowski & Thornton 2007). The ML tree searches and bootstrap estimation of clade support were conducted with RAxML (Stamatakis, Hoover & Rougemont 2008). These analyses used the GTR substitution model with gamma-distributed rate heterogeneity among sites and the proportion of invariable sites estimated from the data. The dataset was partitioned by genes. Support values for the node and clade were estimated from 1000 bootstrap replicates. Bootstrap support (BS) ≥ 70 are considered well supported (Hillis & Bull 1993). Both BI and ML analyses, as well as jModeltest, were performed at the CIPRES Science Gateway (http://www.phylo.org).

RESULTS

Information of DNA sequences. Sequence characteristics of five DNA regions and the concatenated datasets are summarized in Table 1. In the total dataset, the numbers of variable and parsimony informative sites were highest for nrITS, followed by *trnH-psbA*, *trnL-F*, *matK* and *rbcL*. For three selected groups, three spacers (nrITS, *trnH-psbA* and *trnL-F*) were more variable and informative than two coding genes (*matK* and *rbcL*), then *matK* was more than *rbcL*. One exception for *P. delavayi*, alignment of *matK* had only one variable site in the 11 individuals, whereas alignment of *rbcL* had three variable and two informative sites, respectively.

Phylogenetic analyses. The BI tree using the total dataset was presented in Figure 2. The topology was similar to that in previous study (Yu et al. 2015). Two major clades were recovered, named as A and B following Yu et al. (2015). *Pedicularis delavayi* fell into clade A, and *P. siphonantha* lineage was in clade B. Both *P. delavayi* (BS/PP = 100/1.00) and *P. siphonantha* lineage (BS/PP = 96/1.00) were strongly as monophyletic, respectively. In the clade of *P. delavayi*, three Lijiang samples (type locality) formed a group (BS/PP =98/1.00), which was weakly supported sister to the remaining eight samples (PP = 0.62); three Sichuan samples were strongly supported as monophyletic (BS/PP = 92/1.00), and two Yunnan samples

(HW10130 and HW10172) as sister. The *P. siphonantha* lineage split in two groups. One group included clade *P. dolichosiphon* + *P. leptosiphon* (BS/PP = 100/1.00), and monophyletic *P. siphonantha* (BS/PP = 100/1.00) and *P. tenuituba* (= *P. siphonantha* var. *stictochila*) (BS/PP = 100/1.00). Another group comprised of the remaining five sampled species (including *P. humilis*) and sample LIDZ1518. Three samples of *P. millina* from Lijiang were monophyletic by moderate supporting (BS/PP = 55/0.88), then the Lijiang sample (LIDZ1584) of *P. sigmoidea* was resolved as sister (BS/PP = 99/1.00), followed by the Eryuan sample (YWB2015059) of *P. sigmoidea* (BS/PP = 100/1.00). *Peducularis humilis* nested with sample LIDZ1518 (BS/PP = 84/0.79), with long branch length, and *P. variegata* was sister to them (BS/PP = 90/1.00).

Morphological comparisons. Morphologically, *P. delavayi* is similar to *P. siphonantha* by in having large and bi-lobed middle lobe of lower-lip, and semi-circle and crestless beak. However, the middle lobe of *P. delavayi* was significantly incurved (Figs. 1C-G; vs. spreading in *P. siphonantha*, Fig. 1A), which was crushed in herbarium specimens (e.g. Fig. S1). Based on comparisons of flowering specimens, we found that *P. delavayi* also differed from *P. siphonantha* by having a long petiole (~ 50 mm) and pedicel (~ 40mm), a furfuraceous surface on the abaxial leaf blade, a ridged corolla tube, a folded lower-lip of the corolla, and four pubescent filaments. In addition, we found that the type of *P. neolatituba* was very close to specimens of *P. delavayi* and should be reduced to a synonym of *P. delavayi*.

Pedicularis milliana (Fig. 1C) is very similar to *P. siphonantha* by having smooth corolla tube, spreading corolla lower-lip, large and bi-lobed middle lobe, and semi-circle and crestless beak. Because the distribution of *P. delavayi* overlaps with *P. milliana* in northwestern Yunnan, Tsoong (1963) might have misidentified *P. milliana* as *P. delavayi*. Therefore, he downgraded *P. delavayi* to a variety under *P. siphonantha*. Clearly, *P. milliana* was separated from *P. siphonantha*, which is strongly supported by phylogenetic analyses. In addition, sample LIDZ1518 was similar to *P. milliana* in having spreading corolla lower-lip, large and bi-lobed middle lobe, and semi-circle beak, and to *P. sigmoidea* in having spreading corolla lower-lip, large and bi-lobed middle lobe, and crested beak. However, phylogenetic analyses indicating it was a separated lineage, close *P. humilis* and *P. variegata*.

DISCUSSION

Phylogenetic delimitation of *P. siphonantha* group. *Pedicularis siphonantha* was firstly described from Nepal (Don, Hamilton & Wallich 1825), which has been recognized as endemic to the Himalayan region (Pennell 1935; Li 1949). According to current taxonomic treatments (Tsoong 1963; Yang, Holmgren & Mill 1998), *P. siphonantha* var. *delavay* and var. *stictochila* occur in the Hengduan Mountains region, i.e. northwestern Yunnan, western Sichuan, and southeastern Qinghai. *Pedicularis siphonantha* var. *dolichosiphon* was discovered in Muli region of southern Sichuan (Handel-Mazzetti 1923), then upgraded to an independent species by Li (1949). In the Chinese edition of *Flora Reipublicae Popularis Sinicae*, Tsoong (1963) did not mention *P. dolichosiphon*, or he might have overlooked this species. According to current phylogenetic analyses, *P. siphonantha* was polyphyletc, delimitation of *P. siphonantha* group needed to revise. Firstly, *P.*

siphonantha var. *delavayi* was close to *P. obliquigaleata* in clade A, whereas the other taxa of *P. siphonantha* were included in the *P. siphonantha* lineage. Therefore, *P. siphonantha* var. *delavayi* should be reinstated as an independent species. Then, the remaining three infraspecific taxa of *P. siphonantha* (var. *dolichosiphon*, var. *siphonantha* and var. *stictochila*) and *P. leptosiphon* formed a clade, and var. *dolichosiphon* was strongly supported as sister to *P. leptosiphon*. Of them, *P. siphonantha* var. *siphonantha* has a semicircle beak, and the other three taxa have S-shaped beak. Integrating geographical distribution, we agree with the treatment by Li (1949) to adopt var. *dolichosiphon* and var. *stictochila* as independent species as *P. dolichosiphon* and *P. tenuituba*, respectively.

Infraspecific delimitation of *P. siphonantha* was not fully resolved. In a taxonomical revision, Prain (1890) included *P. hookeriana* Wall. ex Benth. as a synonym of *P. siphonantha* var. *siphonantha*, and *P. elephas* Boiss. and *P. punctata* Decne. as synonyms of var. *brevituba* Prain. Nevertheless, some taxonomists treated *P. hookeriana* and *P. punctata* as independent species (Pennell 1943; Yamazaki 1988; Husain, Garg & Agnihotri 2010), and have placed *P. elephas* close to *P. rhinanathoides* Schrenk (Maximowicz 1888; Pennell 1943). A comprehensive phylogeny of *Pedicularis* showed that both *P. hookeriana* and *P. punctata* fell into the clade of *P. siphonantha* from the Himalayas (R. Ree, Personal Communication). In the early of 1900s, Bonati added two varieties under *P. siphonantha*, var. *prostrata* Bonati (1904) from Sikkim and var. *birmanica* Bonati (1921) from upper Burma. In a revision of *Pedicularis* from Bhutan, Mill (2001) pointed out that *P. siphonantha* var. *prostrata* was easily confused with *P. hookeriana*, whereas this variety had broader and ovate leaves and shorter corolla tubes. For *P. siphonantha* var. *birmanica*, we found its type materials were close to that of *P. humilis*.

Parallel evolution of long-tubular corollas in *Pedicularis*. During revision of *Pedicularis*, Li (1948, 1949, 1951) and Tsoong (1955, 1963) hypothesized that long-tubular corollas were independently evolved at least six and ten times, respectively. Phylogenetic inferences supported their hypotheses that long-tubular corollas were independently derived from short-tubular corollas at least eight times (Ree 2005), or up to 21 times (Yu et al. 2015). Long-tubular species occurred in seven of 13 clades, plus two unresolved species *P. batangensis* Franch. & Bur. and *P. flexuosa* Hook. f. (Yu et al. 2015). Series *Longiflorae* Prain included more than 20 long-tubular species from the Himalaya-Hengduan Mountains region (Li 1949; Tsoong 1963; Yamazaki 1988; Mill 2001). Species of series *Longiflorae* fell into clade 3, however, this series was supported as monophyletic (Yu et al. 2015; this study). Phylogenetic analyses tended to split series *Longiflorae* into four groups: a) *P. siphonantha* lineage, b) *P. delavayi*, c) *P. longiflora*, and 4) *P. armata* – *P. cranolopha* group (including a short-tubular species, *P. fletcherii*). From morphological similarity and geographical distribution, *P. delavayi* and *P. longiflora* were close to *P. siphonantha* lineage and *P. armata* – *P. cranolopha* group, respectively. However, phylogenetic evidence indicated that the four groups may evolve independently.

Evolution of long-tubular corollas in *Pedicularis* were hypothesized to adopt long-tongued pollinators (Li 1951). However, pollination observations showed that long-tubular species were exclusively pollinated by bumblebees (Macior & Sood 1991; Macior & Tang 1997; Macior, Tang & Zhang 2001; Wang & Li 2005; Huang & Fenster 2007; Huang, Wang & Sun 2016). Long-tubular corollas are associated with beaked galea, and beaked species rewards pollinators for pollen alone (Macior & Sood 1991; Yu et al. 2008). Due to anthers are tightly enclosed by the beaked galea, long-tongued Lepidoptera are impossible to dislodge pollen from the tightly enclosed anthers. Only bumblebees can open concealed anthers from the beaked galea using forelegs, and release pollen by vibrating wings in high speed, i.e. buzz-pollination (Yu, Li & Wang 2012). When long-tongued pollinators driving evolution of long-tubular corollas was rejected, an alternative hypothesis for enhancing pollination attractiveness was proposed (Macior & Sood 1991; Macior, Tang & Zhang 2001). However, pollinator attraction hypothesis was not supported by experiments on *P. siphonantha* (corrected as *P. milliana* herein) and *P. tricolor* Hand.-Mazz. (Huang, Wang & Sun 2016). Pollination treatments indicated that elongation of corolla tube (and pistil length) may put more selective pressure for male-to-male competition during the pollen germination (Yang & Wang 2015). Moreover, plants growing in more fertilized conditions can produce longer corolla tube (Huang, Wang & Sun 2016). We suggested that evolution of long-tubular corollas may have some advantages in high altitudes, because most of long-tubular species occur in alpine meadow over 3000 m a.s.l. in the Himalya-Hengduan Mountains region (Tsoong 1956, 1961). Such ecological factors may independently drive elongation of corolla tube in different lineages. Subsequent diversification of lineage may be mainly induced by geographical isolation. *Pedicularis siphonantha* lineage is one good example to illustrate geographical isolation facilitating species divergence in the Himalya-Hengduan Mountains region (Yu et al. 2015).

Reinstatement of *Pedicularis delavayi*. Phylogenetic analyses strongly support *P. delavayi* as a separated species, which is sister to *P. obliquigaleata* in clade A, not included in *P. siphonantha* lineage in clade B. From floral color and beak shape, *P. delavayi* was easy to misplace into the *P. siphonantha* group. In the revision of Chinese *Pedicularis*, Li (1949) cited dozens of specimens for *P. delavayi*; however, some Sichuan specimens were *P. tenuituba*, and some Yunnan specimens were *P. milliana*. Subsequently, Tsoong (1963) might be failed to check diagnostic characters of *P. delvayi*, or might misidentify *P. milliana* or *P. tenuituba* as *P. delavayi*, thus he downgraded *P. delavayi* as a variety in *P. siphonantha*. Unfortunately, Tsoong's incorrect treatment has been widely adopted by current Chinese Floras (Yang, Holmgren & Mill 1998; Wang 2006), checklists (Wang & Wu 1994; Guan 1998; Xu, Li & Sun 2013) and other publications (Cai et al. 2004; Yu, Zhang & Wang 2008). Moreover, illustrations and/or voucher specimens of "*P. siphonantha* var. *delvayi*" from northwestern Yunnan were *P. milliana*, or mixed with *P. milliana* (e.g. Wang & Wu 1994; Guan 1998; Cai et al. 2004; Yu, Zhang & Wang 2008; Xu, Li & Sun 2013). Some herbarium specimens of *P. tenuituba* from Sichuan were misidentified as "*P. siphonantha* var. *delvayi*". Phylogenetic analyses supported *P. tenuituba* sister to *P. siphonantha*, and *P. milliana* sister to *P. sigmoidea*. To further clarify phylogenetic relationship between *P. milliana* and *P. sigmoidea* needs to extensively sample more populations of them in northwestern Yunnan. According to morphological and phylogenetic evidence, we propose to reinstate *P. delavayi* as an independent species. Full description and line drawing (Fig. 3) were provided.

Pedicularis neolatituba P. C. Tsoong was described from Songpan, northern Sichuan, which had short plant (less than 10 cm), long pedicel (up to 4 cm) and basal circinate-incurved galea (Tsoong 1963). In protologue, Tsoong proposed this species similar to three long-pedicelled species, *P. franchetiana*, *P. mussotii*, and *P. mychophila*, then established series *Neolatitubae* P. C. Tsoong. After checking the type specimen of *P. neolatituba* (Fig. S2), we found that it was difficult to distinguished from specimens of *P. delavayi*. *Pedicularis delavayi* also has long pedicel, anterior cleft and mid-upper part inflated calyx, basal twisted galea, semi-circle beak, ciliate corolla lobes and pubescent filaments. The plant height is variable in different specimens. Therefore, we proposed to reduce *P. neolatituba* as a new synonymy of *P. delavayi.*

Taxonomic treatments

Pedicularis delavayi Franch. ex Maxim., Bull. Acad. Imp. Sci. Saint-Pétersbourg 32: 531, pl. 1, fig. 7. 1888 ≡ *Pedicularis siphonantha* var. *delavayi* (Franch. ex Maxim.) P. C. Tsoong, Fl. Reipubl. Popularis Sin. 68: 374. 1963 — **Type:** CHINA. Yunnan: Lijiang (Li-kiang), Yulong Snow Mountain (Suee Shan), alt. 4,000m, 14 Aug. 1886, *J. M. Delavay s.n.* (holotype: LE!, barcode 01010308; isotypes: P!, barcode 02987194, K!, barcode 000708729).

Synonymy: *Pedicularis neolatituba* P. C. Tsoong, in Fl. Reipubl. Popularis Sin. 68: 418–419, pl. 72, f. 1–3. 1963. ***Syn. nov.*** — **Type:** CHINA. Sichuan: Songpan (Dongrergo), alt. 4,700m, 9 Aug 1922, *H. Smith 3162* (holotype: PE!, barcode 00033070; isotype: PE!, barcode 00119661).

Perennial herb, barely 10 cm tall, drying black or not. Roots fleshy, fusiform. Stems 1 to several, unbranched and erect or ± ascending, 2–10 cm, with lines of hairs. Basal leaves numerous, mostly membraneous and no leaf blade when beginning to flowering, blades development delayed; petiole up to 5 cm, winged, glabrescent; leaf blades lanceolate-oblong, 10–30 mm, sparely pubescent on both surfaces, abaxially furfuraceous, pinnatipartite; leaf segments 5–10 pairs, triangular-ovate to oblong-ovate, margin dentate; leafe veins sparely pubescent. Cauline leaves alternate or pseudo-opposite; petiole 0.5–5 cm, sparely pubescent; leaf blades and segments similar to basal ones. Flowers alternate and axillary, dense, flowering ± synchronous; pedicel 0.5–4 cm, sparely pubescent. Calyx tube 0.8–1 cm, 1/3–2/5 cleft anteriorly, mid-upper part inflated in flowering, sparsely long-pubescent; calyx lobes 3 or 5, rarely 2, lateral lobes leaflike, and posterior lobe ± entire or absent. Corolla purple-red, base whitish, and white spots on the base of galea and the center of lower lip; corolla tube 3–6.5 cm, slender, glabrescent, ridged; galea strongly twisted apically; beak slender, semicircular or slightly S-shaped, bent upward, to 1.2 cm; lower lip ciliate, 1.5–2.0 × 1.5–1.8 cm, lobes emarginate, middle lobe smaller and involute; filaments attached near tube throats, pubescent. Capsule obliquely oblong, apiculate, 1.4–1.7 × 0.4–0.6 cm; seed black, linear-ovate.

Distribution and Habitat. — Pedicularis delavayi is endemic to the Hengduan Mountains regions. After re-examination of the herbarium specimens and extensive field expeditions, we confirmed that this species occurs in Northwest Yunnan (Deqin, Lijiang, and Shangri-La counties), and West and North Sichuan (Daocheng, Jiulong, Kangding, Luding, Miangning, Muli, Songpan, and Xiangcheng counties). This species mainly grows in alpine meadows or at the margin of alpine shrub, at the altitude over 3600 m a.s.l.

Phenology. — According to field collection and herbarium records, flowering individuals were collected from early June to early August. Fruiting specimens conserved at the herbaria were difficult to identify. In August, 2007 and 2008, we collected fruiting individuals with mature seeds at Daxueshan Mountain of Shangri-Lain, Northwest Yunnan.

Conservation status. — *Pedicularis delavayi* is not common, and it is restricted to alpine meadows. Its habitats may be threatened by human activities in pasture and tourism. This species can be considered Least Concern (LC) according to IUCN Red List criteria.

Selected specimens examined. — CHINA. Yunnan: Deqin, *L.-M. Gao et al., 25794* (KUN); Lijiang, Yulong Mt. *H.-D. Li & H. Tang LHD2014-01* (KUN)*, LHD201-20* (KUN), *LHD201340* (KUN); Shangi-La, *W.-B.Yu 015* (KUN), *W.-B.Yu et al. HW10130* (KUN), *HW10172* (KUN), *HW10200* (KUN), *LIDZ1258* (KUN). Sichuan: Daocheng, Bowa Mt., *Sichuan Vegetation Exped. 1923* (CDBI); Jiulong, *Qing-Quan Wang 20508* (CDBI), *W.-B.Yu et al., LIDZ1116* (KUN), *YWB201507224* (KUN), *YWB201507260* (KUN); Kangding, Zheduo Mt., *Ru Jiang & Cun-Li Jin 02086* (KUN, PE), W.B.Yu et al., HW10316 (KUN); Xiangcheng, Wuming Mt., *Fu-Sheng Yang Y0071* (PE).

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Table 1(on next page)

Sequence characteristics of nrITS and four plastid DNA regions.

Table 1. Sequence characteristics of nrITS and four plastid DNA regions.

Parameters	nrITS	Plastid genes				Total dataset
		matK	*rbcL*	*trnH-psbA*	*trnL-F*	
No. of accessions	75	73	72	52	74	75
Aligned length (bp)	625	705	624	644	1045	3643
Variable sites/Parsimony informative sites						
Total dataset	211/144	161/85	60/37	200/89	234/108	866/463
P. delavayi + *P. siphonantha* lineage	65/33	52/29	19/14	78/37	63/33	277/146
P. delavayi	5/1	1/0	3/2	6/2	6/4	21/9
P. siphonantha lineage	52/21	42/20	15/11	67/30	52/22	228/104

Figure 1

Field photos of *P. delavayi* Franch. ex Maxim., *P. milliana* W. B. Yu & H. Wang and *P. siphonatha* D. Don.

A. *P. siphonantha*; B. *P. milliana*; C-I. *P. delavayi*: C, G-I, from Daxue Mtn., D from Hong Mtn., E from Yulong Mtn., F from Wuxu Lake. A and B were taken by Zhi-Kun Wu; E by Hai-Dong Li; C, D, and F-I by Wen-Bin Yu.


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Figure 2(on next page)

Phylogeny of *Pedicularis siphonantha* group inferred from Bayesian Inference (BI), Maximum Likelihood (ML) methods using nuclear and plastid datasets.

Topology shows the majority rule consensus of the BI tree using the total datasets. BI posterior probability (PP) ≥ 0.50 and ML bootstrap support (BS) ≥ 50 were annotated on the branch. PP ≥ 0.95 and/or BS ≥ 70 were drawn with thicker lines.

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Figure 3

Line drawing of *Pedicularis delavayi* Franch. ex Maxim. based on the gathering *W.-B. Yu 015* (KUN) from Daxue Mountain, Shangri-La, NW Yunnan.

A. Habit; B. leave; C. calyx tube; D. calyx tube open; E. corolla lower lp; F. stamens and style.

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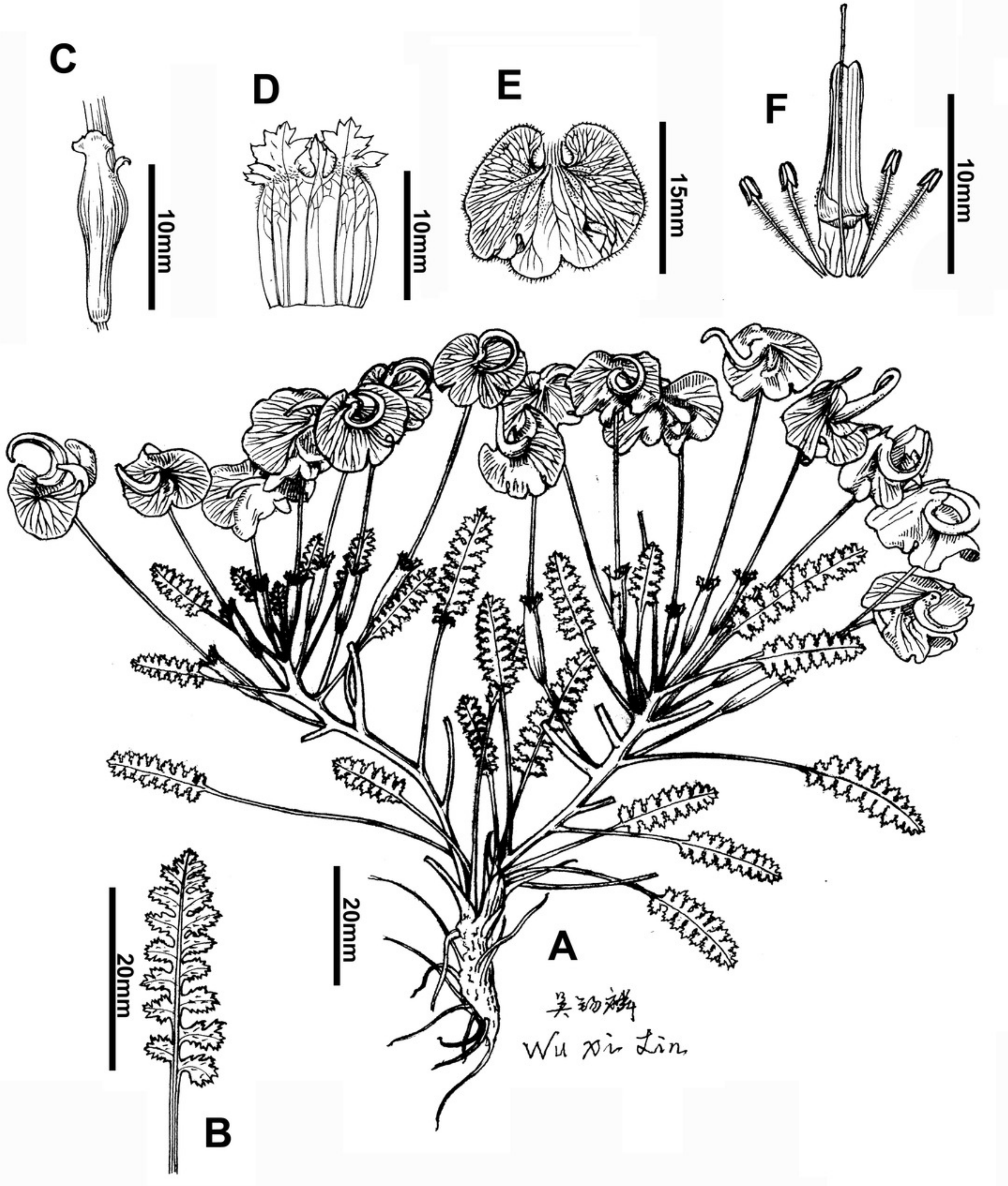
C
10mm
D
10mm
E
15mm
F
10mm
A
20mm
20mm
B
吴锡麟
Wu xi Lin