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Floral nectary morphology and evolution in *Pedicularis* (Orobanchaceae)

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Intricate associations between floral morphology and pollinator foraging behaviour are common. In this context, the presence and form of floral nectaries can play a crucial role in driving floral evolution and diversity in flowering plants. However, the reconstruction of the ancestral state of nectary form is often hampered by a lack of anatomical studies and well-resolved phylogenetic trees. Here, we studied 39 differentially pollinated *Pedicularis* spp., a genus with pronounced interspecific variation in colour, shape and size of the corolla. Anatomical and scanning electron microscopy observations revealed two nectary forms [bulged ($N = 27$) or elongated ($N = 5$)] or the absence of nectaries ($N = 7$). In a phylogenetic context, our data suggest that: (1) the bulged nectary should be the ancestral state; (2) nectaries were independently lost in some beaked species; and (3) elongated nectaries evolved independently in some clades of beakless species. Phylogenetic path analysis showed that nectary presence is indirectly correlated with beak length/pollinator behaviour through an intermediate factor, nectar production. No significant correlation was found between nectary type and nectar production, beak length or pollinator behaviour. Some beaked species had nectary structures, although they did not produce nectar. The nectary in beaked species may be a vestigial structure retained during a recent rapid radiation of *Pedicularis*, especially in the Himalaya–Hengduan Mountains of south-western China. © 2015 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2015, **178**, 592–607.

ADDITIONAL KEYWORDS: anatomy – ancestral state reconstruction – nectary form – pollinator behaviour – rudimentary structure.

INTRODUCTION

Floral nectaries are specialized structures that produce and secrete nectar (Fahn, 1979; Nepi, Ciampolini & Pacini, 1996; Pacini & Nicolson, 2007). Nectar itself is mainly exuded through modified stomata or, in some cases, through small epidermal pores, epidermal ruptures or the cuticle (Fahn, 1979, 1988; Pacini, Nepi & Vesprini, 2003). As nectar is a key reward for pollinators, nectar secretion and composition are often influential with regard to pollinator types and diversity (e.g. Baker & Baker, 1983; Bernardello, Galetto & Anderson, 2000; Nepi *et al*., 2006). Access to floral nectaries is largely dependent on corolla shape and size, and can trigger differential foraging behaviour of pollinators. In turn, pollinators exert selective pressure on flowers, which may result in functional constraints with respect to the combinations of floral traits, including the presence of nectaries (Stanton & Galloway, 1990; Galen, 1996; Macior, Tang

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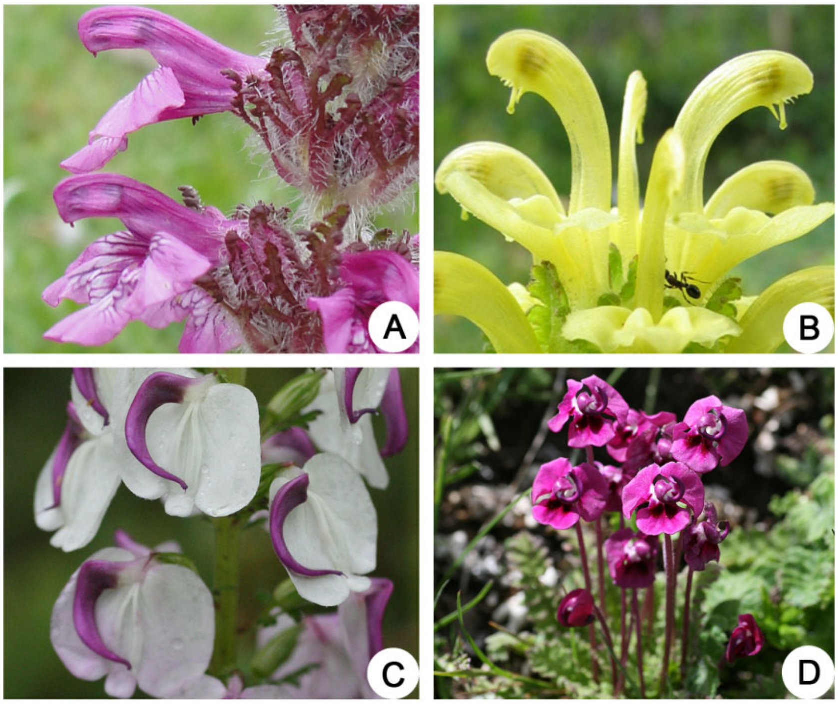

Figure 1. Four corolla forms of *Pedicularis* (Li, 1951). A, Short-tubed with beakless and toothless galea, e.g. *P. rupicola*. B, Short-tubed with beakless and toothed galea, e.g. *P. lutescens*. C, Short-tubed with beaked galea, e.g. *P. oxycarpa*. D, Long-tubed with beaked galea, e.g. *P. siphonantha*.

& Zhang, 2001; Wang & Li, 2005; Huang & Fenster, 2007). In order to understand the direction of evolutionary change, e.g. the loss or gain of nectaries, ancestral character states need to be reconstructed (Petanidou, Goethals & Smets, 2000; Fahn & Shimony, 2001; Bernardello, 2007). However, to date, for the genus *Pedicularis* L. (Orobanchaceae), the general lack of integrated anatomical/morphological, genetic and pollination data has impeded such progress.

For the present investigation, we focused on the species-rich genus *Pedicularis* (> 700 species) (Li, 1951; Mill, 2001) for which multiple data types from previous studies can be utilized in concert with novel results for the thorough exploration of nectary evolution. Further, this genus displays pronounced interspecific variation in the colour, shape and size of the corolla, which can be classified into four corolla types: (1) short-tubed with beakless, toothless galea (i.e. the hoodlike upper lip); (2) short-tubed with beakless, toothed galea; (3) short-tubed with beaked galea; and (4) long-tubed with beaked galea (Li, 1951; Yu *et al*., 2008, 2013a) (Fig. 1).

Pedicularis spp. are primarily pollinated by foraging bumblebees (*Bombus* spp.). In general, corolla forms are highly associated with pollinator foraging position, which can be nototribical (upright) and/or sternotribical (inverted) (e.g. Macior, 1982; Wang, Li & Cai, 2003; Yu *et al*., 2008). To date, long-tubed and long-beaked *Pedicularis* spp. are known to be visited only by pollen-foraging bumblebees, even though one would assume to find visits by other nectar-feeding lepidopteran insects with long probosces (Macior, 1990; Macior & Sood, 1991; Macior & Tang, 1997; Wang & Li, 2005). Hence, it has been argued that nectar-foraging pollinators have imposed particular constraints on the evolution of corolla tube length in *Pedicularis* (Ree, 2005; Huang & Fenster, 2007). On the one hand, corolla tube elongation may have evolved to attract pollinators and/or extend the buzz-pollination mechanism beyond the foliage of the plant (Wang & Li, 2005), which would otherwise interfere with its function (e.g. Macior & Tang, 1997). On the other, it is possible that longer styles function to prevent heterospecific pollen tubes from reaching the ovules (Ree, 2005).

In the last decade, molecular phylogenetic studies have revealed considerable homoplasy of floral morphology in *Pedicularis*, supporting the notion that the corolla has been subject to recurrent pollinator-mediated selection, resulting in the convergent evolution of specialized floral forms (i.e. long-tubed/beaked) from generalized ancestors (i.e. short-tubed/beakless) (Ree, 2005; Eaton *et al*., 2012; Tkach *et al*., 2014). This notion has been further supported by pollination/floral biology studies in *Pedicularis*, which have found that only short-tubed, beakless species produce nectar to reward pollinators (e.g. Macior, 1988, 1995; Macior & Tang, 1997; Wang *et al*., 2003). Although it thus seems plausible that nectariferous corollas represent the ancestral state, this hypothesis has not been investigated in a phylogenetic context and alternative outcomes are possible, especially in the light of the high evolutionary lability of floral form (Ree, 2005).

The understanding of nectary morphology and nectar secretion in *Pedicularis* is limited to date. Some pollination ecology and morphogenetic studies have found that nectaries in *Pedicularis* and in a number of other genera of Orobanchaceae are generally located at the base of the ovary and are either bulged (also called 'annular', 'disc' or 'ring-like') or elongated in shape (Knuth & Muller, 1906; Mattfeld, 1922; Sprague, 1962; Tsoong, 1963; Kwak, 1977, 1978, 1979; Meier-Weniger, 1977; Kwak, Holthuijzen & Prins, 1985; Bernardello, 2007; Huang & Fenster, 2007; Sulborska, Konarska & Chmielewski, 2014). Nectar production and sugar content have been examined in a few *Pedicularis* spp. (Macior, 1988; Huang & Fenster, 2007) and nectar has been documented to be exuded through modified stomata in the holoparasitic *Orobanche alsatica* Kirschl. (Sulborska *et al*., 2014).

In *Pedicularis*, nectar production is generally associated with beak length and pollinator foraging behaviour (Macior, 1988; Ree, 2005; Tang & Xie, 2006; Huang & Fenster, 2007). However, correlations between traits (or factors) of interest are often caused by intermediate traits (or factors) because of their non-independence resulting from shared ancestry, and may conceal intrinsic relationships (Gonzalez-Voyer & von Hardenberg, 2014). Nectar is produced and secreted from the nectary, but the mere presence of a nectary does not necessarily indicate nectar production. Therefore, correlations between nectar production and beak length/pollinator behaviour and between nectary presence/type and beak length/pollinator behaviour need to be investigated in a phylogenetic framework.

In this study, our main goals were to: (1) characterize the variation in nectary anatomy and morphology for *Pedicularis* taxa; (2) infer evolutionary patterns for nectary presence/type in a phylogenetic context; and (3) investigate the interrelationships among traits (i.e. nectary presence/type, nectar production, beak length and pollinator behaviour) from a phylogenetic standpoint by testing the following hypotheses: (1) nectar production relates directly to nectary type/beak length/pollinator behaviour; and (2) nectary presence/type relates directly or indirectly (through other traits such as nectar production) to beak length/pollinator behaviour.

MATERIAL AND METHODS

Mature flowers of 39 species, representing the four basic corolla types of *Pedicularis*, were field-collected in north-western Yunnan, China. For each species, 20–30 flowers from five to six individuals were randomly collected and fixed in formalin–acetic acid–alcohol (FAA) solution. Voucher specimens were deposited at the herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN; see Table A1). Tsoong's (1963) system for the infrageneric classification of *Pedicularis* was followed.

For anatomical observations, the calyx and corolla were removed, and nectary presence, shape and position relative to the ovary were examined under a dissecting microscope (Olympus SZX12). The remaining ovary and pedicel were dehydrated through an ethanol series and embedded in paraffin (Li, 1997). Cross- and longitudinal sections were cut at 6–8 μm using a rotary microtome with a steel blade (Leica RM2135), mounted serially and stained with haematoxylin/Fast Green FCF (Conn, Darrow & Emmel, 1960). The periodic acid–Schiff (PAS) reaction was performed to assess the presence of polysaccharides (McManus, 1948; Feder & O'Brien, 1968; O'Brien & McCully, 1981). Slides were sealed with Permount mounting medium and observed and photographed using light microscopy (LM) (Olympus Vanox) (Li, 1997).

For scanning electron microscopy (SEM), the ovary and pedicel were dehydrated using an ethanol and isoamyl acetate series before critical point drying with liquid CO_2 (Bio-Tec, CPD 030). After coating with gold, samples were examined under SEM (Hitachi S-4800).

To reconstruct the evolutionary history of nectary forms in *Pedicularis*, our analysis was limited to 42 species for which nectary information was available. In addition to the 39 species from this study, we included published data for *P. densiflora* Benth., *P. sceptrum-carolinum* L. and *P. sylvatica* L. (Knuth & Muller, 1906; Sprague, 1962; Kwak, 1977, 1979). A nearly complete global molecular phylogenetic analysis confirmed the representativeness of our restricted phylogenetic tree, with respect to the inclusion of the early-branching derived clades.

DNA sequences of 42 *Pedicularis* spp. were generated in this study, following the method of Yu *et al*. (2011), or obtained from previously published studies (Yu *et al*., 2011, 2013b) (Table A1). DNA sequences of five outgroup species were downloaded from GenBank. Sequences were assembled and edited using Sequencher version 4.1.4, and aligned using MAFFT version 7.0 (Katoh & Standley, 2013). Five DNA loci [internal transcribed spacer (ITS), *matK*, *rbcL*, *trnH-psbA* and *trnL-trnF*] were used to build a maximum likelihood (ML) tree with RAxML on the

CIPRES Science Gateway (http://www.phylo.org). The evolution of nectary forms was inferred by mapping character states onto the ML tree with parsimony optimization using the program Mesquite version 2.75 (Maddison & Maddison, 2011).

In order to examine intrinsic correlations among nectary presence/type, nectar production, beak length and pollinator behaviour, the phylogenetic generalized least-squares (PGLS) and d-separation methods for path analysis was performed using the statistical program R version 3.1.2 (R Development Core Team, 2013). Data on pollinator behaviour were taken from previous studies (Fig. 6, see below). Directed acyclic graphs were drawn based on the above-mentioned hypotheses. Coded traits and the phylogenetic tree were processed and incorporated following Gonzalez-Voyer & von Hardenberg (2014) and the online guidelines to path analysis (http://www.mpcm-evolution.org).

RESULTS

Nectary anatomy and morphology

Among the 39 studied species, 32 possessed nectaries on the abaxial side at the base of the superior ovary. The remaining seven species lacked nectariferous tissue (Table 1). Only the two previously known morphological nectary forms were found, i.e. bulged or elongated. Considerable intraspecific plasticity in nectary dimensions was apparent in both forms.

The bulged nectary was found in 27 species (Figs 2A–L, 3A–D) and varied from small protuberances (e.g. Fig. 2B) to large semicircular bulges (e.g. Fig. 2J), but never formed a complete ring. Modified stomata occurred across the upper part of the bulged nectary (Figs 2B, J, 3B).

Five species, *P. cymbalaria* Bonati, *P. deltoidea* Franch. ex Maxim. (Fig. 3E–H), *P. lutescens* Franch. ex Maxim. (Fig. 3I–L), *P. rex* C.B.Clarke ex Maxim. and *P. thamnophila* (Hand.-Mazz.) H.L.Li (Fig. 4A–D), possessed an elongated nectary. Variations of the elongated nectary were also found: the nectary of *P. rex* and *P. thamnophila* was straight, whereas that of *P. cymbalaria*, *P. deltoidea* and *P. lutescens* was rather falcate. The elongated nectary was occasionally even longer than the ovary, with modified stomata scattered near the apex of the nectary (Figs 3F, J, 4B).

All observed forms of the nectary consisted of epidermis, nectary parenchyma and vascular bundles. The epidermis was single-layered, glabrous and covered by a cuticle. The vascular bundle supporting the nectary originated from the ovary and comprised both phloem and spiral thickened xylem (Figs 2C, G, K, 3G). Nectary parenchyma and epidermal cells were intensely stained as a result of accumulated starch (Figs 2C, D, G, H, K, L; 3C, D, G, H, K, L; 4C, D). Modified stomata were present on a specified region of the nectary surface (see above), with two kidney-shaped guard cells. Guard cells were either slightly elevated or approximately isobathic to the neighbouring epidermal cells or, in some cases, sunken. The modified stomata were solitary, being surrounded by adjacent non-specialized epidermal cells (Fig. 5A–L).

Evolution of nectary forms

In the ML tree based on five DNA regions (Table A2), monophyly of *Pedicularis* was strongly supported [maximum likelihood bootstrap percentage (MLBP) = 100%] (Fig. 6). Opposite- and whorled-leaved species were distinctly separated from alternate-leaved ones and formed a well-supported clade (MLBP = 73%). Series *Lyratae* H.L. Li and series *Reges* H.L. Li and section *Cyathophora* H.L. Li were strongly supported as monophyletic (MLBP = 96%, 100% and 100%, respectively).

For the evolutionary analysis of the floral nectary, the five outgroups were removed because they had no nectary information (Fig. 6). Our results showed that the bulged nectary form was probably the ancestral state in *Pedicularis*, as it was found in early-branching *Pedicularis* spp., such as *P. aloensis* Hand.-Mazz., *P. densiflora* and *P. sceptrum-carolinum*. Further, the elongated nectary was gained twice, i.e. in series *Reges* (*P. rex* and *P. thamnophila*) and series *Lyratae* (*P. cymbalaria*, *P. deltoidea* and *P. lutescens*). The evolution of nectary forms was equivocal throughout the branch to the clade consisting of series *Lyratae* and *P. alopecuros* Franch. ex Maxim. The elongated nectary was a synapomorphy in series *Lyratae* in two equally parsimonious reconstructions and a synapomorphy for the clade formed by series *Lyratae* and *P. alopecuros* in one reconstruction. Nectary loss occurred six times in the examined samples and was an autapomorphy for *P. alopercuros*, *P. confertiflora* Prain, *P. elwesii* Hook.f., *P. lachnoglossa* Hook.f and *P. vialii* Franch. ex Hemsl.

Phylogenetic path analysis

On the one hand, for nectary presence, nectar production, beak length and pollinator behaviour, path analysis supported significant direct correlation between nectar production and beak length ($P < 0.001$; standardized path coefficient, −1.00), nectar production and pollinator behaviour ($P < 0.001$; standardized path coefficient, 0.54), beak length and pollinator behaviour ($P < 0.001$; standardized path coefficient, −0.46) and nectary presence and nectar production ($P < 0.01$; standardized path coefficient, 0.45) (Fig. 7A). Alternatively, direct correlation between nectary presence and

Table 1. Summary of nectary information of the 41 studied taxa of *Pedicularis*

Taxa	Series	Nectary form
Pedicularis aloensis Hand.-Mazz.	Series *Aloënses* H.L.Li	Bulged
P. alopecuros Franch. ex Maxim.	Series *Brevifoliae* Prain	No nectary
P. axillaris Franch. ex Maxim.	Series *Axillares* Maxim.	Bulged
P. comptoniaefolia Franch.	Series *Rigidae* Prain	Bulged
P. confertiflora Prain	Series *Debiles* Prain	No nectary
P. cyathophylla Franch.	Series *Cyathophyllae* H.L.Li	Bulged
P. cymbalaria Bonati	Series *Lyratae* Maxim.	Elongated
P. deltoidea Franch. ex Maxim.	Series *Lyratae* Maxim.	Elongated
P. densispica Franch. ex Maxim.	Series *Abrotanifoliae* Limpr.	Bulged
P. dichotoma Bonati	Series *Dichotomae* H.L.Li	Bulged
P. dolichocymba Hand.-Mazz.	Series *Dolichocymbae* P.C.Tsoong	Bulged
P. dunniana Bonati	Series *Rudes* Bonati	Bulged
P. elwesii Hook.f.	Series *Pseudomacranthae* P.C.Tsoong	No nectary
P. filicula Franch. ex Maxim.	Series *Filiculae* H.L.Li	Bulged
P. glabrescens H.L.Li	Series *Verticillatae* Maxim.	Bulged
P. gracilis Wallich ex Benth.	Series *Graciles* Maxim.	No nectary
P. integrifolia Hook.f.	Series *Integrifoliae* Prain	No nectary
P. labordei Vaniot ex Bonati	Series *Microphyllae* Prain	Bulged
P. lachnoglossa Hook.f.	Series *Lachnoglossae* Prain	No nectary
P. longiflora var. *tubiformis* (Klotzsch) P.C.Tsoong	Series *Longiflorae* Prain	Bulged
P. lutescens Franch. ex Maxim.	Series *Lyratae* Maxim.	Elongated
P. macrosiphon Franch.	Series *Muscicolae* Maxim.	Bulged
P. oxycarpa Franch. ex Maxim.	Series *Oxycarpae* Prain	Bulged
P. pseudomelampyriflora Bonati	Series *Melampyriflorae* Prain	Bulged
P. pygmaea subsp. *deqinensis* H.Wang	Series *Verticillatae* Maxim.	Bulged
P. rex C.B.Clarke ex Maxim.	Series *Reges* H.L.Li	Elongated
P. rhinanthoides Schrenk ex Fisch. & C.A.Mey.	Series *Rhinanthoides* Prain	Bulged
P. rhinanthoides subsp. *tibetica* (Bonati) P.C.Tsoong	Series *Rhinanthoides* Prain	Bulged
P. roylei Maxim.	Series *Verticillatae* Maxim.	Bulged
P. rupicola Franch. ex Maxim.	Series *Verticillatae* Maxim.	Bulged
P. salviaeflora Franch.	Series *Salviaeflorae* Prain	Bulged
P. siphonantha D.Don	Series *Longiflorae* Prain	Bulged
P. siphonantha var. *stictochila* H.Wang & W.B.Yu	Series *Longiflorae* Prain	Bulged
P. superba Franch. ex Maxim.	Series *Superbae* Maxim.	Bulged
P. tachanensis Bonati	Series *Oliganthae* Prain	Bulged
P. tenuisecta Franch. ex Maxim.	Series *Microphyllae* Prain	Bulged
P. thamnophila (Hand.-Mazz.) H.L.Li	Series *Reges* H.L.Li	Elongated
P. tongolensis Franch.	Series *Craspedotrichae* H.L.Li	Bulged
P. tricolor Hand.-Mazz.	Series *Longiflorae* Prain	Bulged
P. umbelliformis H.L Li	Series *Paucifoliatae* Prain	Bulged
P. vialii Franch. in Forbes & Hemsl.	Series *Subsurrectae* Prain	No nectary

beak length/pollinator behaviour was not significant ($P > 0.05$). Nectary presence was indirectly related to beak length/pollinator behaviour through the mediation of nectar production (standardized indirect effects, −0.49 and 0.44, respectively). On the other hand, for nectary type, nectar production, beak length and pollinator behaviour, direct correlations were still significant from the path analysis between nectar production and beak length ($P < 0.001$; standardized path coefficient, −0.84), nectar production and pollinator behaviour ($P < 0.001$; standardized path coefficient, 0.52) and beak length and pollinator behaviour ($P < 0.001$; standardized path coefficient, −0.50). Direct correlation between nectary type and nectar production was not significant ($P > 0.05$).

DISCUSSION

NECTARY ANATOMY AND MORPHOLOGY

The species investigated in this study formed an informative subset of *Pedicularis* spp., including

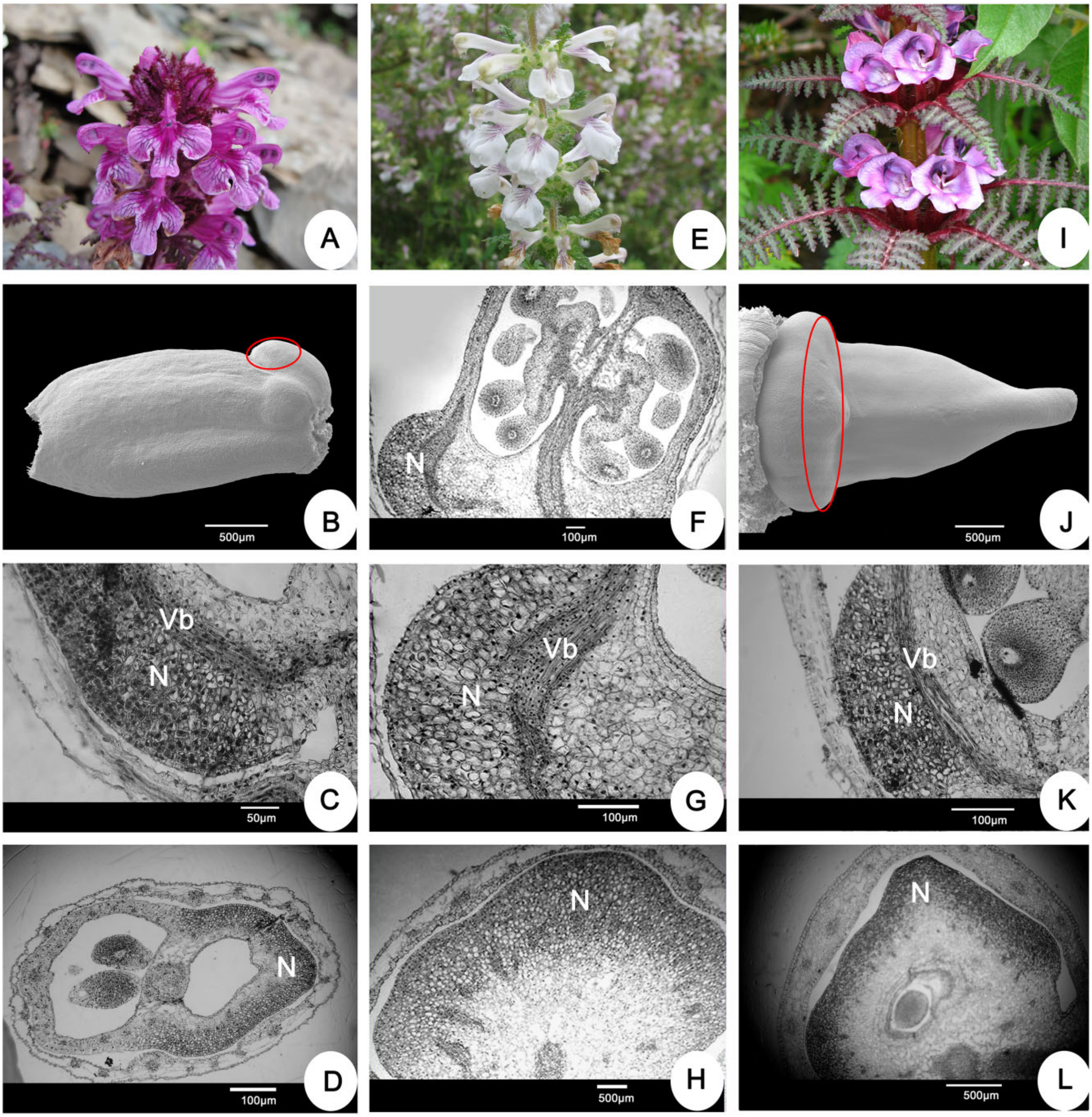

Figure 2. Flower and nectary form of *Pedicularis*. A–D, *Pedicularis rupicola* (short-tubed, beakless, toothless). A, Flowers. B, Ovary with nectary. C, Longitudinal section of nectary. D, Cross-section of nectary. E–H, *Pedicularis pseudomelampyriflora* (short-tubed, beakless, toothed). E, Flowers. F, Ovary with nectary. G, Longitudinal section of nectary. H, Cross-section of nectary. I–L, *Pedicularis superba* (short-tubed with beak). I, Flowers. J, Ovary with nectary. K, Longitudinal section of nectary. L, Cross-section of nectary. N, nectary; Vb, vascular bundle; red circle, stomata region (detailed illustration in Fig. 5).

representatives of the four general corolla types from all major clades across the phylogenetic tree. It is therefore likely that we were able to describe the existing variation in nectary forms present in *Pedicularis*, i.e. bulged or elongated (Tsoong, 1963; Kwak, 1977, 1979; Meier-Weniger, 1977; Bernardello, 2007; Sulborska *et al*., 2014), which have also been reported for other related genera, such as *Melampyrum* L., *Rhinanthus* L. and *Orobanche* L. (Mattfeld, 1922; Kwak, 1977, 1978, 1979; Kwak, Holthuijzen & Prins,

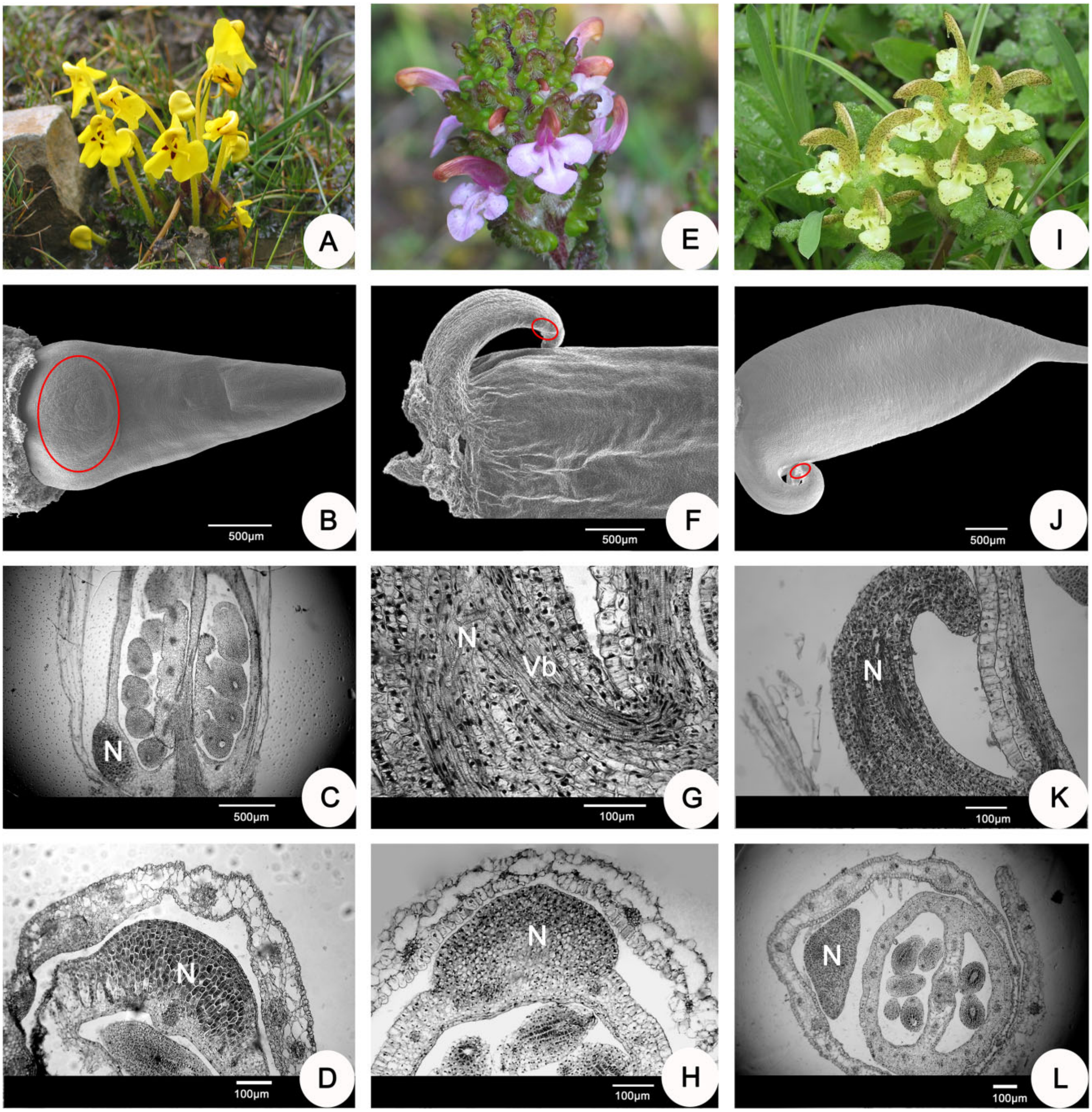

Figure 3. Flower and nectary form of *Pedicularis*. A–D, *Pedicularis longiflora* (long-tubed with beak). A, Flowers. B, Ovary with nectary. C, Longitudinal section of nectary. D, Cross-section of nectary. E–H, *Pedicularis deltoidea* (short-tubed, beakless, toothed). E, Flowers. F, Ovary with nectary. G, Longitudinal section of nectary. H, Cross-section of nectary. I–L, *Pedicularis lutescens* (short-tubed, beakless, toothed). I, Flowers. J, Ovary with nectary. K, Longitudinal section of nectary. L, Cross-section of nectary. N, nectary; Vb, vascular bundle; red circle, stomata region (detailed illustration in Fig. 5).

1985; Sulborska *et al*., 2014). Both nectary forms were found at the base of the ovary (Figs 2–4, *pro parte*). Tsoong (1963) suggested that the elongated nectary in series *Lyratae* might be initiated from the receptacle; however, to date, a receptacle nectary has never been found in *Pedicularis*.

Functional adaptation of the elongated nectary in *Pedicularis* remains unclear and deserves further experimental work. Davis *et al*. (1996) suggested that earlier formed floral organs could impose physical constraints on the morphology and size of the later derived nectary. In *Pedicularis*, nectary formation

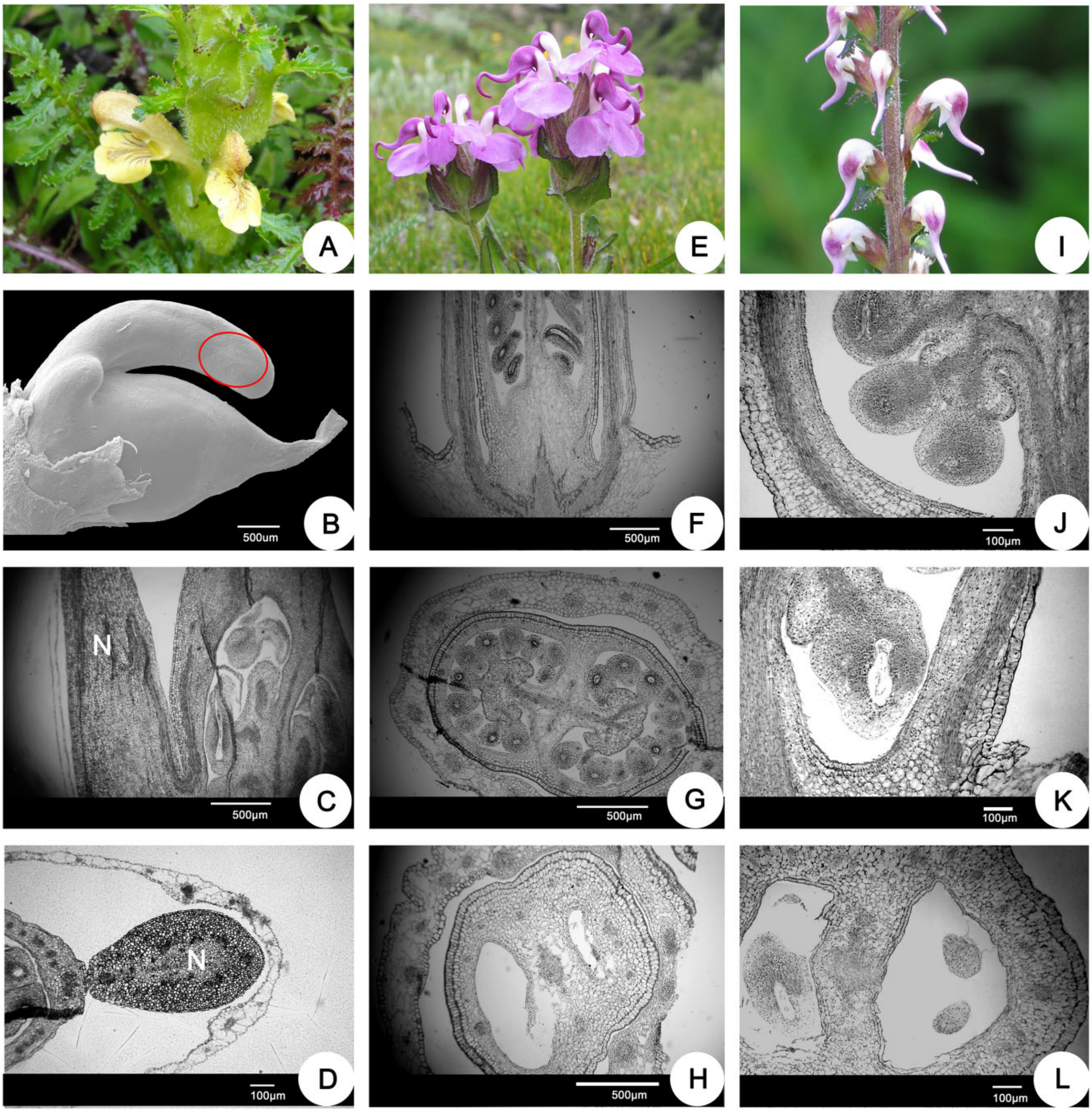

Figure 4. Flower and nectary form of *Pedicularis*. A–D, *Pedicularis thamnophila* (short-tubed, beakless, toothed). A, Flowers. B, Ovary with nectary. C, Longitudinal section of nectary. D, Cross-section of nectary. E–H, *Pedicularis integrifolia* (short-tubed with beak). E, Flowers. F, Longitudinal section of ovary. G, H, Cross-section of ovary. I–L, *Pedicularis vialii* (short-tubed with beak). I, Flowers. J, K, Longitudinal section of ovary. L, Cross-section of ovary. N, nectary; Vb, vascular bundle; red circle, stomata region (detailed illustration in Fig. 5).

appears to be initiated after the establishment of the sepals, petals and ovary (Meier-Weniger, 1977; Cai, Liang & Wang, 2003; Yu *et al*., 2013a). Those species with an elongated nectary, e.g. *P. cymbalaria*, *P. deltoidea*, *P. lutescens*, *P. rex* and *P. thamnophila*, all had short, straight, wide corolla tubes and expanded throats, presumably providing more space for the development of an elongated nectary. However, this hypothesis is challenged by the species that have similar corolla architecture but only a bulged nectary, e.g. *P. sceptrum-carolinum*, *P. aloenesis*, *P. densiflora*, etc.

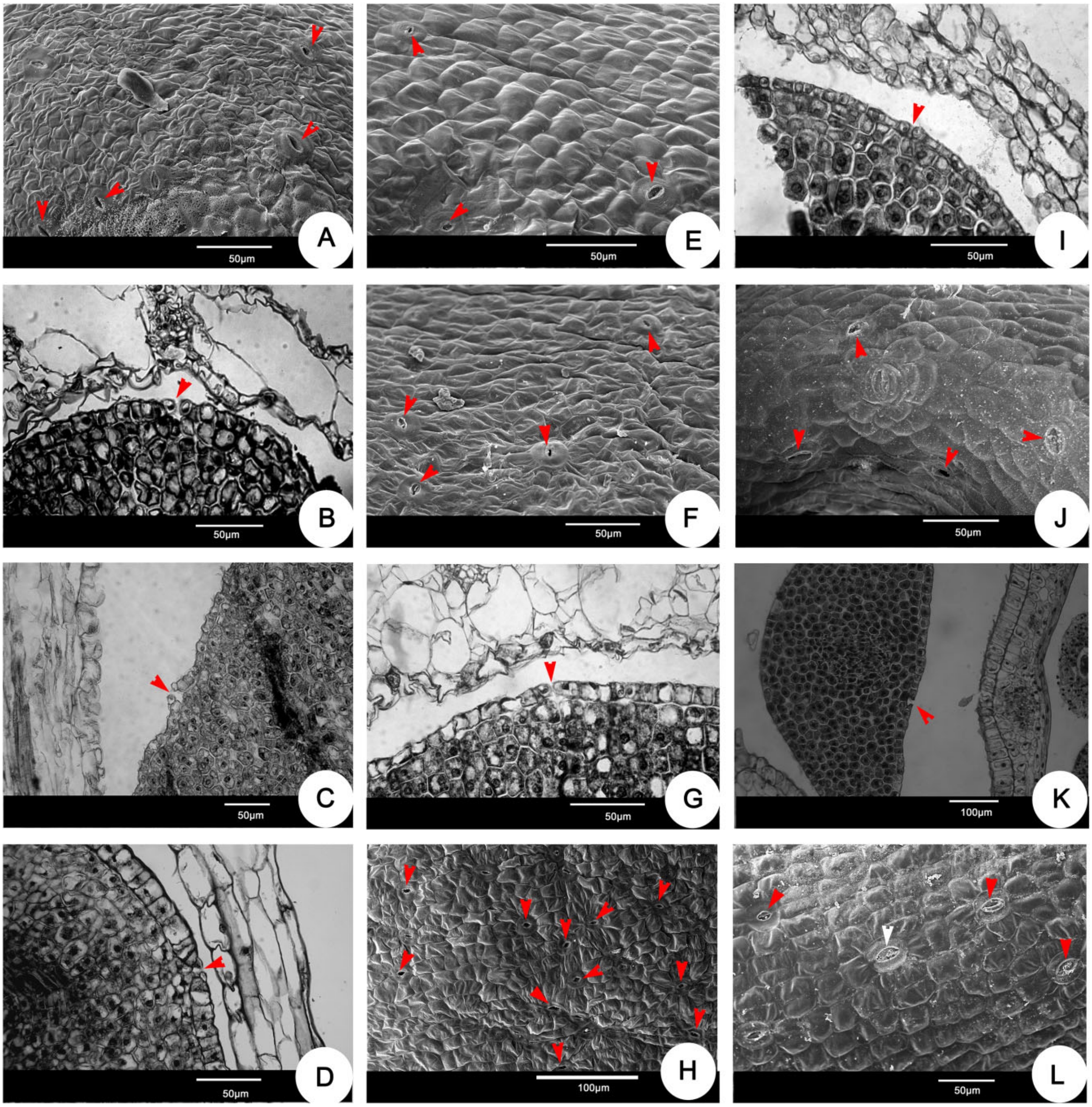

Figure 5. Modified stomata on nectary of *Pedicularis*: A, B, *P. rupicola*; C, *P. salviaeflora*; D, *P. dolichocymba*; E, *P. superba*; F, G, *P. cyathophylla*; H, I, *P. longiflora*; J, K, *P. lutescens*; L, *P. rex*. Red arrow, stomata.

Contrary to expectation, a rudimentary nectary structure was found in some long-tubed species, e.g. *P. siphonantha* D.Don, which have been reported previously to be nectarless (Huang & Fenster, 2007). These nectary structures were probably overlooked in the past because they are inconspicuous when superficially observed with light or electron microscopy. In our study, these structures were revealed mainly in stained microsections. In addition, we found nectary dimensions in *P. siphonantha* to vary from small protuberances to semicircular bulges, which may represent developmental stages of the nectary. Nevertheless, the rudimentary nectary in long-tubed species does not appear to be functional and may be an evolutionary remnant.

All nectariferous *Pedicularis* taxa investigated had modified stomata, as similarly found in the related *Orobanche alsatica* (Sulborska *et al*., 2014). Whereas

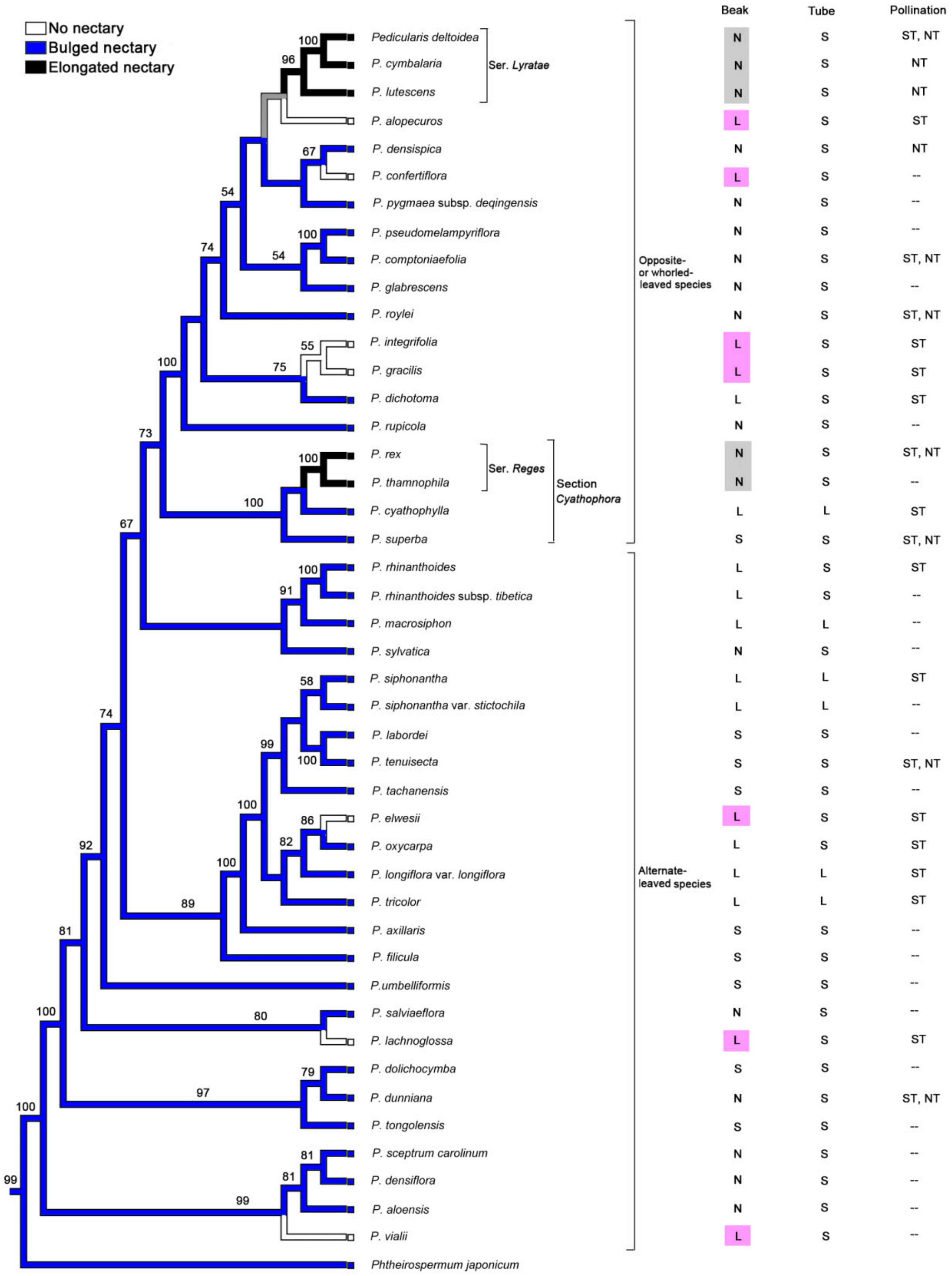

Figure 6. See caption on next page.

Figure 6. Maximum likelihood tree of *Pedicularis* obtained from the combined dataset of internal transcribed spacer (ITS), *matK*, *rbcL*, *trnH-psbA* and *trnL-trnF*, and inference of evolutionary trend of nectary form in *Pedicularis*. Grey line, equivocal evolutionary pattern. Beak, beak length; Tube, tube length; Pollination, pollinator behaviour; N, no beak (beakless); L, long beak/tube; S, short beak/tube; NT, nototribically; ST, sternotribically; –, missing data; grey shaded, state of beak in species with elongated nectary; pink shaded, state of beak in nectarless species. Data on pollinator behaviour were obtained from previous studies (Wang & Li, 1998; Macior *et al*., 2001; Wang *et al*., 2003; Sun *et al*., 2005a, 2005b; Tang & Xie, 2006; Tang, Xie & Sun, 2007; Yang, Gituru & Guo, 2007; Yu, 2007; Yu *et al*., 2012; Huang & Shi, 2013; Armbruster, Shi & Huang, 2014).

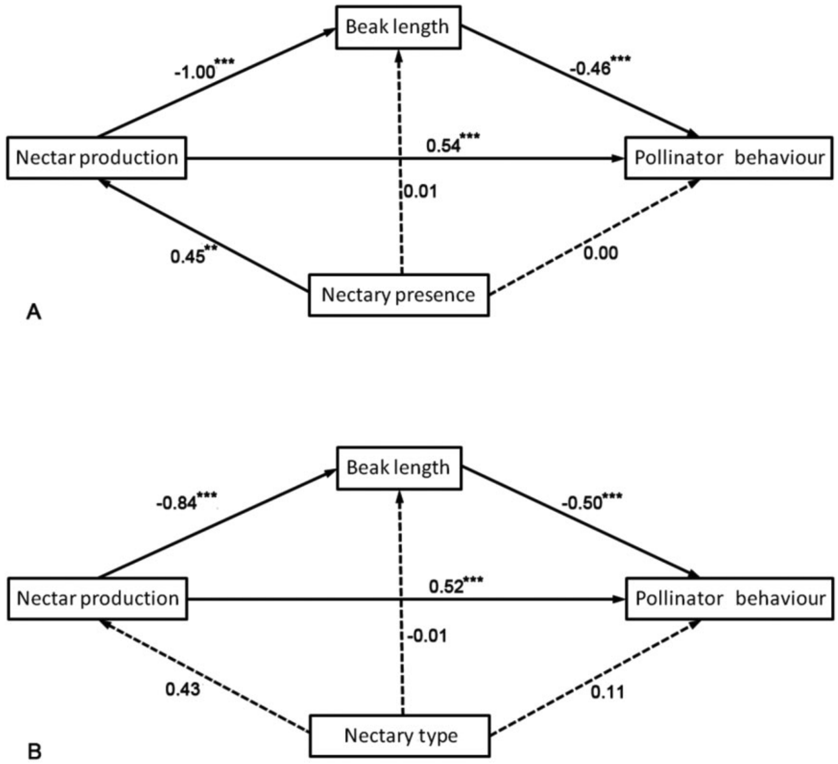

Figure 7. Hypothesized path model. A, Directed acyclic graph controlling nectary presence, nectar production, beak length and pollinator behaviour. B, Directed acyclic graph controlling nectary type, nectar production, beak length and pollinator behaviour. Numbers, standardized path coeffcients; full lines, significant correlation; broken lines, non-significant correlation; $^{**}P < 0.01$; $^{***}P < 0.001$.

the modified stomata were located below other epidermal cells for *O. alsatica*, the relative position of the stomata to the surrounding epidermal cells in *Pedicularis* was elevated, isobathic or embedded. In the broader context, Petanidou *et al*. (2000) and Papp, Csete & Farkas (2013) ascribed this variation in stomatal position to the growing conditions of plants and to habitat-specific water availability, respectively. Although variability in the position of modified stomata was present in *Pedicularis*, our methodological design precluded us from examining the potential influences of habitat ecology and physiology on this.

The nectary parenchyma in *Pedicularis* contained starch grains (Razem & Davis, 1999), which stained purple (Figs 2–4, *pro parte*). It is well documented that starch grains can be hydrolysed to sugar as nectar begins to form (e.g. Nepi *et al*., 1996; Pacini *et al*., 2003). Consequently, it is likely that the sugars are transported to the brown or whitish nectaries through the collateral bundles that comprised both phloem and xylem elements. Using an experimental physiological approach, it would be worthwhile to investigate whether phloem or xylem cells are responsible for sugar transport to the nectary and to what degree nectar concentration relates to bundle type abundance. Both alternatives have been suggested previously and documented for other plants (e.g. Frei, 1955; Beardsell, Williams & Knox, 1989; De la Barrera & Nobel, 2004), but this remains unstudied in *Pedicularis* and other larger groups of Orobanchaceae.

EVOLUTIONARY DIRECTION

The bulged nectary occurred in all four corolla types, whereas the elongated nectary was only found in some of the short-tubed and beakless species. Absence of nectary was observed only in species with a short corolla tube and with a long beaked galea (Fig. 6). In our phylogenetic reconstruction, the nectary was independently lost repeatedly over time along branches that lead to long-beaked species (shaded pink in Fig. 6); however, some long-beaked species have retained the nectary structure, which reflects their ancestral beakless, nectariferous state. An elongated nectary was first found in species belonging to series *Reges*, which forms part of the well-supported monophyletic section *Cyathophora* H.L. Li (Ree, 2005). Hence, the elongated nectary can be assumed to be derived from a bulged nectary.

Path analysis supported the hypothesis that nectar production was directly associated with beak length/pollinator behaviour, and also indicated that nectary presence indirectly affected beak length/pollinator behaviour through the intermediate factor of nectar production. In contrast, nectary type had no correlation with nectar production, beak length or pollinator behaviour. The nectary in some beaked species may only be a vestigial structure, given the absence of nectar production. The significant correlation between nectar production and beak length revealed by path analysis also indicated that there were non-functional nectaries in beaked species. Overall, the general notion of functional constraints in floral state combinations still holds true for *Pedicularis*. However, it is nectar production, and not nectary presence, that is functionally correlated to pollinator behaviour.

EFFECT OF NECTARIES ON POLLINATOR BEHAVIOUR

Relationships between flowers and pollinators are generally established on the basis of rewards, i.e. nectar or pollen (Wang *et al*., 2009). The nectary structure occurs not only in beakless and short-beaked species, but also in some long-beaked, nectarless species that receive only pollen-foraging species. Nevertheless, as we outlined above, species bearing nectaries are not necessarily nectariferous. On the other hand, floral organs may also obstruct pollinators from accessing nectaries. The functional evolution of corolla traits may thus be driven by pollination selection, with a high degree of homoplasy in corolla shape, and not in nectary traits.

In this study, nectary structure, starch grains and modified stomata were all observed in some long-beaked species (including long-tubed species) (Fig. 6). On the one hand, bumblebees are unable to access nectaries in these species because of the floral restriction caused by the long, narrow corolla beak and tube. On the other hand, night-flying long-proboscid insects are not effective pollinators for these long-tubed species (Huang & Fenster, 2007) because pollen grains, which are produced by anthers that are enclosed in a compact beak, can only be collected after they are dislodged by high-speed vibrations of bumblebee wings (i.e. buzz pollination) (Macior, 1982; Yu, Li & Wang, 2012). Thus, an interesting question arises: are these nectary structures functional if they are inaccessible to foragers? Previous studies have indicated that long-beaked *Pedicularis* spp. are nectarless (Macior, 1988; Macior & Tang, 1997; Tang & Xie, 2006; Huang & Fenster, 2007); however, our investigation revealed that some long-beaked species bear a nectary structure. In total, this suggests that the nectaries in these species may be vestigial and do not produce a nectar reward for pollinators. Generally, loss of nectaries may allow a plant to allocate additional resources to floral investments, such as the production of more flowers or larger flowers, or increased floral longevity (Ackerman, 1986; Johnson & Nilsson, 1999; Pansarin *et al*., 2012). As the nectary structure seems to be of no functional value, but of high energy investment, in long-beaked species, selective pressures may promote its loss, which may be what has happened in some long-beaked species. However, other long-beaked species have retained the nectary structure, which suggests that they might have been derived from beakless, nectariferous ancestors during the recent rapid radiation of *Pedicularis* in the Himalaya–Hengduan Mountains of south-western China.

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REFERENCES

Ackerman JD. 1986. Mechanisms and evolution of food-deceptive pollination systems in orchids. *Lindleyana* **1:** 108–113.

Armbruster WS, Shi XQ, Huang SQ. 2014. Do specialized flowers promote reproductive isolation? Realized pollination accuracy of three sympatric *Pedicularis* species. *Annals of Botany* **113:** 331–340.

Baker HG, Baker I. 1983. Floral nectar sugar constituents in relation to pollinator type. In: Jones CE, Little RJ, eds. *Handbook of experimental pollination biology*. NewYork: Van Nostrand Reinhold, 117–141.

Beardsell DV, Williams EG, Knox RB. 1989. The structure and histochemistry of the nectary and anther secretory tissue of the flowers of *Thryptomene calycina* (Lindl) Stapf (Myrtaceae). *Australian Journal of Botany* **37:** 65–80.

Bernardello G. 2007. A systematic survey of floral nectaries. In: Nicolson SW, Nepi M, Pacini E, eds. *Nectaries and nectar*. Dordrecht: Springer, 19–128.

Bernardello G, Galetto L, Anderson GJ. 2000. Floral nectary structure and nectar chemical composition of some species from Robinson Crusoe Island (Chile). *Canadian Journal of Botany* **78:** 862–871.

Cai J, Liang HX, Wang H. 2003. Floral organogenesis of *Pedicularis* (Orobanchaceae) with erostrate corolla. *Acta Botanica Yunnanica* **25:** 671–679.

Conn HJ, Darrow MA, Emmel V. 1960. *Staining procedures used by the biological stain commission*. Baltimore, MD: The Williams and Wilkinson Company.

Davis AR, Fowke LC, Sawhney VK, Low NH. 1996. Floral nectar secretion and ploidy in *Brassica rapa* and *B. napus* (Brassicaceae). II. Quantified variability of nectary structure and function in rapid-cycling lines. *Annals of Botany* **77:** 223–234.

De la Barrera E, Nobel PS. 2004. Nectar: properties, floral aspects, and speculations on origin. *Trends in Plant Science* **9:** 65–69.

Eaton DAR, Fenster CB, Hereford J, Huang SQ, Ree RH. 2012. Floral diversity and community structure in *Pedicularis* (Orobanchaceae). *Ecology* **93:** S182–S194.

Fahn A. 1979. *Secretory tissues in plants*. London: Academic Press.

Fahn A. 1988. Secretory tissues in vascular plants. *New Phytologist* **108:** 229–257.

Fahn A, Shimony C. 2001. Nectary structure and ultrastructure of unisexual flowers of *Ecballium elaterium* (L.) A. Rich. (Cucurbitaceae) and their presumptive pollinators. *Annals of Botany* **87:** 27–33.

Feder N, O'Brien TP. 1968. Plant microtechnique: some principles and new methods. *American Journal of Botany* **55:** 123–142.

Frei E. 1955. Die Innervierung der floralen Nektarien dikotyler Pflanzen-familien. *Berichte der Schweizerischen Botanischen Gesellschaft* **65:** 60–114.

Galen C. 1996. Rates of floral evolution: adaptation to bumblebee pollination in an alpine wildflower, *Polemonium viscosum*. *Evolution* **50:** 120–125.

Gonzalez-Voyer A, von Hardenberg A. 2014. An introduction to phylogenetic path analysis. In: Garamszegi LZ, ed. *Modern phylogenetic comparative methods and their application in evolutionary biology*. Berlin: Springer, 201–229.

Huang SQ, Fenster CB. 2007. Absence of long-proboscid pollinators for long-corolla-tubed Himalayan *Pedicularis* species: implications for the evolution of corolla length. *International Journal of Plant Sciences* **168:** 325–331.

Huang SQ, Shi XQ. 2013. Floral isolation in *Pedicularis*: how do congeners with shared pollinators minimize reproductive interference? *New Phytologist* **199:** 858–865.

Johnson SD, Nilsson LA. 1999. Pollen carryover, geitonogamy, and the evolution of deceptive pollination systems in orchids. *Ecology* **80:** 2607–2619.

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* **30:** 772–780.

Knuth P, Muller H. 1906. *Handbook of flower pollination, Vol. 3*. Oxford: Clarendon Press.

Kwak MM. 1977. Pollination ecology of five hemiparasitic, large flowered Rhinanthoideae with special reference to the pollination behaviour of nectar thieving, short tongued bumblebees. *Acta Botanica Neerlandica* **26:** 97–108.

Kwak MM. 1978. Pollination, hybridization and ethological isolation of *Rhinanthus minor* and *R. serotinus* (Rhinanthoideae: Scrophulariaceae) by bumblebees (*Bombus* Latr.). *Taxon* **27:** 145–158.

Kwak MM. 1979. Effects of bumblebee visits on the seed set of *Pedicularis*, *Rhinanthus* and *Melampyrum* (Scrophulariaceae) in the Netherlands. *Acta Botanica Neerlandica* **28:** 177–195.

Kwak MM, Holthuijzen YA, Prins HHT. 1985. A comparison of nectar characteristics of the bumblebee-pollinated *Rhinanthus minor* and *R. serotinus*. *Oikos* **44:** 123–126.

Li HL. 1951. Evolution in the flowers of *Pedicularis*. *Evolution* **5:** 158–164.

Li ZL. 1997. *Sectioning method of plant*. Beijing: Science Press.

Macior LW. 1982. Plant community and pollinator dynamics in the evolution of pollination mechanisms in *Pedicularis* (Scrophulariaceae). In: Armstrong JA, Powell JM, Richards AJ, eds. *Pollination and evolution: symposium on Pollination Biology held during the 13th International Botanical Congress*. Sydney: Royal Botanic Gardens, 29–45.

Macior LW. 1988. A preliminary study of the pollination ecology of *Pedicularis* in Japan. *Plant Species Biology* **3:** 61–66.

Macior LW. 1990. Pollination ecology of *Pedicularis punctata* Decne.(Scrophulariaceae) in the Kashmir Himalaya. *Plant Species Biology* **5:** 215–223.

Macior LW. 1995. *Pedicularis*, a valuable information recource for plant conservation. In: Sihag RC, ed. *Pollination biology*. *Environmental factors and pollination, Vol. 2*. Hisar: Rajendra Scientific Publishers, 8–19.

Macior LW, Sood SK. 1991. Pollination ecology of *Pedicularis megalantha* D. Don (Scrophulariaceae) in the Himachal Himalaya. *Plant Species Biology* **6:** 75–81.

Macior LW, Tang Y. 1997. A preliminary study of the pollination ecology of *Pedicularis* in the Chinese Himalaya. *Plant Species Biology* **12:** 1–7.

Macior LW, Tang Y, Zhang J. 2001. Reproductive biology of *Pedicularis* (Scrophulariaceae) in the Sichuan Himalaya. *Plant Species Biology* **16:** 83–89.

Maddison WP, Maddison DR. 2011. *Mesquite: a modular system for evolutionary analysis. Version 2.75.* Mesquite website [online]. Available at: http://mesquiteproject.org (accessed 3 June 2013).

Mattfeld J. 1922. Zwei neue Orobanchen aus Peru. *Notizblatt des Königlichen Botanischen Gartens und Museums zu Berlin* **72:** 182–186.

McManus JFA. 1948. Histological and histochemical uses of periodic acid. *Stain Technology* **23:** 99–108.

Meier-Weniger E. 1977. Die morphogenese der blüte von *Pedicularis recutita* L. (Scrophulariaceae). *Berichte der Deutschen Botanischen Gesellschaft* **90:** 67–75.

Mill RR. 2001. Notes relating to the Flora of Bhutan: XLIII. Scrophulariaceae (*Pedicularis*). *Edinburgh Journal of Botany* **58:** 57–98.

Nepi M, Ciampolini F, Pacini E. 1996. Development and ultrastructure of *Cucurbita pepo* nectaries of male flowers. *Annals of Botany* **78:** 95–104.

Nepi M, Human H, Nicolson SW, Cresti L, Pacini E. 2006. Nectary structure and nectar presentation in *Aloe castanea* and *A. greatheadii* var. *davyana* (Asphodelaceae). *Plant Systematics and Evolution* **257:** 45–55.

O'Brien TP, McCully ME. 1981. *The study of plant structure: principles and selected methods*. Melbourne: Termarcarphi Pty. Ltd.

Pacini E, Nepi M, Vesprini JL. 2003. Nectar biodiversity: a short review. *Plant Systematics and Evolution* **238:** 7–21.

Pacini E, Nicolson SW. 2007. Introduction. In: Nicolson SW, Nepi M, Pacini E, eds. *Nectaries and nectar*. Dordrecht: Springer, 1–18.

Pansarin ER, Salatino A, Pansarin LM, Sazima M. 2012. Pollination systems in Pogonieae (Orchidaceae: Vanilloideae): a hypothesis of evolution among reward and rewardless flowers. *Flora* **207:** 849–861.

Papp N, Csete S, Farkas Á. 2013. Comparative ecomorphology of the cyathial nectaries in eight European *Euphorbia* species. *Acta Biologica Hungarica* **64:** 45–59.

Petanidou T, Goethals V, Smets E. 2000. Nectary structure of Labiatae in relation to their nectar secretion and characteristics in a Mediterranean shrub community – does flowering time matter? *Plant Systematics and Evolution* **225:** 103–118.

R Development Core Team. 2013. *R: a language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.

Razem FA, Davis AR. 1999. Anatomical and ultrastructural changes of the floral nectary of *Pisum sativum* L. during flower development. *Protoplasma* **206:** 57–72.

Ree RH. 2005. Phylogeny and the evolution of floral diversity in *Pedicularis* (Orobanchaceae). *International Journal of Plant Sciences* **166:** 595–613.

Sprague EF. 1962. Pollination and evolution in *Pedicularis* (Scrophulariaceae). *Aliso* **5:** 181–209.

Stanton ML, Galloway LF. 1990. Natural selection and allocation to reproduction in flowering plants. In: Mangel M, ed. *Sex allocation and sex change: experiments and models*. Providence, RI: American Mathematical Society, 1–50.

Sulborska A, Konarska A, Chmielewski P. 2014. Micromorphology of flowers and the structure of floral nectaries in *Orobanche alsatica* Kirschl. *Modern Phytomorphology* **6:** 23.

Sun SG, Guo YH, Gituru RW, Huang SQ. 2005a. Corolla wilting facilitates delayed autonomous self-pollination in *Pedicularis dunniana* (Orobanchaceae). *Plant Systematics and Evolution* **251:** 229–237.

Sun SG, Liao K, Xia J, Guo YH. 2005b. Floral colour change in *Pedicularis monbeigiana* (Orobanchaceae). *Plant Systematics and Evolution* **255:** 77–85.

Tang Y, Xie HS. 2006. A pollination ecology study of *Pedicularis* Linnaeus (Orobanchaceae) in a subalpine to alpine area of northwest Sichuan, China. *Arctic, Antarctic and Alpine Research* **38:** 446–453.

Tang Y, Xie JS, Sun H. 2007. The pollination ecology of *Pedicularis rex* subsp. *lipkyana* and *P. rex* subsp. *rex* (Orobanchaceae) from Sichuan, southwestern China. *Flora* **202:** 209–217.

Tkach N, Ree RH, Kuss P, Röser M, Hoffmann MH. 2014. High mountain origin, phylogenetics, evolution, and niche conservatism of arctic lineages in the hemiparasitic genus *Pedicularis* (Orobanchaceae). *Molecular Phylogenetics and Evolution* **76:** 75–92.

Tsoong PC. 1963. Scrophulariaceae (Pars II). In: Chien SS, Chun WY, eds. *Flora reipublicae popularis sinacae, Vol. 68*. Beijing: Science Press, 1–378.

Wang H, Li DZ. 1998. A preliminary study of pollination biology of *Pedicularis* (Scrophulariaceae) in northwest Yunnan, China. *Acta Botanica Sinica* **40:** 204–210.

Wang H, Li DZ. 2005. Pollination biology of four *Pedicularis* species (Scrophulariaceae) in northwestern Yunnan, China. *Annals of the Missouri Botanical Garden* **92:** 127–138.

Wang H, Li WL, Cai J. 2003. Correlations between floral diversity and pollination patterns in *Pedicularis* (Scrophulariaceae). *Acta Botanica Yunnanica* **25:** 63–70.

Wang H, Yu WB, Chen JQ, Blackmore S. 2009. Pollen morphology in relation to floral types and pollination syndromes in *Pedicularis* (Orobanchaceae). *Plant Systematics and Evolution* **277:** 153–162.

Yang CF, Gituru RW, Guo YH. 2007. Reproductive isolation of two sympatric louseworts, *Pedicularis rhinanthoides* and *Pedicularis longiflora* (Orobanchaceae): how does the same pollinator type avoid interspecific pollen transfer? *Botanical Journal of the Linnean Society* **90:** 37–48.

Yu WB. 2007. *Reproductive strategies of Pedicularis (Orobanchaceae) in the Hengduan Mountains region*. Kunming: Kunming Institue of Botany, Chinese Academy of Sciences.

Yu WB, Cai J, Li DZ, Mill RR, Wang H. 2013a. Floral ontogeny of *Pedicularis* (Orobanchaceae), with an emphasis on the corolla upper lip. *Journal of Systematics and Evolution* **51:** 435–450.

Yu WB, Cai J, Wang H, Chen JQ. 2008. Advances in floral divergence and reproductive adaptation in *Pedicularis* L.(Orobanchaceae). *Chinese Bulletin of Botany* **25:** 392–400.

Yu WB, Huang PH, Li DZ, Wang H. 2013b. Incongruence between nuclear and chloroplast DNA phylogenies in *Pedicularis* section *Cyathophora* (Orobanchaceae). *PLoS ONE* **8:** e74828.

Yu WB, Huang PH, Ree RH, Liu ML, Li DZ, Wang H. 2011. DNA barcoding of *Pedicularis* L. (Orobanchaceae): evaluating four universal barcode loci in a large and hemiparasitic genus. *Journal of Systematics and Evolution* **49:** 425–437.

Yu WB, Li DZ, Wang H. 2012. Highly efficient pollination by bumblebees ensures seed production in *Pedicularis lachnoglossa* (Orobanchaceae), an early-flowering Himalayan plant. *Journal of Systematics and Evolution* **50:** 218–226.

APPENDIX

Table A1. Voucher information for taxa included in the phylogenetic analysis

Taxa	Voucher	Sampling origin	ITS	*mat*K	*rbc*L	*trn*H-*psb*A	*trn*L-*trn*F
Pedicularis aloensis Handel-Mazzetti	H Wang *et al*. 04169	Shangrila, Yunnan	KF277524	Y	Y	Y	KF277603
P. alopecuros Franchet ex Maximowicz	W B Yu *et al*. LIDZ0994	Ninglang, Yunnan	JF977467	JF955061	JF942950	JN045885	KF277604
P. axillaris Franchet ex Maximowicz	W B Yu *et al*. LIDZ1290	Xiangcheng, Sichuan	JF977478	JF955072	JF942961	JN045896	KC733345
P. comptoniaefolia Franchet	PPBG 08721	Lijiang, Yunnan	JF977498	JN120029	JF942981	JN120045	KF277619
P. confertiflora Prain	W B Yu *et al*. LIDZ1310	Qiaojia, Yunnan	JF977505	JF955098	JF942988	JN045921	KF277620
P. cyathophylla Franchet	W B Yu *et al*. LIDZ1268	Shangrila, Yunnan	JF977518	JF955111	JF943001	JN045934	KC733332
P. cymbalaria Bonati	C L Xiang *et al*. HP9804	Luquan, Yunnan	JF977529	JF955122	JF943012	JN045945	KF277629
P. deltoidea Franchet ex Maximowicz	W B Yu *et al*. LIDZ1309	Qiaojia, Yunnan	JF977541	JF955134	JF943024	JN045957	KF277637
P. densiflora Bentham	L Lu *et al*. 1924	Siskiyou County, CA, USA	Y	Y	Y	—	Y
P. densispica Franchet ex Maximowicz	PPBG 08722	Lijiang, Yunnan	JF977547	JF955140	JF943030	JN045963	KF277638
P. dichotoma Bonati	S D Zhang & H J He 08811	Shangrila, Yunnan	JF977548	JF955141	JF943031	JN045964	KF277639
P. dolichocymba Handel-Mazzetti	S D Zhang & H J He 08827	Deqin, Yunnan	JF977554	JF955147	JF943037	JN045970	KC733347
P. dunniana Bonati	W B Yu *et al*. HW10138	Shangrila, Yunnan	JF977558	JF955151	JF943041	JN045974	KF277644
P. elwesii J. D. Hooker	W B Yu *et al*. LIDZ1259	Shangrila, Yunnan	JF977561	JF955154	JF943044	JN045977	KF277645
P. filicula Franchet ex Maximowicz	W B Yu LIDZ1520	Dongchuan, Yunnan	KF011709	KF011854	KF011753	KF011797	KC733348
P. glabrescens H. L. Li	W B Yu *et al*. LIDZ1272	Shangrila, Yunnan	JF977568	JF955161	JF943051	JN045984	KF277653
P. gracilis Wallich ex Bentham	S D Zhang & H J He 081304	Cuona, Xizang	JF977576	JF955169	JF943059	JN045992	KF277655
P. integrifolia J. D. Hooker	S D Zhang & H J He 08920	Zuogong, Xizang	JF977588	JF955181	JF943071	JN046004	KF277664
P. labordei Vaniot ex Bonati	W B Yu *et al*. LIDZ1302	Fumin, Yunnan	JF977601	JF955193	JF943084	JN046017	KF277666
P. lachnoglossa J. D. Hooker	W B Yu *et al*. LIDZ1094	Kangding, Sichuan	JF977602	JF955194	JF943085	JN046018	KF277667
P. longiflora var. *tubiformis* (Klotzsch) P. C. Tsoong	W B Yu *et al*. LIDZ1091	Kangding, Sichuan	JF977623	JF955215	JF943106	JN046037	KF277676
P. lutescens Franchet ex Maximowicz	W B Yu *et al*. YWB005	Lijiang, Yunnan	JF977629	JF955221	JF943112	JN046043	KF277678
P. macrosiphon Franchet	L. M. Gao *et al*. 07486	Gongshan, Yunnan	JF977640	JF955232	JF943123	JN046054	KF277682
P. oxycarpa Franchet ex Maximowicz	W B Yu *et al*. LIDZ1299	Qiaojia, Yunnan	JF977665	JF955256	JF943148	JN046079	KF277700
P. pseudomelampyriflora Bonati	S D Zhang & H J He 08873	Mangkang, Xizang	JF977669	JF955260	JF943152	JN046083	KF277708
P. pygmaea subsp. *deqinensis* H. Wang	S D Zhang & H J He 08828	Deqin, Yunnan	KF277575	Y	Y	—	KF277710
P. rex C. B. Clarke ex Maximowicz	W B Yu *et al*. LIDZ1193	Litang, Sichuan	JF977680	JF955271	JF943163	JN046094	KC733306
P. rhinanthoides Schrenk ex Fischer & C. A. Meyer	H Wang *et al*. 04249	Shangrila, Yunnan	JF977689	JF955280	JF943172	JN046103	KC733352
P. rhinanthoides subsp. *tibetica* (Bonati) P. C. Tsoong	S D Zhang & H J He 08938	Bangda, Xizang	JF977687	JF955278	JF943170	JN046101	KC733352
P. roylei Maximowicz	W B Yu *et al*. LIDZ1219	Litang, Sichuan	JF977713	JN046127	JF943196	JF955304	KF277718
P. rupicola Franchet ex Maximowicz	W B Yu *et al*. LIDZ1236	Xiangcheng, Sichuan	JF977714	JF955305	JF943197	JN046128	KF277720
P. salviaeflora Franchet	W B Yu YWB269	Songming, Yunnan	JF977717	JF955308	JF943200	JN046131	KF277722
P. sceptrum-carolinum L.	C H Zhou 1264	Antu, Jilin	Y	Y	Y	—	Y
P. siphonantha D. Don	H Wang *et al*. 04205	Deqin, Yunnan	JF977724	JF955315	JF943207	JN046138	KC733351
P. siphonantha var. *stictochila* H. Wang & W. B. Yu	W B Yu *et al*. LIDZ1270	Shangrila, Yunnan	JF977730	JF955320	JF943213	JN046144	KF277728
P. superba Franchet ex Maximowicz	W B Yu *et al*. LIDZ1578	Lijiang, Yunnan	JF977739	JF955329	JF943222	JN046153	KC733343
P. sylvatica L.	Peregrym OP7	Ukraine	KF277588	JN894577	JN893223	—	KF277738
P. tachanensis Bonati	W B Yu *et al*. LIDZ1062	Mianning, Sichuan	JF977743	JF955333	JF943226	JN046157	KF277740
P. tenuisecta Franchet ex Maximowicz	PPBG 08719	Lijiang, Yunnan	JF977747	JN120037	JF943230	JN120054	KF277744
P. thamnophila (Handel-Mazzetti) H. L. Li	W B Yu *et al*. LIDZ1016	Muli, Sichuan	JF977753	JF955342	JF943236	JN046166	KC733321
P. tongolensis Franchet	W B Yu *et al*. LIDZ1251	Xiangcheng, Sichuan	JF977760	JF955349	JF943243	JN046173	KF277748
P. tricolor Handel-Mazzetti	W B Yu *et al*. YWB069	Xiangcheng, Sichuan	JF977767	JF955356	JF943250	JN046180	KF277755
P. umbelliformis H. L. Li	W B Yu *et al*. HW10134	Shangrila, Yunnan	JF977774	JF955363	JF943257	JN046187	KF277757
P. vialii Franchet in Forbes & Hemsley	W B Yu *et al*. HW10191	Shangrila, Yunnan	JF977783	JF955372	JF943266	JN046196	KF277765
Agalinis tenella Pennell	NCBI		EU827871	GU943733	GU943648	EU827954	EU828200
Agalinis tenuifolia (Vahl) Raf.	NCBI		EU827874	GU943737	GU943651	EU827957	EU828203
Castilleja arvensis Cham. & Schltdl.	NCBI		EF103698	JQ588566	JQ593063	—	EF103845
Orthocarpus tenuifolius (Pursh) Benth.	NCBI		KC480375	JX091328	—	-	EF093890
Phtheirospermum japonicum (Thunb.) Kanitz	NCBI		JF746383	JX091332	JQ910067	JQ910099	JQ910129

NCBI, National Center for Biotechnology Information.

Y stands for sequences that have been submitted to NCBI, but the accession number has not been returned.

Table A2. Sequence information for five DNA regions examined from 44 samples representing 42 *Pedicularis* taxa

DNA region	Total length	Variable sites	Parsimony informative sites	Ratios of variable/ parsimony informative sites (%)
ITS	618	298	209	48.2/33.8
*mat*K	734	217	105	29.6/14.3
*rbc*L	624	75	42	12.0/6.7
*trn*H-*psb*A	810	297	153	36.7/18.9
*trn*L-F	1044	280	137	26.8/13.1