

## PHYLOGENY AND THE EVOLUTION OF FLORAL DIVERSITY IN *PEDICULARIS* (OROBANCHACEAE)

Richard H. Ree<sup>1</sup>

Department of Organismic and Evolutionary Biology, Harvard University, 22 Divinity Avenue, Cambridge, Massachusetts 02138, U.S.A.

*Pedicularis* has a conflicted infrageneric taxonomic history in which floral and vegetative characters have alternately been interpreted as primary indicators of common ancestry. Recent classifications favor subgenera delimited by similarity in leaf arrangement, on the basis of the premise that corolla morphology has been subject to pollinator-mediated selection, resulting in the convergent evolution of specialized floral forms from generalized ancestors. In this study, molecular phylogenetic analysis of *Pedicularis* reveals a low concordance of robust clade support and taxonomic groups. Nevertheless, the basic premise of higher homoplasy in floral characters relative to leaf arrangement is confirmed, although the hypothesized direction of change (from short-tubed, beakless, toothed corollas to long-tubed, beaked, toothless forms) is not uniformly upheld. In spite of the high evolutionary lability of floral form in *Pedicularis*, some character state combinations are rarely or never observed, suggesting functional constraints. Correlation analysis shows a significant association between curved-rostrate corollas (a marker for nectar production) and long corolla tubes, indicating that access to nectar by pollinators has acted as a constraint on the evolution of corolla tube length.

**Keywords:** Bayesian analysis, biodiversity hotspot, China, constraint, floral evolution, homoplasy, *Pedicularis*, Orobanchaceae, stochastic mapping, phylogeny.

### Introduction

*Pedicularis* L. (Orobanchaceae) is a lineage of hemiparasitic flowering plants distributed throughout the northern hemisphere, primarily in cold, high-latitude or montane habitats. With more than 500 described species, it has been noted as one of the largest genera of angiosperms in the north temperate zone (Pennell, in Li 1948) and is characterized by dramatic interspecific morphological variation, particularly in the galea (the hoodlike upper lip of the corolla), which can be variously elongated (beaked), curved, toothed, and crested. Conspicuous variation is also seen in the length of the corolla tube, which in some species can be greater than 11 cm. The greatest morphological diversity and species richness in *Pedicularis* is confined to a relatively small region of eastern Asia. About two-thirds of the species are endemic to the Hengduan Mountains region, situated south of the Qinghai-Tibetan plateau at the juncture of the Chinese provinces of Sichuan, Yunnan, and Xizang (Hong 1983). This region has been recognized as one of 25 biodiversity hotspots in the world on the basis of its vascular plant diversity, endemism, and threat of degradation (Boufford and Van Dijk 2000).

Because of the relative inaccessibility of the Hengduan region, many aspects of the basic biology of many species of *Pedicularis* are poorly known. Aspects of pollination ecology have been studied, but phylogenetic relationships within this

lineage are poorly known. This study presents a phylogenetic analysis of *Pedicularis*, with the aim of resolving fundamental differences in classification systems that have been proposed and providing insight into the evolution of floral diversity in this group.

### Taxonomic Background

Within the traditional family Scrophulariaceae (figworts), *Pedicularis* has been placed with other hemiparasitic genera in tribe Rhinanthae Benth. (=Euphrasiae Benth.) of subfamily Rhinanthoideae (von Wettstein 1897). More recent studies of chloroplast DNA sequences (dePamphilis et al. 1997; Young et al. 1999) have shown that neither Rhinanthae nor Rhinanthoideae are monophyletic and that *Pedicularis* falls within a clade that includes the hemiparasitic figworts, the holoparasitic Orobanchaceae (broomrapes), and the nonparasitic *Lindenbergia*. This clade, characterized by having ventral corolla lobes external to the dorsal lobes during aestivation (Pennell 1935; Armstrong and Douglas 1989), is now recognized as Orobanchaceae (Olmstead et al. 2001).

Species of *Pedicularis* are mostly perennial herbs and generally have dissected leaf margins, a campanulate calyx, and a bilabiate corolla possessing a galea. The galea, while present in other members of Orobanchaceae (e.g., *Siphonostegia* Benth.), is more highly developed and conspicuously variable in *Pedicularis* than in any other lineage of Orobanchaceae. A strongly galeate corolla and compressed, ovoid (subglobose to lanceolate) capsular fruit are potential synapomorphies of *Pedicularis* within the orobanch clade.

Several classification systems for *Pedicularis* have been proposed (Steven 1823; Bentham 1835, 1846; Bunge 1846;

<sup>1</sup> Current address: Department of Botany, Field Museum of Natural History, 1400 South Lake Shore Drive, Chicago, Illinois 60605, U.S.A.; e-mail rree@fieldmuseum.org.

Manuscript received December 2004; revised manuscript received March 2005.

Maximowicz 1888; Prain 1890; Bonati 1918; Limpricht 1924; Hurusawa 1947, 1948, 1949; Li 1948, 1949; Tsoong 1955, 1956, 1963). In the system of Maximowicz (1888), four basic corolla types in *Pedicularis* were established to categorize interspecific variation: (1) beakless, toothed, and short tubed; (2) beakless, toothless, and short tubed; (3) beaked, toothless, and short tubed; and (4) beaked, toothless, and long tubed. Subsequent authors have retained these categories for their heuristic value while acknowledging that their boundaries are necessarily blurred because of continuous variation in the component characters. The nonfloral characters that have received the most attention are phyllotaxy (leaves spiral, opposite, or whorled) and inflorescence structure (terminal inflorescences or flowers solitary in the axils of leaves).

Much of the difference between systems can be attributed to whether the primary taxa within *Pedicularis* are differentiated by floral or vegetative characters. Prior to the system of Li (1948, 1949), taxonomists emphasized floral characters. For example, the system of Steven (1823) contains six groups, five of which are based on floral characters; only one (*Verticillatae*) is based on phyllotaxy. Similarly, the system of Maximowicz (1888), the first system based on a substantial number of known species (250), retains *Verticillatae*, but all other species are grouped based on the corolla types described above. The subsequent systems of Prain (1890), Bonati (1918), and Limpricht (1924) were based almost entirely on the same floral characters emphasized by Maximowicz.

Taxonomic revisions of *Pedicularis* have generally taken the form of regional treatments on account of the group's size. Of these, two revisions of the genus in China, where the majority of species occur, have been the most influential in the development of a classification system for *Pedicularis*. The system of Li (1948, 1949) represents a drastic departure from earlier systems in its arrangement of *Pedicularis* into three subgenera based on vegetative characters: (1) *Cyclophyllum*, characterized by verticillate or opposite leaves; (2) *Allophyllum*, characterized by spiral phyllotaxy, erect stems, and terminal inflorescences; and (3) *Poecilophyllum*, characterized by spiral (or subopposite) phyllotaxy, lax, spreading stems, and axillary flowers. Li's rationale for this classification structure rested on the hypothesis that pollinator-mediated natural selection acting independently on different lineages of *Pedicularis* produced greater homoplasy in floral characters, i.e., the repeated evolution of similar specialized floral forms. In contrast, vegetative characters such as phyllotaxy were subject to lesser selection pressure and therefore exhibit less convergence, and for this reason they are more reliable for diagnosing primary subgeneric groups.

The system of Tsoong (1955, 1956, 1963) is similar to that of Li in that each subgenus is uniform with respect to phyllotaxy but heterogeneous with respect to floral morphology. It also expanded considerably the set of sectional names that have become commonly used in regional treatments of *Pedicularis*. In justifying his system, Tsoong concurred with Li that floral traits such as tube length and galea type were unreliable taxonomic markers because of convergent evolution.

It is worth noting that despite their differences, all classification systems have retained the species of section *Cyathophora* as a cohesive unit. *Cyathophora* is a distinctive group of four series (six species) that is characterized by perfoliate

leaf bases, i.e., leaf bases that are fused together to form a cuplike structure around the stem at each node. Within *Cyathophora*, all four of Maximowicz's corolla types are represented, rendering it incongruous with those systems that placed primary emphasis on floral characters. In recognition of this group, all authors have treated floral characters as subordinate to vegetative morphology.

### Pollination

*Pedicularis* is pollinated almost exclusively by bumblebees (*Bombus* Latr.) and has been shown to be dependent on them for seed set (Macior 1995b), although evidence of autogamy exists for some Arctic species (Mosquin and Martin 1967; Kaigorodova 1976; Williams and Batzli 1982; Eriksen et al. 1993). The ecology of these interactions has been documented in many regions, e.g., North America (Macior 1982 and references therein; Macior 1986b, 1986c, 1993, 1995a, 1995b, 1996), Europe (Kwak 1977, 1979), Japan (Macior 1988), and continental Asia (Macior 1990; Macior and Sarvesh 1991; Macior and Tang 1997). It is interesting to note that the geographic ranges of *Bombus* and *Pedicularis* are similar in that both groups are distributed circumboreally, with a concentration of species diversity in the Himalayan region (Hong 1983; Williams 1996).

Several general patterns have emerged from the pollination studies of *Pedicularis* cited above. *Bombus* queens tend to forage for nectar, while workers tend to forage for pollen. Pollination can be nototribic (i.e., the insect enters the flower upright) or sternotribic (i.e., the insect enters the flower upside down). Nectar-foraging bees typically forage nototribically, while pollen-foraging bees have been observed foraging sternotribically, grasping the galea while upside down or resting laterally. Pollen is scraped or vibrated out of the anthers, which are concealed in the galea. The role of apical teeth on the galea may be to facilitate the transfer of pollen from the dorsum of the pollinator to the stigma (Sprague 1962; Macior 1982), but no experimental evidence has been gathered to test this. In nectarless species, curvature of the beak appears to function in optimally positioning the stigma to facilitate contact with the dorsal or ventral midline of the pollinating insect's body or the crevice between the head and thorax, where grooming is unable to remove pollen (Macior 1982). Analyses of corbicular pollen loads (Macior 1968, 1970, 1973, 1975, 1977, 1983, 1986a, 1986b, 1986c, 1995a, 1995b, 1996; Macior and Tang 1997) have found that in general, bumblebee visitors to *Pedicularis* do not exhibit a high level of species fidelity, but pure *Pedicularis* pollen loads tend to be observed in higher frequencies from nectarless, beaked species than from nectariferous species.

The long corolla tubes exhibited by certain species of *Pedicularis* in Asia were previously thought to be adaptations for visitation by long-tongued lepidopteran pollinators (Li 1951; Sprague 1962), but studies have shown that long-tubed species are nectarless and provide pollen only for foraging bumblebee visitors (Macior 1990; Macior and Sarvesh 1991; Macior and Tang 1997). Long corolla tubes may function in elevating the stigma and anthers away from potentially obstructing vegetative parts (Macior 1990; Macior and Sarvesh 1991; Macior and Tang 1997). This hypothesis is based on the observation

that most long-tubed species are rosette plants or have lax stems that bear flowers close to the ground. One exception to this pattern is *P. cyathophylla*, which has an erect stem along which long-tubed flowers are borne in the axils of the leaves; in this case, the function of the tube may be to elevate the reproductive parts away from the perfoliate leaf bases.

*Hypotheses of Floral Character Evolution*

Li (1948, 1949) hypothesized a sequence of transitions between corolla types, repeated in different groups, from toothless to toothed to beaked, culminating in the long-tubed type. Li envisioned a definite polarity of character change: repeatedly, teeth have been lost, galea beaks have been gained, and corolla tubes have evolved from short to long (fig. 1). These are hypotheses that may be tested in a phylogenetic context. More recent evidence for the association between beaked galeas and higher pollinator fidelity and behavioral specificity indicates an adaptive significance of the beaked type and solidifies the expectation that it is phylogenetically derived relative to the beakless type.

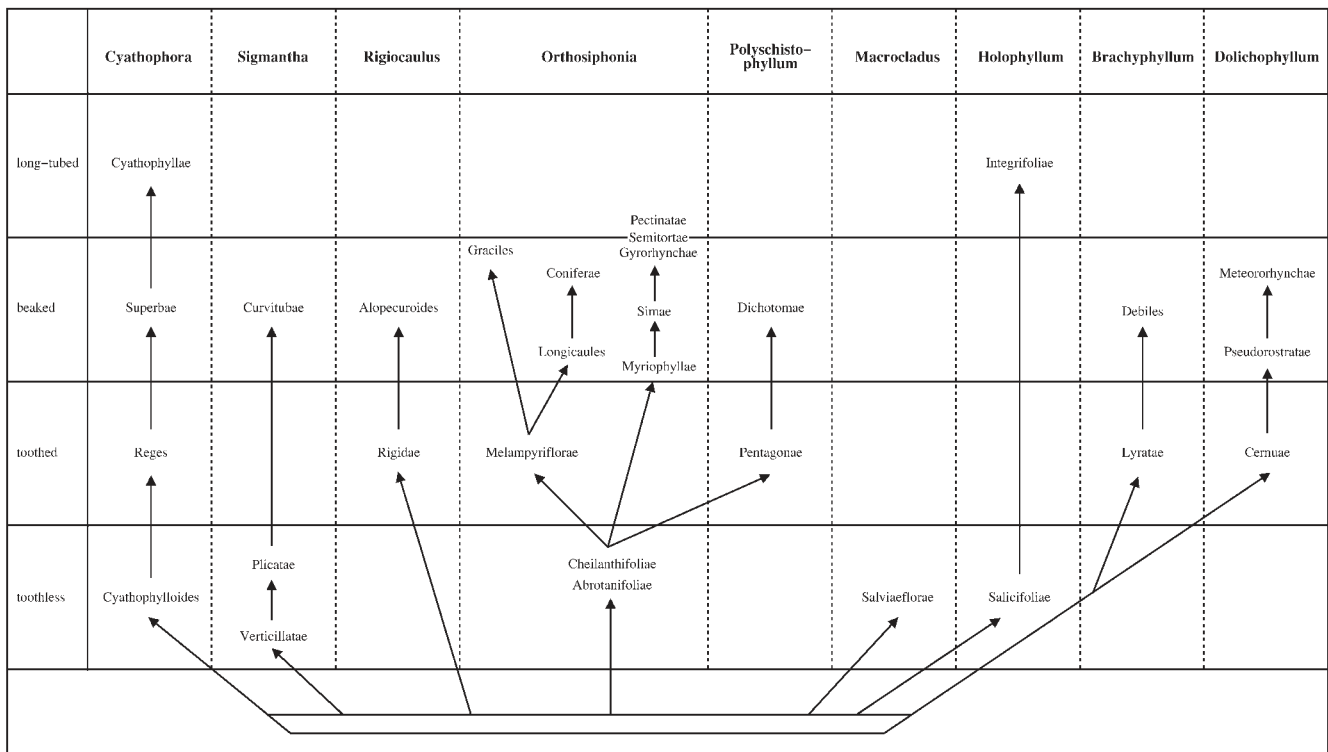
Another hypothesis concerns the association between nectar production and corolla tube length in *Pedicularis*. If long corolla tubes inhibit access to nectar by bumblebee visitors and are therefore selectively disfavored relative to short tubes, the transition from producing nectar to providing only pollen as a reward would release a constraint on corolla tube

length. This would result in a historical correlation between the short-tubed and nectariferous states. Similarly, the evolutionary history of long tubes should covary with the lack of nectar production.

To evaluate these hypotheses in a parametric statistical framework, stochastic mapping is a promising new technique, originally developed to study nucleotide mutations (Nielsen 2002) and extended to morphological characters (Huelsenbeck et al. 2003). Like parsimony-based ancestral state reconstruction, stochastic mapping estimates the history (location and direction) of character state changes on a phylogeny. Unlike parsimony, it does so via simulation, using an explicit transformation model for a continuous-time Poisson process. Stochastic mapping utilizes information from branch lengths, so it is less prone to underestimating the actual number of changes that occurred (Felsenstein 1978) than parsimony. Because it is based on a stochastic model, the method also provides estimates of the uncertainty (variance) associated with inferences of character evolution on a given phylogeny (Nielsen 2002) and, if applied to a set of candidate trees, can be used to make inferences while allowing for uncertainty in the phylogeny itself (Huelsenbeck et al. 2003).

*Objectives*

In this study, chloroplast and nuclear ribosomal DNA sequences are used to infer phylogenetic relationships within



**Fig. 1** Schematic diagram of *Pedicularis* subgenus *Cyclophyllum* Li. Columns correspond to sections, rows are corolla types, and names within the grid are series. Li's view of parallel evolution in the corolla is clearly indicated by the arrangement of series according to corolla type. The hypothesized direction of evolution (indicated by arrows) is also unambiguous, with toothed corollas derived from toothless, beaked from nonbeaked, and long tubed from short tubed. Li (1949) provided similar diagrams for the other subgenera in his system, *Allophyllum* and *Poecilophyllum*. Redrawn from Li (1948).

*Pedicularis* and determine the correspondence between strongly supported clades and previously described taxa. There are two main objectives. The first is to reconstruct phylogenetic relationships for *Pedicularis* using molecular data from different genomes and compare the results to previous classification systems. Of primary interest is the question of whether phyllotaxy, a vegetative trait used extensively by taxonomists, is more useful for diagnosing subgeneric lineages than floral traits. The second objective is to infer the history of floral and vegetative character evolution in this group and evaluate the hypotheses outlined above. Specifically, stochastic mapping is applied to infer directional trends in character evolution and test the hypothesis that the evolution of longer corolla tubes is constrained by nectar production.

## Material and Methods

### Taxon Sampling

Seventy-one species of *Pedicularis* were sampled that are broadly representative of the taxonomic and morphological breadth of the group. The sample includes 20 sections and 43 series based on the treatments by Tsoong (1963) and Yang et al. (1998) for species from China, Yamazaki (1988) for species from Nepal, Yamazaki (1993) for species from Japan, and Tsoong (1955) for species from Europe. It includes multiple representatives of each of the subgenera in Li's (1948, 1949) classification. The majority are endemic to central Asia (south-central China and Nepal). A total of 15 outgroup taxa from Orobanchaceae were also included, in addition to *Kigelia africana* Benth. (Bignoniaceae) and *Hemimeris sabulosa* L. (Scrophulariaceae). Outgroups were selected partly on the basis of previous phylogenetic analyses of Orobanchaceae (Young et al. 1999). All species included in this study, in addition to specimen voucher information and GenBank accession numbers, are listed in table 1.

### DNA Sequencing

Sequences of the internal transcribed spacer region (ITS) of the nuclear ribosomal DNA repeat (including the 5.8S subunit) and partial sequences of the chloroplast gene maturase K (*matK*) were analyzed. Both markers are widely used in plant phylogenetics. All of the *Pedicularis* sequences presented here are new, as are the sequences of ITS and *matK* for *Phtheirospermum tenuisectum* Bureau & Franchet. The ITS sequence for *Seymeria pectinata* Pursh was obtained from N. Young (unpublished data). The remaining outgroup *matK* sequences were downloaded from GenBank.

Genomic DNA was isolated from silica gel-dried leaves obtained from fresh field-collected specimens, using either a CTAB extraction method (Doyle and Doyle 1990) or a DNEasy plant extraction kit (Qiagen). Extracted DNA was purified using a GeneClean II kit (Bio 101) or used directly in double-stranded PCR. The ITS region was amplified and sequenced using the primers ITS2, ITS3 (in some cases ITS3B), ITS4, and ITS5 (White et al. 1990; Baum et al. 1994). A fragment of *matK* approximately 1200 base pairs in length was amplified and sequenced using primers *trnK11*, *trnK53F*, *matK323F*, and *matK510R* (Young et al. 1999) in

addition to *matK319R* and *trnK-79R* (5' to 3' sequence, CCACAATAAAAGCAAAYCCCTCTG and CATAGTGCGA-TANAGYCAAAAACAAG, respectively; N. Young, personal communication). PCR reactions were done in 50- $\mu$ L volumes. For ITS, reactions typically contained 5  $\mu$ L reaction buffer (GibcoBRL), 2  $\mu$ L MgCl<sub>2</sub>, 5  $\mu$ L DMSO, 2  $\mu$ L 10 mM dNTP solution, 2.5  $\mu$ L 10 mM solution of each primer (ITS5 and ITS2), 0.25  $\mu$ L Taq DNA polymerase (GibcoBRL), and 3  $\mu$ L of genomic DNA template. Optimal amplification of ITS was achieved with the following thermocycler settings: 36 cycles of 1 min 35 s denaturation at 94°C, 55 s annealing at 52°C, and 1 min 52 s extension at 72°C, followed by a final extension step of 1 min 11 s at 72°C.

For *matK*, reactions typically contained 5  $\mu$ L reaction buffer (GibcoBRL), 3  $\mu$ L MgCl<sub>2</sub>, 3  $\mu$ L DMSO, 2  $\mu$ L 10 mM dNTP solution, 3  $\mu$ L 10 mM solution of each primer (ITS5 and ITS2), 0.25  $\mu$ L Taq DNA polymerase (GibcoBRL), and 2  $\mu$ L of genomic DNA template. Optimal amplification of *matK* was achieved with the following thermocycler settings: initial denaturation for 1 min 30 s at 94°C, 38 cycles of 30 s denaturation at 94°C, 45 s annealing at 48°C, and 1 min 30 s extension at 72°C, followed by a final extension step of 10 min at 72°C.

PCR products were purified using the QiaQuick kit spin column protocol (Qiagen), and both strands were sequenced with minimum 80% overlap of the total sequence length using an ABI model 377 automated sequencer (Applied Biosystems) according to the manufacturer's instructions using BigDye cycle-sequencing reagents (Applied Biosystems). Base calls were verified and sequences were assembled using the Phred/Phrap/Consed sequence finishing software package (Ewing and Green 1998; Ewing et al. 1998; Gordon et al. 1998). Finished sequences were aligned using ClustalX (version 1.64b; Thompson et al. 1994, 1997) over a range of gap-opening and extension costs, with minor subsequent editing. In the final alignment, indels ranging in length from 1 to 26 bases were introduced into the ITS alignment in 30 regions. The *matK* alignment required indels in 35 regions, ranging in length from 1 to 21 bases. A total of 80 and 137 gaps were introduced into the ITS and *matK* alignments, respectively.

### Phylogenetic Analysis

For phylogeny reconstruction, the simplest acceptable nucleotide substitution model was chosen for each gene region by the Akaike Information Criterion (AIC) (Akaike 1974). AIC scores for 24 commonly used models were calculated using MrModeltest (version 1.1b; Nylander 2002), and the model with lowest AIC score was selected. In each case, this was the GTR+I+ $\Gamma$  model, which has a separate parameter for change between each of the six pairs of nonidentical nucleotides and parameters for the proportion of invariant sites and the shape of the gamma distribution describing rate heterogeneity among sites. Phylogenetic relationships were inferred from the DNA sequence alignments using MrBayes (version 3.0b; Huelsenbeck and Ronquist 2001), which uses a Bayesian Markov-chain Monte Carlo (MCMC) procedure to sample tree topologies and other model parameters from their posterior densities. Prior probabilities of nucleotide frequencies were specified as a Dirichlet distribution with



$u_A = u_C = u_G = u_T = 1$ . Priors for the  $\gamma$  shape parameter and proportion of invariant sites were drawn from uniform distributions ( $\{0.05, 50\}$  and  $\{0.05, 1\}$ , respectively).

The ITS and *matK* data sets were analyzed separately and in combination. The combined analyses included the union of the ITS and *matK* data sets (97 taxa) and used a mixed model, in which nucleotide substitution parameters (GTR rate matrix, invariant sites, and rate heterogeneity) were estimated separately for each gene partition. For some outgroup taxa (*Castilleja*, *Harveya*, *Seymeria*, and *Orobancha*), ITS and *matK* sequences from different species were combined to form placeholders for those genera. Each MCMC analysis was run for a minimum of 3,000,000 generations, with one cold and four incrementally heated chains. After 1,000,000 burn-in generations (inspected visually by plots of likelihood against generation), chain stationarity was assumed, and trees and parameters were sampled every 100 generations. Posterior support for clades was determined by clade frequencies in the MCMC sample. Conflicts in phylogenetic signal between nuclear and plastid markers was assessed by identifying strongly supported (posterior probability  $\geq 0.95$ ) clades that were incompatible between genes.

### Character Evolution

Evolutionary histories of morphological characters were inferred using stochastic mapping (Nielsen 2002; Huelsenbeck et al. 2003). For a detailed description of this method, the reader is referred to the original articles, but a brief summary is provided here. The method takes as input character state data for extant species and a phylogenetic tree of those species and uses a model parameterized by a matrix of instantaneous rates of change between states ( $Q$ ), a vector of stationary frequencies for character states ( $\pi$ ), and tree length, measured as the expected amount of change in the character of interest. The relative lengths of branches in the input tree are retained, since they represent relative evolutionary opportunity as inferred from the molecular data, but their absolute values are scaled to yield the prior expectation of tree length for the character in question, not for the nucleotide characters used to reconstruct the tree.

The method works by simulating character evolution as a Poisson process on the tree according to the model (with parameter values drawn from prior distributions), given the fractional likelihoods of ancestral states at internal nodes. A simulated mapping of character state changes on the tree that recovers the observed data at the tips is considered to be a plausible inference of character history. Iteration of the simulation procedure thus yields mappings that are proportionate to their posterior probability in the same way that MCMC estimates the posterior density of phylogenetic trees. Subsequent analysis of the generated mappings allows the estimation of posterior probabilities associated with hypotheses of character evolution (e.g., the probability of state change along a particular phylogenetic branch).

Each character was coded categorically with two states, 0 and 1. States were assigned as follows: phyllotaxy, whorled (0) or spiral (1); corolla tube, short (0) or long (1); galea beak, absent (0) or present (1); curve-rostrate galea, absent (0) or present (1); and galea teeth, absent (0) or present (1).

For corolla tube length, measurements were taken from herbarium specimens ( $N \geq 5$  measurements per species) or from the literature. These data form a bimodal distribution, with a distinct separation at 25 mm; this was selected as the cutoff between “short” and “long” tubes (fig. 2).

To account for uncertainty in the phylogeny of *Pedicularis* as inferred from the molecular data, character histories were mapped on 20,000 trees sampled from the posterior density by MCMC from the combined mixed-model analysis. Specifically, for each character, one mapping consistent with the observed states was generated for each tree. In the simulations, the stationary frequency of state 0,  $\pi_0$ , was drawn from a uniform  $\{0, 1\}$  prior distribution, and branch lengths were scaled to yield a total tree length equal to 1, the minimum number of changes expected for a variable binary coded character. Tree length was fixed rather than allowed to vary (Schulz and Churchill 1999) in order to focus the analysis on uncertainty in the phylogeny itself; also, it is difficult to rationalize prior distributions for tree length, particularly with regard to the variance (Huelsenbeck et al. 2003).

To test the hypothesis that corolla tube length is correlated with nectar production, a proxy marker for nectar production was needed because direct observations of nectar production are not available for many of the species included in the study. Field studies have found that beakless galeas and galeas with short, straight beaks are consistently associated with nectar production, while species with long, curved beaks always lack nectar and produce only pollen (Macior 1990; Macior and Tang 1997, and references therein). On the basis of this evidence, species with long ( $>4$  mm) curved beaks were scored as nectarless. Following Huelsenbeck et al. (2003), stochastic mapping was used to estimate the proportion of evolutionary history in which lineages of *Pedicularis* had both long tubes and lacked nectar. The summary statistic  $d$  was used to quantify the difference between the observed and expected association of corolla tube length and nectar production over the set of MCMC-generated trees from the phylogenetic reconstruction. Simulations on this same set of trees under the assumption of character independence (i.e., simulated characters were not constrained to match the observed data) were conducted to estimate the posterior predictive  $P$  distribution for  $d$ . All computational aspects of the character evolution analysis presented here were implemented in the Python programming language by the author, and the source code and programs used are available on request.

## Results

### Clade Support within *Pedicularis*

In the results of all analyses, there is a backbone region in the phylogeny of *Pedicularis* where branch lengths are short and relationships are unresolved or poorly supported. In general, the number of strongly supported clades, and the strength of clade support, is higher in the combined-data analysis than in either single-gene analysis. Congruence in phylogenetic signal between genes is moderate: most clades with strong support in one single-gene analysis are also recovered, if not strongly supported, in the other. Direct conflicts are found in clades 1 and 4.

Table 1

## Taxa Included in Phylogenetic Analyses

Taxon	Species	Voucher	Location	ITS	matK
<i>Pedicularis:</i>					
<i>Cyclophyllum:</i>					
<i>Astenocaulus:</i>					
<i>Flexuosae</i>	<i>P. flexuosa</i> Hooker	YO 9830153 (TI)	Nepal	AY949687	
	<i>P. oxyrhyncha</i> Yamazaki	RHR 9908 (A)	Nepal	AY949651	AY949724
<i>Brachyphyllum:</i>					
<i>Debiles</i>	<i>P. confertiflora</i> Prain	DEB 28271 (A)	Sichuan	AY949678	AY949744
<i>Lyratae</i>	<i>P. lyrata</i> Prain ex Maximowicz	DEB 27706 (A)	Sichuan	AY949648	AY949722
	<i>P. dolichoglossa</i> H. L. Li	DEB 29236 (A)	Yunnan	AY949639	AY949716
<i>Cyathophora:</i>					
<i>Cyathophyllae</i>	<i>P. cyathophylla</i> Franchet	DEB 28478 (A)	Sichuan	AY949668	AY949738
<i>Reges</i>	<i>P. rex</i> C. B. Clarke ex Maximowicz	DEB 28416 (A)	Sichuan	AY949643	AY949719
	<i>P. thamnophila</i> (Handel-Mazzetti) H. L. Li	DEB 28967 (A)	Sichuan	AY949697	AY949761
<i>Superbae</i>	<i>P. superba</i> Franchet ex Maximowicz	DEB 29154 (A)	Yunnan	AY949684	AY949749
<i>Dolichophyllum:</i>					
<i>Tatsienenses</i>	<i>P. tatsienensis</i> Bureau & Franchet	DEB 28186 (A)	Sichuan	AY949660	AY949730
<i>Holophyllum:</i>					
<i>Integrifoliae</i>	<i>P. integrifolia</i> J. D. Hooker	DEB 28273 (A)	Sichuan	AY949699	AY949763
<i>Notbosigmantha:</i>					
<i>Graciles</i>	<i>P. gracilis</i> Wallich	NF 2418 (MAK)	Nepal	AY949657	
	<i>P. pennelliana</i> Tsoong	RHR 9912 (A)	Nepal	AY949655	AY949726
<i>Orthosiphonia:</i>					
<i>Abrotanifoliae</i>	<i>P. densispica</i> Franchet ex Maximowicz	DEB 28415 (A)	Sichuan	AY949642	AY949718
<i>Cheilanthifoliae</i>	<i>P. cheilanthifolia</i> Schrenk	DEB 26627 (A)	Sichuan	AY949637	AY949714
<i>Molles</i>	<i>P. mollis</i> Wallich ex Bentham	NF 2415 (MAK)	Nepal	AY949646	
	<i>P. mollis</i> Wallich ex Bentham	DEB 29681 (A)	Xizang	AY949671	AY949739
<i>Myriophyllae</i>	<i>P. chamissonis</i> Steven	NF 919 (MAK)	Japan	AY949631	AY949709
	<i>P. alaschanica</i> Maximowicz	DEB 26947 (A)	Qinghai	AY949680	AY949746
	<i>P. scolopax</i> Maximowicz	DEB 26806 (A)	Qinghai	AY949670	
	<i>P. cristatella</i> Pennell & H. L. Li	DEB 27815	Sichuan	AY949638	AY949715
<i>Pilostachyae</i>	<i>P. ternata</i> Maximowicz	DEB 26594 (A)	Qinghai	AY949694	AY949758
<i>Semitortae</i>	<i>P. semitorta</i> Maximowicz	DEB 28435 (A)	Sichuan	AY949692	AY949756
<i>Sigmantha:</i>					
<i>Cheilanthifoliae</i>	<i>P. anas</i> Maximowicz	DEB 27904 (A)	Sichuan	AY949700	AY949764
<i>Microchilae</i>	<i>P. metaszetschuanica</i> P. C. Tsoong	DEB 27898 (A)	Sichuan	AY949665	AY949735
<i>Verticillatae</i>	<i>P. verticillata</i> L.	NF 151 (MAK)	Japan	AY949698	AY949762
	<i>P. verticillata</i> L.	RN, 15 July 1999 (A)	Switzerland	AY949706	AY949769
	<i>P. nodosa</i> Pennell	NF 2495 (MAK)	Nepal	AY949669	
	<i>P. roylei</i> Maximowicz	DEB 26959 (A)	Qinghai	AY949675	
	<i>P. kansuensis</i> Maximowicz	DEB 27837 (A)	Sichuan	AY949632	AY949710
	<i>P. likiangensis</i> Franchet ex Maximowicz	DEB 28297 (A)	Sichuan	AY949659	AY949729
	<i>P. lineata</i> Franchet ex Maximowicz	DEB 27942 (A)	Sichuan	AY949664	AY949734
	<i>P. rupicola</i> Franchet ex Maximowicz	DEB 28190 (A)	Sichuan	AY949688	AY949752
	<i>P. szetschuanica</i> Maximowicz	DEB 27708 (A)	Sichuan	AY949690	AY949754
<i>Allophyllum:</i>					
<i>Apocladus:</i>					
<i>Foliosae</i>	<i>P. foliosa</i> L.	RN, 7 July 1999 (A)	Switzerland	AY949679	AY949745
<i>Rostratae</i>	<i>P. tuberosa</i> L.	IS, "summer 1999" (A)	Austria	AY949661	AY949731
	<i>P. kernerii</i> Dalla Torre	IS, "summer 1999" (A)	Italy	AY949652	AY949725
<i>Bicuspidatae:</i>					
<i>Comosae</i>	<i>P. schistostegia</i> Maximowicz	NF 80 (MAK)	Japan	AY949704	AY949767
<i>Botryantha:</i>					
<i>Rhinanthoides</i>	<i>P. rhinanthoides</i> Schrenk ex Fischer & C. Meyer	DEB 27839 (A)	Sichuan	AY949691	AY949755
<i>Haplophyllum:</i>					
<i>Racemosae</i>	<i>P. bifida</i> (Buchanan-Hamilton) Pennell	NF 2460 (MAK)	Nepal	AY949685	AY949750
<i>Lasioglossa:</i>					
<i>Craspedotrichae</i>	<i>P. anserantha</i> Yamazaki	NF 2508 (MAK)	Nepal	AY949636	
	<i>P. trichocymba</i> H. L. Li	DEB 28244 (A)	Sichuan	AY949696	AY949760
<i>Dolichocymbae</i>	<i>P. dolichocymba</i> Handel-Mazzetti	DEB 29181 (A)	Yunnan	AY949633	AY949711

**Table 1**  
(Continued)

Taxon	Species	Voucher	Location	ITS	matK
<i>Gloriosae</i>	<i>P. gloriosa</i> Bisset & S. Moore	NF 2588 (MAK)	Japan	AY949647	
	<i>P. iwatensis</i> Ohwi	NF 0575 (MAK)	Japan	AY949654	
	<i>P. nipponica</i> Makino	NF 655 (MAK)	Japan	AY949663	AY949733
<i>Kialenses</i>	<i>P. streptorhyncha</i> P. C. Tsoong	DEB 27963 (A)	Sichuan	AY949689	AY949753
<i>Lachnoglossae</i>	<i>P. lachnoglossa</i> J. D. Hooker	DEB 28066 (A)	Sichuan	AY949658	AY949728
<i>Lasiophrydes</i>	<i>P. lasiophrys</i> Maximowicz	DEB 27968 (A)	Sichuan	AY949662	AY949732
<i>Rudes</i>	<i>P. rudis</i> Maximowicz	DEB 27790 (A)	Sichuan	AY949686	AY949751
<i>Trichoglossae</i>	<i>P. trichoglossa</i> Hooker	NF 2417 (MAK)	Nepal	AY949702	
	<i>P. rhodotricha</i> Maximowicz	DEB 28979 (A)	Sichuan	AY949674	AY949741
<i>Macrostachys:</i>					
<i>Oxycarpae</i>	<i>P. davidii</i> Franchet	DEB 27481 (A)	Sichuan	AY949703	AY949766
	<i>P. tibetica</i> Franchet	DEB 27836 (A)	Sichuan	AY949644	AY949720
<i>Pedicularis:</i>					
<i>Furfurae</i>	<i>P. furfuracea</i> Wallich ex Bentham	RHR 9904 (A)	Nepal	AY949701	
<i>Resupinatae</i>	<i>P. resupinata</i> (L.) Pursh	NF 1105 (MAK)	Japan	AY949653	
	<i>P. yezoensis</i> Maximowicz	NF 1103 (MAK)	Japan	AY949683	
<i>Phanerantha:</i>					
<i>Macranthae</i>	<i>P. scullyana</i> Prain ex Maximowicz	NF 2440 (MAK)	Nepal	AY949650	
<i>Pseudomacranthae</i>	<i>P. elwesii</i> J. D. Hooker	NF 2432 (MAK)	Nepal	AY949707	
	<i>P. elwesii</i> J. D. Hooker	DEB 29147 (A)	Yunnan	AY949649	AY949723
<i>Rhizophyllum:</i>					
<i>Flammeae</i>	<i>P. oederi</i> Vahl	NF 203 (MAK)	Japan	AY949672	AY949740
	<i>P. oederi</i> Vahl	DEB 26595 (A)	Qinghai	AY949676	AY949742
<i>Pumiliones</i>	<i>P. przewalskii</i> Maximowicz	DEB 26703 (A)	Qinghai	AY949677	AY949743
	<i>P. przewalskii</i> Maximowicz	DEB 28058 (A)	Sichuan	AY949681	AY949747
	<i>P. bella</i> subsp. <i>holophylla</i> (Marquand & Shaw) P. C. Tsoong	DEB 30143 (A)	Xizang	AY949693	AY949757
<i>Rhynchodontae</i>	<i>P. rhynchodonta</i> Bureau & Franchet	DEB 28070 (A)	Sichuan	AY949682	AY949748
<i>Roseae</i>	<i>P. muscoides</i> H. L. Li	DEB 28664 (A)	Sichuan	AY949635	AY949713
<i>Poecilophyllum:</i>					
<i>Dolichomischus:</i>					
<i>Axillares</i>	<i>P. axillaris</i> Franchet ex Maximowicz	DEB 28246 (A)	Sichuan	AY949705	AY949768
<i>Saccochilus:</i>					
<i>Megalanthae</i>	<i>P. cornigera</i> Yamazaki	RHR 9909 (A)	Nepal	AY949634	AY949712
	<i>P. hoffmeisteri</i> Klotzsch	NF 2483 (MAK)	Nepal	AY949640	
	<i>P. megalantha</i> D. Don	NF 2453 (MAK)	Nepal	AY949656	AY949727
<i>Schizocalyx:</i>					
<i>Franchetianae</i>	<i>P. mussotii</i> Franchet	DEB 29117 (A)	Sichuan	AY949667	AY949737
<i>Longiflorae</i>	<i>P. longiflora</i> Rudolph	NF 2500 (MAK)	Nepal	AY949645	AY949721
	<i>P. longiflora</i> Rudolph	DEB 27508 (A)	Sichuan	AY949666	AY949736
	<i>P. cranolopha</i> Maximowicz	DEB 27821 (A)	Sichuan	AY949695	AY949759
	<i>P. decorissima</i> Diels	DEB 27825 (A)	Sichuan	AY949641	AY949717
	<i>P. siphonantha</i> D. Don	NF 2443 (MAK)	Sichuan	AY949673	
<i>Outgroups:</i>					
<i>Orobanchaceae</i>	<i>Ptheiospermum tenuisectum</i> Bureau & Franchet	DEB 28207 (A)	Sichuan	AY949708	AY949770
	<i>Seymeria pectinata</i> Pursh				AF051999
	<i>Harveya squamosa</i> Kuntze			AF120225	
	<i>Harveya bolusii</i> Kuntze				AF051983
	<i>Harveya purpurea</i> Harvey				AF051984
	<i>Hyobanche atropurpurea</i> Bolus				AF051986
	<i>Hyobanche sanguinea</i> L.			AF120220	AF051987
	<i>Lathraea clandestina</i> L.				AF051989
	<i>Melampyrum sylvaticum</i> L.				AF051991
	<i>Tozzia alpina</i> L.				AF052001
	<i>Striga asiatica</i> (L.) Kuntze				AF052000
	<i>Schwalbea americana</i> L.				AF051998
	<i>Castilleja sulphurea</i>			AF478944	
	<i>Castilleja liniariifolia</i> Bentham				AF051981
	<i>Lindenbergia phillipinensis</i> Bentham				AF051990

**Table 1**  
(Continued)

Taxon	Species	Voucher	Location	ITS	matK
	<i>Boschniakia hookeri</i> Walpers				AF051979
	<i>Boschniakia strobilacea</i> A. Gray				AF051980
	<i>Orobanche minor</i>			AF437315	
	<i>Orobanche corymbosa</i> (Rydb.) Ferris				AF051993
Bignoniaceae	<i>Kigelia africana</i> Bentham				AF051988
Scrophulariaceae	<i>Verbascum thapsis</i> L.				AF052002
	<i>Hemimeris sabulosa</i> L.				AF051985
Veronicaceae	<i>Veronica arvensis</i> L.				AF052003

Note. Table includes voucher information for specimens that were used to generate new sequences, including collector's initials, number or date, and herbarium of deposition in parentheses. GenBank accession numbers are provided for ITS and *matK*; those for new sequences are in bold. YO = Y. Omori et al.; RHR = R. H. Ree; DEB = D. E. Boufford et al.; NF = N. Fujii; RN = R. Nyffeler; IS = Ivana Stehlik. Herbarium acronyms are from Holmgren et al. (1990).

The results of the Bayesian phylogenetic analysis of ITS+*matK* show that in general, correspondence between clades and taxa is low (fig. 3). Of the 17 series represented by more than one species in this study, the monophyly of only seven are supported: *Graciles*, *Megalanthae*, *Oxycarpae*, *Reges*, *Trichoglossae*, *Lyratae*, and *Gloriosae*. Only two of the 16 sections represented (*Brachyphyllum* and *Cyathophora*), and none of the three subgenera described by Li (1948, 1949), are found to be strictly monophyletic. However, several strongly supported clades do appear that are noteworthy from a taxonomic, morphological, and/or geographic standpoint.

**Clade 1.** This group is distinguished by opposite or whorled leaves and short corolla tubes and includes both beaked and nonbeaked species (fig. 4A). Most of the opposite/whorled-leaved species sampled fall into this clade; the only exceptions are *Cyathophora* (clade 3), *Gloriosae* (clade 6), *P. mollis*, and *P. flexuosa*. Disregarding these, the clade generally corresponds to subgenus *Cyclophyllum* Li. All sampled species of series *Verticillatae* are included here, but these are not monophyletic. This suggests homoplasy in a distinctive character of *Verticillatae*, namely that of a short, basally de-curved corolla tube.

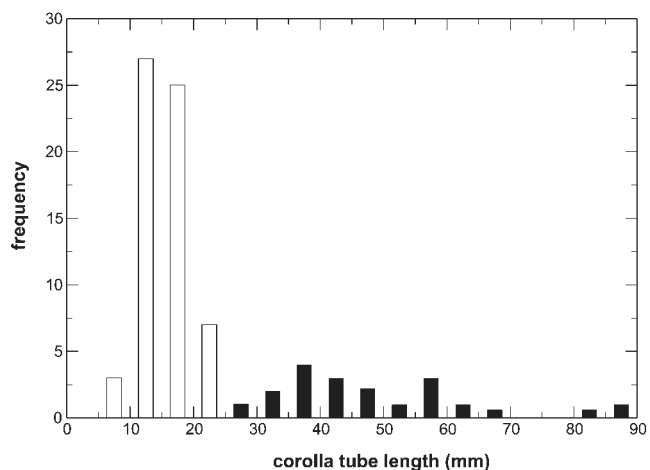
In this clade, several phylogenetic conflicts between genes are apparent. For the clade comprising *P. kansuensis*, *P. nodosa*, and *P. chamissonis*, ITS places *P. chamissonis* at the base of a clade comprising all taxa above and including *P. ternata* in figure 3. For the clade comprising *P. dolichoglossa*, *P. lyrata*, and *P. confertiflora*, *matK* places *P. confertiflora* as the sister group to *P. chamissonis* + *P. kansuensis*. The clade comprising *P. oxyrhyncha* and its sister group conflicts with ITS, which places *P. oxyrhyncha* and *P. tatsienensis* in a basal polytomy with the rest of clade 1. For the clade comprising *P. metaszetchuanica* and its sister group, ITS places *P. metaszetchuanica* near the base of clade 1, outside of the clade including *P. ternata*. Finally, in contrast to ITS and the combined analysis, *matK* places *P. integrifolia* outside of the clade marked by the ancestor of *P. gracilis* and *P. scolopax*.

**Clade 2.** This corresponds to series *Megalanthae*, a western Himalayan group characterized by flowers with long (up to 11 cm) corolla tubes that are commonly resupinate near the distal end, resulting in the ventral corolla lip hanging

over and covering the beaked galea, ostensibly as a means of sheltering visiting pollinators from frigid alpine weather conditions.

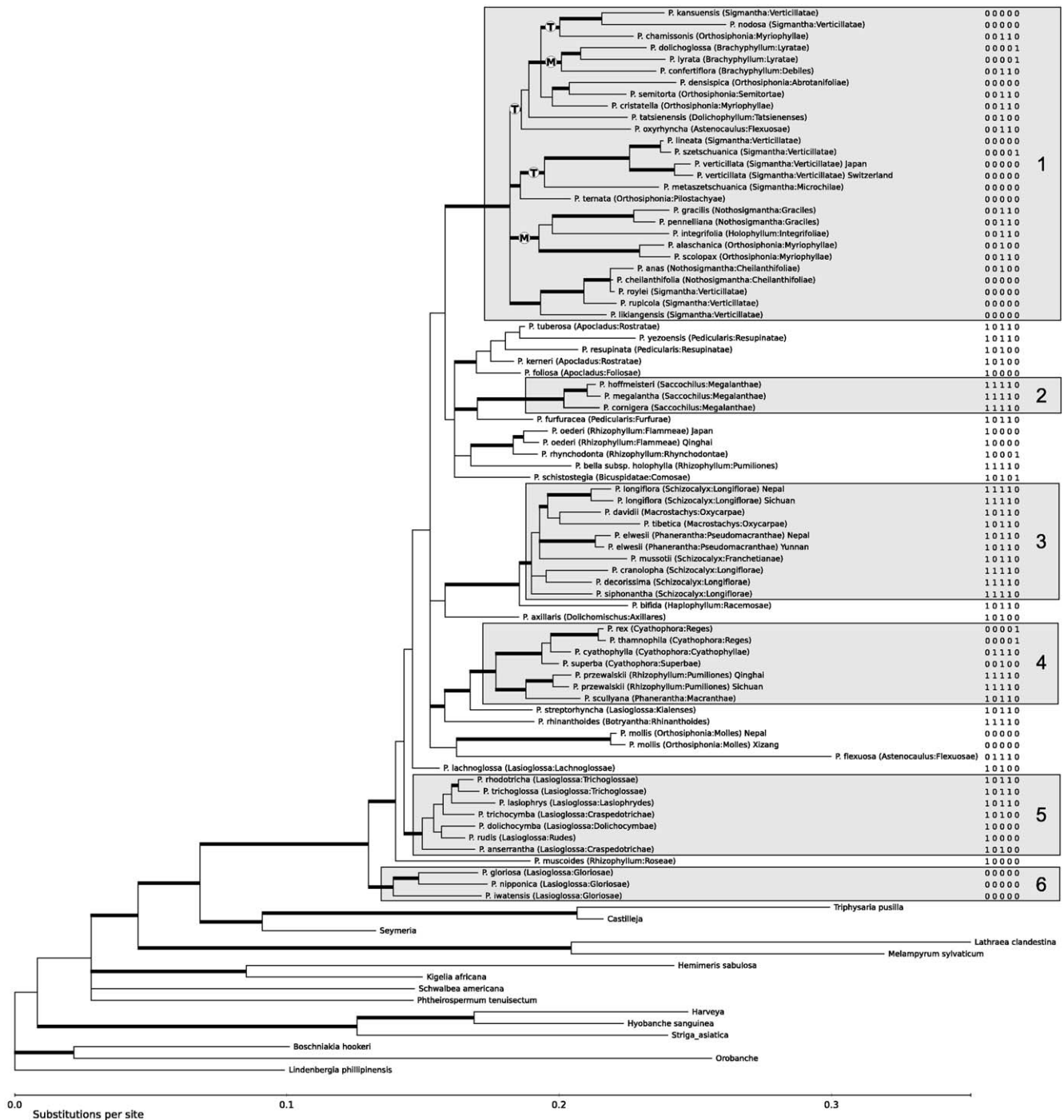
**Clade 3.** This group includes all representatives of series *Longiflorae* sampled. *Longiflorae*, named by Prain for its personate long corolla tube, is a group that has remained intact throughout taxonomic revisions of *Pedicularis*. In this study, however, strong support is found for short-tubed species (*P. davidii*, *P. tibetica*, *P. elwesii*, and *P. mussotii*) being nested within this clade. All species in this clade have beaked, curved-rostrate corollas (fig. 4B), as does its closest relative, *P. bifida*.

**Clade 4.** This clade corresponds to section *Cyathophora*, a group distinguished morphologically by conspicuously perfoliate leaf bases (fig. 4C). It includes six species endemic to south-central China that exhibit extreme interspecific floral variation. *Pedicularis rex* and *P. thammophila* have short-tubed, beakless, toothed, yellow to cream-colored corollas, while the flowers of *P. superba* and *P. cyathophylla* are short tubed, beaked, toothless, and colored pink to reddish-purple.



**Fig. 2** Histogram of mean corolla tube length for species included in this study. Filled versus open bars indicate the scoring of tube length as a binary character.



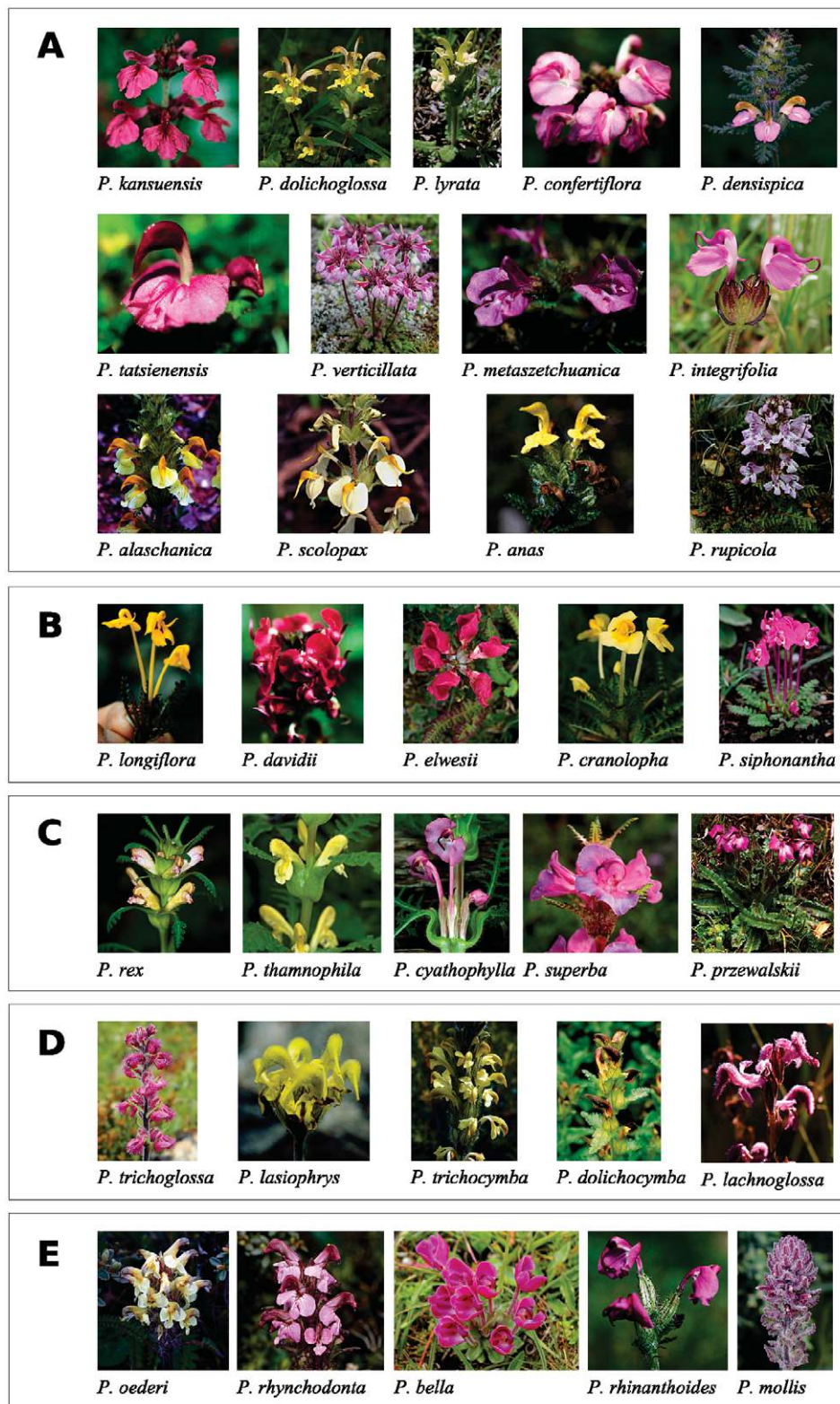


**Fig. 3** Majority rule consensus of trees visited by the combined ITS + *matK* Markov-chain Monte Carlo analysis. Clades with Bayesian posterior support  $\geq 0.95$  are drawn with thicker lines. Multiple sequences from the same species are differentiated by locality. Noteworthy clades discussed in the text are in numbered boxes. Conflicts in phylogenetic signal between genes are marked by circles, in which T and M indicate clades contradicted by single-gene analyses of ITS and *matK*, respectively. In those cases, the single-gene analysis yielded a strongly supported clade that is not compatible with the marked clade. Binary coded morphological characters are shown on the right in the following order: phyllotaxy, corolla tube, galea beak, curve-rostrate galea, and galea teeth.

The long-tubed corolla type is represented by *P. cyathophylla*. The group has been recognized in all classification systems for *Pedicularis* and has traditionally been placed with other verticillate-leaved species; however, the molecular

evidence presented here indicates that *Cyathophora* is nested among spiral-leaved species.

Within this clade, *matK* places *P. cyathophylla* sister to *P. rex* + *P. thamnophila*, and this relationship is retained in



**Fig. 4** Photographs of representative species of *Pedicularis* sampled in this study. A, Species in clade 1, which corresponds most closely to subgenus *Cyclophyllum*. B, Species in clade 3, which includes all sampled species of series *Longiflorae*. C, Species in clade 4, section *Cyathophora*, and a closely related species, *P. przewalskii*. D, Species in clade 5 and *P. lachnoglossa*, which form the Himalayan representatives of section *Lasioglossa*. E, Representative species in the analysis not associated with the major clades discussed in the text. All photos are of species occurring in the Hengduan Mountains region.

the combined analysis. ITS alone groups *P. cyathophylla* + *P. superba* together.

**Clade 5.** All members of this spiral-leaved clade belong to section *Lasioglossa*. Distinctive morphological features that unite them include taproots; single upright stems; narrow, coriaceous leaves with pinnatifid to dentate margins; leaf bases that clasp the stem; and navicular to beaked corollas, often with hairs along the margins and/or upper surface of the galea (fig. 4D). Toothed and long-tubed corollas are absent from this clade. Other species of *Lasioglossa* that were sampled but do not fall in this clade are *P. streptorhyncha*, *P. lachnoglossa*, and those of series *Gloriosae* (clade 6); these species lack taproots and clasping leaf bases. *Gloriosae* also differs in being distributed in Japan, while the others are Himalayan.

**Clade 6.** This clade consists of *P. nipponica*, *P. iwatensis*, and *P. gloriosa* and corresponds to series *Gloriosae*. These species, in addition to *P. ochiana* (not sampled), are endemic to Japan, but *Gloriosae* also includes the widespread *P. sceptrum-carolinum*, distributed throughout Europe and Asia, and *P. odontochila*, endemic to the Qinling Mountains of Shaanxi, China. Species of *Gloriosae* in Japan have opposite or whorled leaves, but other species in this group have spiral leaves (*P. sceptrum-carolinum* can have “pseudo-whorls”). *Gloriosae* is in section *Lasioglossa*, i.e., is not part of the opposite/whorled-leaved subgenus *Cyclophyllum*.

#### Character Evolution

Analysis of the evolutionary histories of the key morphological characters that have been most important in *Pedicularis* classification (leaf arrangement, corolla tube length, galea beak, and galea teeth) yielded, for each character, a set of 20,000 stochastic mappings, one for each tree sampled in the combined data MCMC run. These mappings represent random draws from the posterior distribution of trees and character histories and thus provide estimates of the posterior probabilities associated with distinct combinations of gains and losses (table 2). The effect of uncertainty in the phylogenetic tree topology is summarized by the most frequent distinct histories for each character. Here, I define a distinct history to be a set of stochastic mappings that are identical with respect to the identities of all clades that are subtended by a change in character state (a clade’s identity is simply the set of taxa within it). In other words, mappings belonging to the same distinct history share the same numbers of gains and losses and are also identical in the phylogenetic locations of those changes. However, the trees on which they occur may differ in parts where no changes are mapped. Here, distinct histories are shown on the majority-rule consensus of the trees associated with them.

**Phyllotaxy.** Analysis of spiral leaves shows a single gain in the ancestor of *Pedicularis* and two to five subsequent losses (fig. 5). A change back to verticillate leaves occurred along the branch leading to clade 1 (in 2% of cases, the change occurs along the branch leading to a clade comprising clade 1 and *P. mollis* + *P. flexuosa*). A shift from spiral to verticillate is also always observed along the branch subtending *Cyathophora*. In 49.5% of cases, simulated histories realized two independent changes to spiral phyllotaxy in the

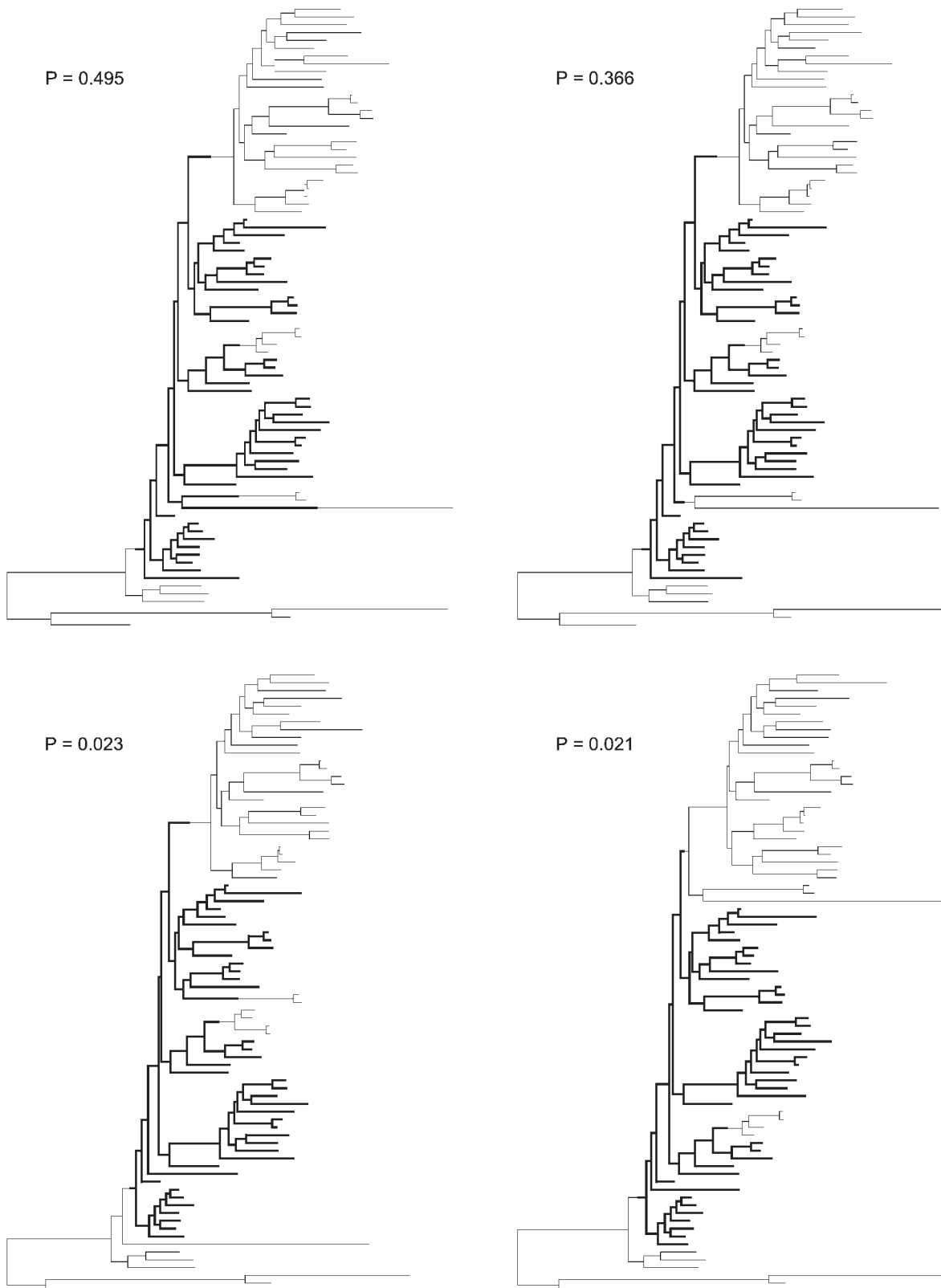
**Table 2**  
Frequencies of Change in Morphological Characters from Stochastic Mapping Simulations

Character	Gains	Losses	Frequency	Cumulative frequency	
Phyllotaxy (spiral leaves)	1	4	0.509	0.509	
	1	3	0.399	0.907	
	1	2	0.021	0.928	
	0	6	0.019	0.947	
	1	5	0.017	0.964	
	Corolla tube (long)	9	0	0.431	0.431
		8	0	0.411	0.843
		8	1	0.043	0.885
		7	3	0.023	0.909
		9	1	0.017	0.926
10		0	0.015	0.941	
7		4	0.014	0.955	
Galea beak (present)		2	11	0.249	0.249
		2	10	0.200	0.449
		3	9	0.099	0.547
	4	9	0.073	0.621	
	2	12	0.070	0.690	
	3	10	0.066	0.756	
	3	11	0.031	0.787	
	4	10	0.022	0.809	
	5	8	0.019	0.828	
	4	8	0.015	0.844	
Galea teeth (present)	2	13	0.013	0.857	
	5	9	0.011	0.868	
	10	5	0.011	0.878	
	2	9	0.010	0.889	
	11	5	0.009	0.897	
	9	6	0.008	0.905	
	3	12	0.008	0.913	
	6	8	0.006	0.920	
	9	7	0.006	0.926	
	1	13	0.006	0.932	
Galea teeth (present)	3	8	0.006	0.938	
	8	7	0.005	0.943	
	8	6	0.005	0.947	
	4	11	0.004	0.951	
	5	0	0.877	0.877	
	5	1	0.048	0.925	
	6	0	0.044	0.969	

Note. Unique combinations of numbers of gains and losses of the indicated state are shown in descending order of frequency, up to a cumulative frequency of 0.95.

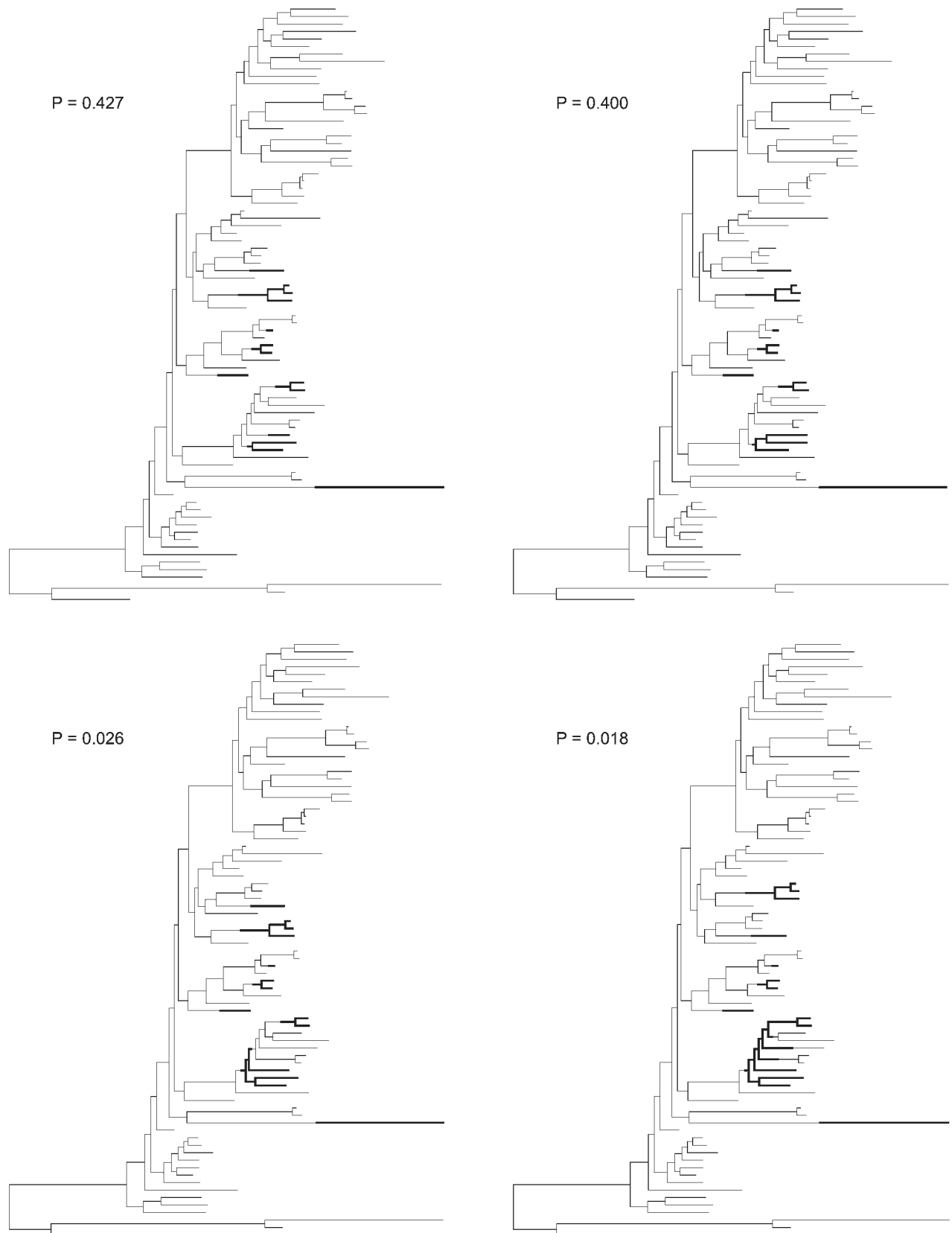
*P. mollis* + *P. flexuosa* clade, despite both species having that trait; this is greater than the 36.6% of cases that show the more parsimonious mapping of a single shift. However the positions of these species are not strongly supported by the molecular data. *Pedicularis flexuosa* in particular has a long branch, and its phylogenetic placement is uncertain.

**Corolla tube.** Long tubes have been derived eight or nine times in *P. bella*, *Megalanthae*, *P. cyathophylla*, *P. przewalskii*, *P. rhinanthoides*, and *P. flexuosa*. Losses (reversal to short tubes) are inferred in <10% of the posterior distribution of mappings. In those cases, they occur mostly along the branch leading to clade 3 (fig. 6). These results indicate that once gained, long corolla tubes were seldom, if ever, lost,



**Fig. 5** Four most probable distinct mappings of phyllotaxy on the phylogeny of *Pedicularis*. Thick branches represent the presence of spiral leaves. Each distinct mapping is shown on a majority rule consensus of the trees associated with it; i.e., for each tree associated with a distinct mapping, simulations of phyllotaxy yielded an identical set of gains and losses. Identical mappings were determined on the basis of the taxon bipartitions defined by the branches on which character change occurred.





**Fig. 6** Four most probable distinct mappings of corolla tube length on the phylogeny of *Pedicularis* (see fig. 5). Thick branches represent the presence of long tubes.

and they provide support for Li's hypothesis that long tubes are a derived character state.

**Galea.** Beaked galeas have been gained one to three times near the base of *Pedicularis* and subsequently lost several times (fig. 7). Of the four characters mapped, the galea beak exhibits the greatest variation in how gains and losses are distributed over the tree; e.g., the four most probable mappings account for only 50% of the posterior density (fig. 8). Galea teeth have been gained five or six times; in only 5% of cases, a single loss is inferred.

#### *Correlated Evolution of Nectar Production and Tube Length*

Summary statistics for the correlation analysis of nectar production and corolla tube length are presented in figure 9. Short corolla tubes are significantly associated with nectar production, as are long tubes with the lack of nectar ( $P = 0.015$  in each case). Associations between the other state pairs are not statistically significant.

## Discussion

#### *Taxonomic Implications of Molecular Phylogenetic Results*

This is the largest phylogenetic study of *Pedicularis* to date, and it represents a broad taxonomic and morphological sample, primarily but not solely of species from the eastern Himalayan biodiversity hotspot (Wilson 1992; Meyers 1988). However, the species included represent only about 20% of the total species diversity in the lineage, and some key geographic regions in the distribution of *Pedicularis* are unsampled or only poorly sampled here, such as North America, Europe, and the Arctic. For these reasons, the phylogenetic relationships presented here should be regarded as preliminary.

The monophyly of *Pedicularis* is strongly supported in all analyses, refuting the suggestion of a diphyletic origin of the group (Tsoong 1955). A more rigorous test of this would require more exhaustive outgroup sampling, but ongoing phylogenetic studies of Orobanchaceae have not found taxa more closely related to *Pedicularis* than *Castilleja* (J. Bennett and S. Mathews, personal communication), a group sampled in this study. Within *Pedicularis*, the molecular data cannot firmly resolve basal relationships and thus do not yet clarify the persistent uncertainty that has characterized the taxonomic literature regarding the identity and relationships of primary subgeneric groups. In general, strict agreement between the molecular results and taxonomic groups is limited, as was found in a smaller-scale study of *Pedicularis* using only ITS (Yang et al. 2003). This suggests a need for a critical re-evaluation of the characters used to delimit taxa, particularly those floral characters that have been heavily emphasized at lower taxonomic rank.

The discovery of clades that are morphologically distinctive but have not yet been named (clade 1, clade 5) may prove useful in guiding future taxonomic revisions of the group. It is noteworthy that morphologically distinct clades that are resolved by the molecular data are, more often than not, united by vegetative similarities, not floral characters. For example, clade 1 has whorled leaves; *Cyathophora* has perfoliate leaf bases; clade 5 has clasping leaf bases and tap-

roots. One exception is *Megalanthae*, whose most conspicuous distinguishing features are a long corolla tube and inverted, hoodlike lower corolla lip.

Li (1949, 1951) placed spiral-leaved species of *Pedicularis* in two subgenera. *Allophyllum*, characterized by erect stems and terminal inflorescences, is shown not to be monophyletic in this study. The same is true for *Poecilophyllum*, characterized by lax stems and axillary flowers. Although support for the basal region of the phylogeny is low, most of the analyses reported here suggest that the spiral-leaved species form a paraphyletic grade, within which opposite/whorled-leaved clades (*Cyathophora* and clade 1) are nested.

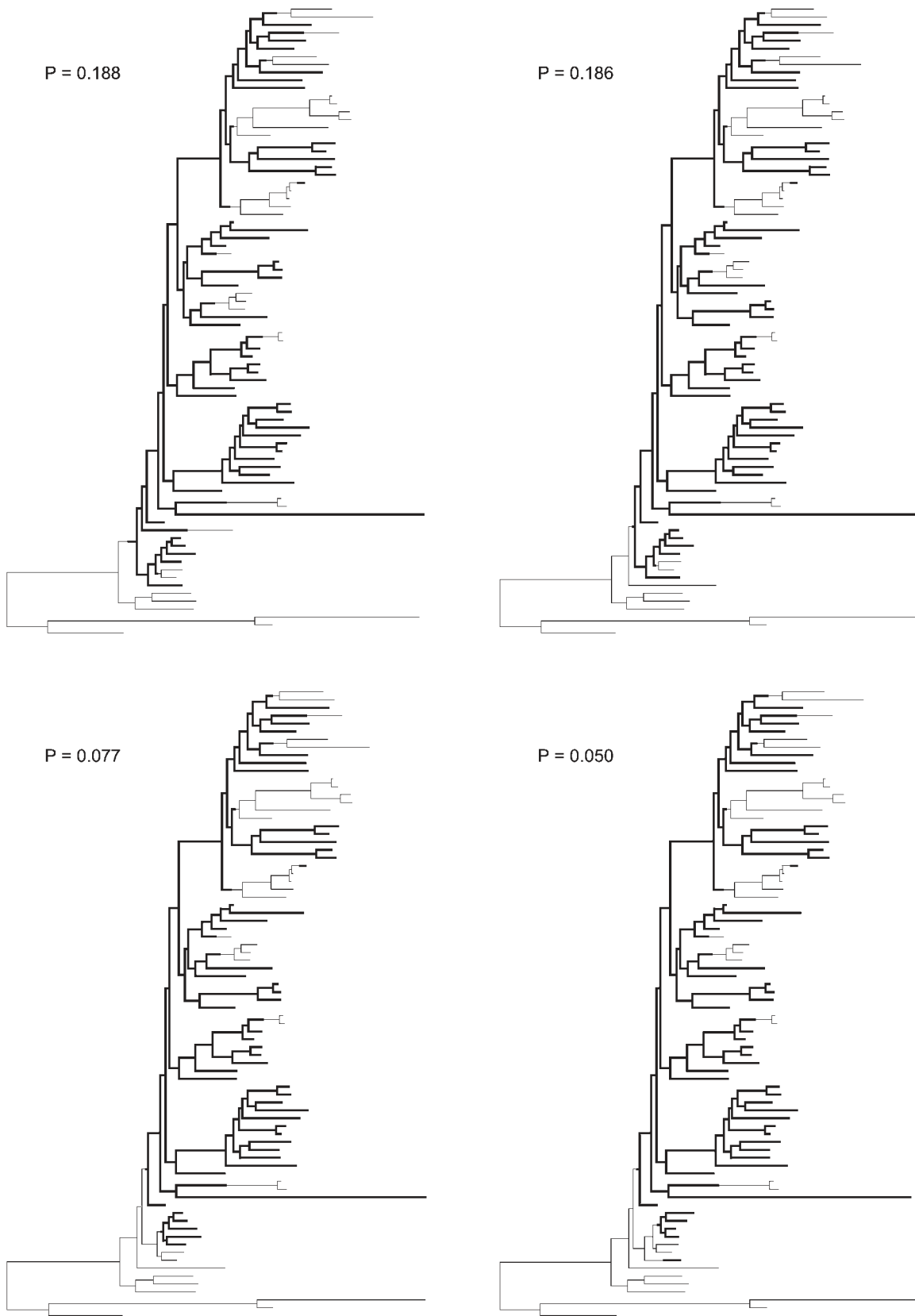
The conflicts observed between phylogenetic trees inferred from ITS and *matK* warrant further investigation. Because Bayesian posterior probabilities may tend to overestimate clade support (e.g., Suzuki et al. 2002) or support erroneous clades if phylogenetic branches are short (Alfaro et al. 2003), it is possible that the observed conflicts are methodological artifacts or stochastic error. Possible biological causes include hybridization, interspecific chloroplast introgression, or cryptic paralogy of ITS sequences (see Bailey et al. 2003 for review).

#### *Floral Homoplasy, Pollination, and Constraint*

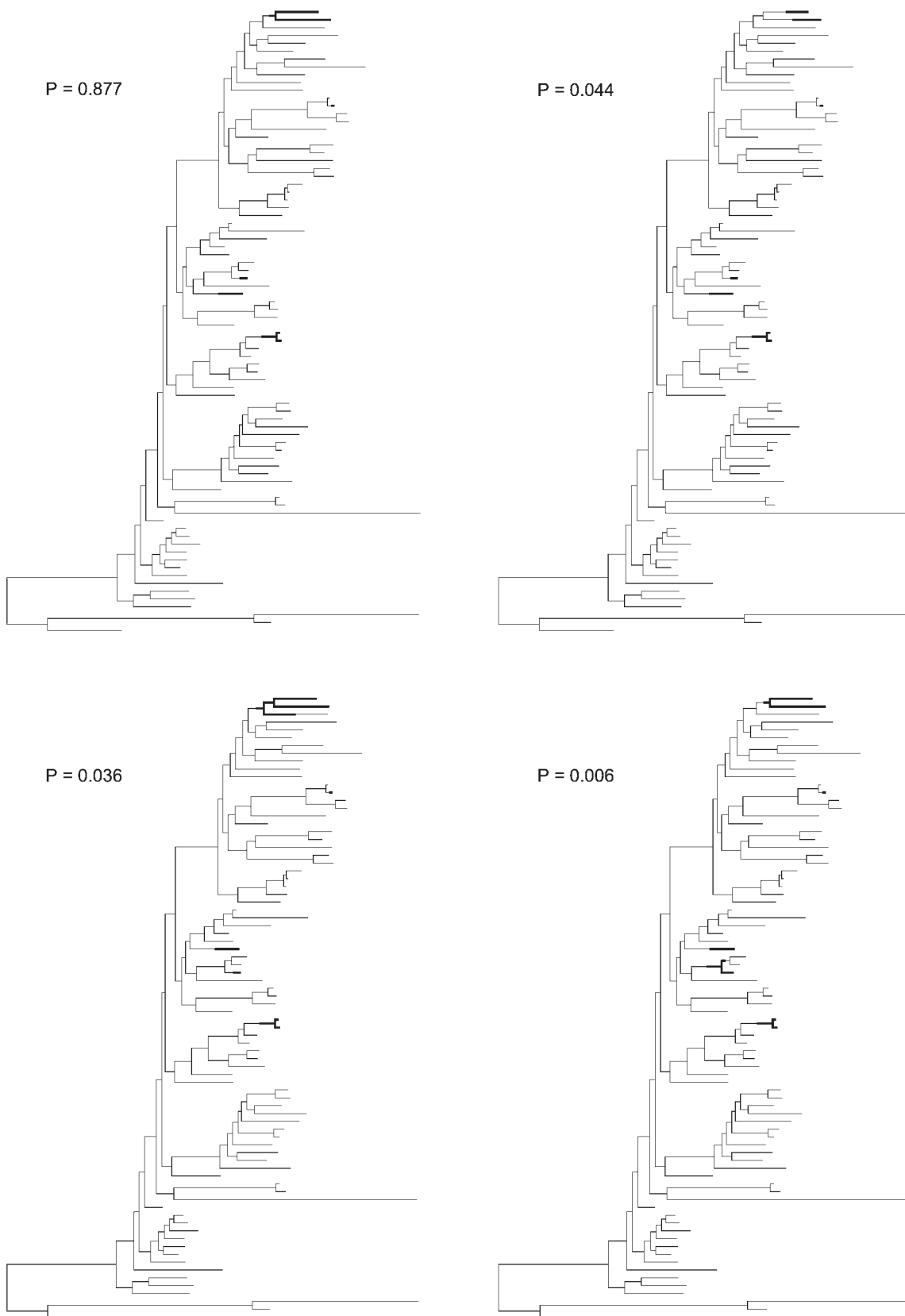
Systematic relationships within *Pedicularis* have generally been postulated in reference to variation in leaf arrangement, inflorescence structure, and corolla characters, with most classification systems placing more emphasis on floral characters in the delimitation of higher-level taxa. A view widely expressed in the literature is that floral characters have played a key role in speciation in *Pedicularis*. Li (1948, 1949) based the structure of his taxonomic system on the idea that convergent adaptations to pollinator interactions influenced floral evolution as the lineage spread from its putative center of origin in north-central Asia (Limpricht 1924). Macior and Tang (1997) cited the sympatric occurrence of species with divergent pollination traits (e.g., species with and without nectar), the absence of hybrids, and diversity in both floral form and pollinator behavior as indicators that ecological, pollinator-mediated prezygotic isolation mechanisms have driven speciation in *Pedicularis*.

Generally speaking, floral characters show a high degree of homoplasy, as originally hypothesized by Li (1948, 1949). Stochastic mapping shows that long corolla tubes have been independently derived multiple times, suggesting a specialized function of this trait, but fitness experiments are needed to clarify what ecological advantage is conferred by longer tubes. Galea teeth have also been gained and not lost, a result that contradicts previous hypotheses of the polarity of that character. Of the floral characters analyzed, the most labile and diverse in form is the galea beak, which from our current knowledge about pollination mechanisms in *Pedicularis* appears to be of considerable importance in influencing pollinator behavior.

Phyllotaxy exhibits less homoplasy overall compared with floral characters. This result supports the underlying rationale of Li's (1948, 1949) taxonomic system, but the view that phyllotaxy is more useful than floral characters in diagnosing primary lineages of *Pedicularis* is supported only to the extent that the clade 1 is almost congruent with Li's

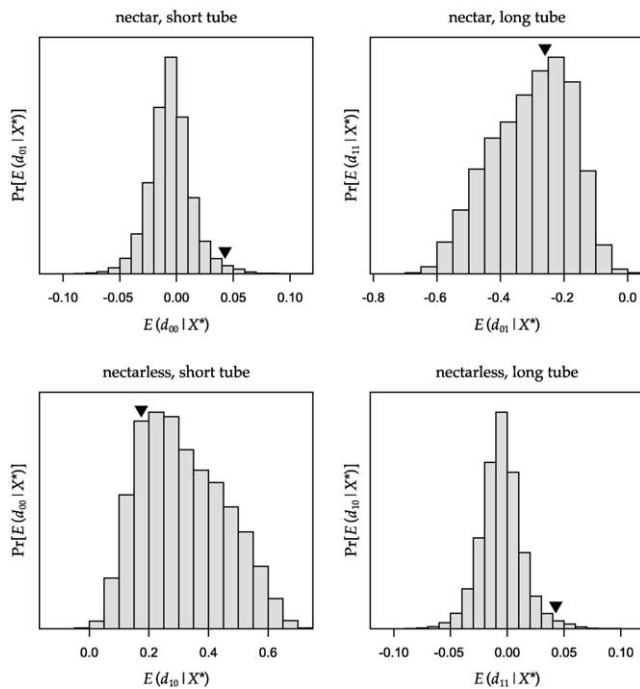


**Fig. 7** Four most probable distinct mappings of galea beak state on the phylogeny of *Pedicularis* (see fig. 5). Thick branches represent the presence of a beak.



**Fig. 8** Four most probable distinct mappings of galea teeth on the phylogeny of *Pedicularis* (see fig. 5). Thick branches represent the presence of teeth.





**Fig. 9** Posterior predictive probability distributions for the association between nectar production and corolla tube length. Triangles indicate the position of the test statistic for each pair of states.

subgenus *Cyclophyllum*. The phylogenetically conserved pattern of phyllotaxy in *Pedicularis* may be due to greater genetic constraints suppressing switches between spiral and opposite leaves and/or stabilizing selection pressure favoring stasis within lineages.

On the basis of the functional importance of the galea beak in pollination, one might expect that once arisen in a lineage, stabilizing selection would maintain the beaked state, because specialization in pollinator behavior associated with an elaborate beak morphology would prohibit transitions back to the beakless state. This view is not corroborated by this study. The posterior distribution of character mappings shows a wide range in frequencies of gains and losses of beaked galeas (table 2), but the predominant directionality of change has been from beaked to beakless. More complete taxon sampling and increased phylogenetic resolution should reduce the uncertainty regarding actual numbers of gains and losses that occurred.

Under what ecological conditions might reversals to beakless corolla types be favored? If beaks do in fact represent a specialization for modifying pollinator behavior to limit nonconspecific pollen transfer, a more generalized morphology might be favored under conditions where competition for pollinators is low and pollinators are rare, as might be the case if a lineage is expanding into new habitats not already occupied by congeners. In other words, the evolution of beaks may be favored as a means to avoid local pollen competition but be easily lost if those competitive forces are removed. One clear case of character change from beaked to beakless is in *Cyathophora*. Of the six species in this group, the beakless species (*P. rex*, *P. thamnophila*, and *P. cyatho-*

*phyloides*) are more common and widespread than the beaked species. Their ranges extend from the Hengduan Mountain region into southeast Xizang (Tibet) and north Myanmar, while the beaked species are restricted to southwest Sichuan and north Yunnan. It has not been tested whether derived beakless forms also re-evolved nectar production, but over three field seasons in the Hengduan Mountains, the author has only observed pollen foraging, not nectar foraging, by bumblebees on *P. rex*.

One of the most intriguing aspects of floral diversity in *Pedicularis* is the occurrence of conspicuous gaps in the floral morphospace defined by the presence or absence of long corolla tubes, galea beaks, and galea teeth. No species possess beakless corollas with long corolla tubes or beaked galeas with apical teeth. This study demonstrates a significant historical association between beakless galeas and nectar production (fig. 9), suggesting that visitation by short-tongued insects such as *Bombus* imposes a constraint on corolla tube length.

The selective factors that have driven increased tube length in disparate lineages of *Pedicularis* remain unknown. The hypothesis that long tubes function in raising the reproductive parts of the flower above potentially obstructing vegetation, facilitating access by pollinators (Macior and Tang 1997), may apply in alpine habitats, where long-tubed species are typically rosette plants, with flowers borne on or near the ground. It is less likely to explain the elongated tube of *P. cyathophylla*, which bears flowers along an upright stem. Another hypothesis is that long styles impose sexual selection on microgametophytes via pollen tube competition (Stephenson and Bertin 1983; Armbruster 1996). This would predict high levels of pollen deposition on stigmas in communities with long-tubed species. Reported frequencies of pollinator visits to species of *Pedicularis* in the Himalaya vary widely, from 47 insects collected per person-hour for *P. punctata* (Macior 1990) to fewer than 0.5 insects/person-hour (Macior and Tang 1997).

Homoplasy observed in floral characters is consistent with the hypothesis that coadaptation with pollinators has driven parallel evolution in the corolla of *Pedicularis* (Li 1948, 1949). While it is tempting to invoke pollinator-mediated selection as a strong influence in the diversification of *Pedicularis*, inferences about speciation mode are beyond the scope of the data presented here. A perhaps more tenable question concerns not the origin but the maintenance of diversity in *Pedicularis*: to what extent does pollinator fidelity and pollen transfer efficiency play a role in the structuring of sympatric assemblages of *Pedicularis*? If reproduction in *Pedicularis* is generally pollinator limited, and pollination efficiency substantially affects fitness, corolla morphology might influence community assembly rules in *Pedicularis* by dictating which species are most likely to avoid competition for pollinators: i.e., nectariferous with nectarless species, red-flowered species with yellow, and so on. The phylogenetic structure of sympatric assemblages would also enable inferences to be made about the geographic scale of speciation in *Pedicularis*.

### Acknowledgments

I thank N. Fujii for providing DNA of samples from Nepal and Japan; P. Endress, I. Stehlik, and R. Nyeffeler for

samples of European species; and N. Young for *matK* primers and the *Seymeria pectinata* ITS sequence. R. Nielsen and J. Bollback provided helpful advice on implementing stochastic mapping. The manuscript was improved from comments by J. Wen, C. Bell, P. Fine, the associate editor, and

an anonymous reviewer. This study was supported by grants from the National Science Foundation (NSF; DEB-9972612) and the American Society of Plant Taxonomists to R. Ree, NSF grant DEB-9705795 to M. J. Donoghue, and NSF grant DEB-0321846 to D. E. Boufford.

### Literature Cited

- Akaike H 1974 A new look at the statistical model identification. *IEEE Trans Automatic Control* 19:716–723.
- Alfaro ME, S Zoller, F Lutzoni 2003 Bayes or bootstrap? a simulation study comparing the performance of Bayesian Markov Chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. *Mol Biol Evol* 20:255–266.
- Armbruster WS 1996 Evolution of floral morphology and function: an integrative approach to adaptation, constraint, and compromise in *Dalechampia* (Euphorbiaceae). Pages 241–272 in DG Lloyd, SCH Barrett, eds. *Floral biology: studies in floral evolution in animal-pollinated plants*. Chapman & Hall, New York.
- Armstrong JA, AW Douglas 1989 The ontogenetic basis for corolla aestivation in Scrophulariaceae. *Bull Torrey Bot Club* 116:378–389.
- Bailey CD, T Carr, SA Harris, CE Hughes 2003 Characterization of angiosperm nrDNA polymorphism, paralogy, and pseudogenes. *Mol Phylogenet Evol* 29:435–455.
- Baum DA, KJ Sytsma, PC Hoch 1994 A phylogenetic analysis of *Epilobium* (Onagraceae) based on nuclear ribosomal DNA sequences. *Syst Bot* 19:363–388.
- Bentham G 1835 Scrophularineae Indicae. J. Ridgway, London.
- 1846 Scrophulariaceae. Parisiis, n.p.
- Bonati G 1918 Le genre *Pedicularis* L. Morphologie, classification, distribution géographique, évolution et hybridation. PhD thesis. Université de Nancy.
- Boufford DE, PP Van Dijk 2000 South-central China. Pages 339–351 in RA Mittermeier, N Myers, CG Mittermeier, eds. *Hotspots: earth's biologically richest and most endangered terrestrial ecoregions*. CEMEX, Mexico City.
- Bunge A 1846 Ueber *Pedicularis comosa* L. und die mit ihr verwandten Arten. *Bull Phys Math Acad Sci St Petersburg* 1:369–384.
- dePamphilis CW, ND Young, AD Wolfe 1997 Evolution of plastid gene *rps2* in a lineage of hemiparasitic and holoparasitic plants: many losses of photosynthesis and complex patterns of rate variation. *Proc Natl Acad Sci USA* 94:7367–7372.
- Doyle JJ, JL Doyle 1990 Isolation of plant DNA from fresh tissue. *Focus* 12:13–15.
- Eriksen B, U Molau, M Svensson 1993 Reproductive strategies in two arctic *Pedicularis* species (Scrophulariaceae). *Ecography* 16: 154–166.
- Ewing B, P Green 1998 Basecalling of automated sequencer traces using phred II: error probabilities. *Genome Res* 8:186–194.
- Ewing B, L Hillier, M Wendl, P Green 1998 Basecalling of automated sequencer traces using phred I: accuracy assessment. *Genome Res* 8: 175–185.
- Felsenstein J 1978 Cases in which parsimony or compatibility methods will be positively misleading. *Syst Zool* 27:401–410.
- Gordon D, C Abajian, P Green 1998 Consed: a graphical tool for sequence finishing. *Genome Res* 8:195–202.
- Holmgren PK, NH Holmgren, LC Barnett 1990 Index herbariorum. I. The herbaria of the world. 8th ed. New York Botanical Garden, Bronx.
- Hong D 1983 The distribution of Scrophulariaceae in the Holarctic with special reference to the floristic relationships between eastern Asia and eastern North America. *Ann Mo Bot Gard* 70:701–713.
- Huelsenbeck J, R Nielsen, J Bollback 2003 Stochastic mapping of morphological characters. *Syst Biol* 52:131–158.
- Huelsenbeck JP, FR Ronquist 2001 MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17:745–755.
- Hurusawa I 1947 Genus *Pedicularis* Linnaeus. *J Jpn Bot* 21: 159–166.
- 1948 Genus *Pedicularis* Linnaeus. *J Jpn Bot* 22:11–16, 70–76, 178–185.
- 1949 Genus *Pedicularis* Linnaeus. *J Jpn Bot* 23:20–24, 106–113.
- Kaigorodova MS 1976 The ecology of flowering and pollination of three *Pedicularis* species in the upper reaches of rivers Sob and Yelets (Polar Urals). *Bot Zh* 61:726–730.
- Kwak M 1977 Pollination ecology of five hemiparasitic large-flowered Rhinanthoideae with special reference to the pollination behaviors of nectar-thieving, short-tongued bumblebees. *Acta Bot Neerl* 26:97–108.
- 1979 Effects of bumblebee visits on the seed set of *Pedicularis*, *Rhinanthus*, and *Melampyrum* (Scrophulariaceae) in the Netherlands. *Acta Bot Neerl* 28:177–195.
- Li H 1948 A revision of the genus *Pedicularis* in China. I. *Proc Acad Sci Phila* 100:205–378.
- 1949 A revision of the genus *Pedicularis* in China. II. *Proc Acad Sci Phila* 101:1–214.
- 1951 Evolution in the flowers of *Pedicularis*. *Evolution* 5: 158–164.
- Limpricht W 1924 Studien über die Gattung *Pedicularis*. *Repert Spec Nov* 20:161–265.
- Maciore LW 1968 Pollination adaptation in *Pedicularis canadensis*. *Am J Bot* 55:1031–1035.
- 1970 Pollination ecology of *Pedicularis* in Colorado. *Am J Bot* 57:716–728.
- 1973 Pollination ecology of *Pedicularis* on Mount Rainier. *Am J Bot* 60:863–871.
- 1975 The pollination ecology of *Pedicularis* (Scrophulariaceae) in the Yukon Territory. *Am J Bot* 62:1065–1072.
- 1977 Pollination ecology of *Pedicularis* (Scrophulariaceae) in Sierra Nevada of California. *Bull Torrey Bot Club* 104:148–154.
- 1982 Plant community and pollinator dynamics in the evolution of pollination mechanisms in *Pedicularis* (Scrophulariaceae). Pages 29–45 in JA Armstrong, JM Powell, AJ Richards, eds. *Pollination and evolution*. Royal Botanic Gardens, Sydney.
- 1983 The pollination dynamics of sympatric species of *Pedicularis* (Scrophulariaceae). *Am J Bot* 70:844–853.
- 1986a Floral resource sharing by bumblebees and hummingbirds in *Pedicularis* (Scrophulariaceae) pollination. *Bull Torrey Bot Club* 113:101–109.
- 1986b Pollination ecology and endemic adaptation of *Pedicularis howellii* Gray (Scrophulariaceae). *Plant Species Biol* 1: 163–172.
- 1986c Pollination ecology and endemism of *Pedicularis pulchella* Pennell (Scrophulariaceae). *Plant Species Biol* 1:173–180.
- 1988 A preliminary study of the pollination ecology of *Pedicularis* (Scrophulariaceae) in Japan. *Plant Species Biol* 3: 61–66.
- 1990 Pollination ecology of *Pedicularis punctata* Decne. (Scrophulariaceae) in the Kashmir Himalaya. *Plant Species Biol* 5: 215–223.

- 1993 Pollination ecology of *Pedicularis palustris* L. (Scrophulariaceae) in North America. *Plant Species Biol* 8:35–44.
- 1995a Pollination ecology of *Pedicularis* in the Teton Mountain region. *Plant Species Biol* 10:77–82.
- 1995b Pollination ecology of *Pedicularis parryi* ssp. *purpurea* (Parry) Carr (Scrophulariaceae). *Plant Species Biol* 10:163–168.
- 1996 Pollination ecology of *Pedicularis bracteosa* in the montane-subalpine ecotone. *Plant Species Biol* 11:165–171.
- Macior LW, KS Sarvesh 1991 Pollination ecology of *Pedicularis megalantha* D. Don (Scrophulariaceae) in the Himachal Himalaya. *Plant Species Biol* 6:75–81.
- Macior LW, Y Tang 1997 A preliminary study of the pollination ecology of *Pedicularis* in the Chinese Himalaya. *Plant Species Biol* 12:1–8.
- Maximowicz CJ 1888 Diagnoses plantarum novarum Asiaticarum. *Bull Acad Sci St Petersburg* 32:427–629.
- Meyers N 1988 Threatened biotas: “hot spots” in tropical forests. *Environmentalist* 8:187–208.
- Mosquin T, JEH Martin 1967 Observations on the pollination biology of plants on Melville Island, NWT, Canada. *Can Field-Nat* 81:201–205.
- Nielsen R 2002 Mapping mutations on phylogenies. *Syst Biol* 51:729–739.
- Nylander JAA 2002 MrModeltest, version 1.1b. Department of Systematic Zoology, Uppsala University, Sweden.
- Olmstead RG, CW dePamphilis, AD Wolfe, ND Young, WJ Elisons, PA Reeves 2001 Disintegration of the Scrophulariaceae. *Am J Bot* 88:348–361.
- Pennell FW 1935 The Scrophulariaceae of eastern temperate North America. Monographs 1. Academy of Natural Sciences, Philadelphia.
- Prain D 1890 The species of *Pedicularis* of the Indian Empire and its frontiers. Bengal Secretariat, Calcutta.
- Schulz TR, GA Churchill 1999 The role of subjectivity in reconstructing ancestral states: a Bayesian approach to unknown rates, states, and transformation asymmetries. *Syst Biol* 48:6651–6664.
- Sprague E 1962 Pollination and evolution in *Pedicularis* (Scrophulariaceae). *Aliso* 5:181–209.
- Stephenson AG, RI Bertin 1983 Male competition, female choice, and sexual selection in plants. Pages 110–149 in L Real, ed. *Pollination biology*. Academic Press, Orlando, FL.
- Steven C 1823 Monographia *Pedicularis*. *Mem Soc Nat Moscou* 6:1–60.
- Suzuki Y, GV Glazko, M Nei 2002 Overcredibility of molecular phylogenies obtained by Bayesian phylogenetics. *Proc Natl Acad Sci USA* 99:16138–16143.
- Thompson JD, TJ Gibson, PFF Jeanmougin, DG Higgins 1997 The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 24:4876–4882.
- Thompson JD, DG Higgins, TJ Gibson 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680.
- Tsoong P 1955 A new system for the genus *Pedicularis*. *Acta Phytotaxon Sin* 4:103–147.
- 1956 A new system for the genus *Pedicularis*. *Acta Phytotaxon Sin* 5:41–73, 239–278.
- 1963 *Pedicularis*. *Flora Reipublicae Popularis Sinicae* 68:1–378.
- von Wettstein R 1897 Scrophulariaceae. Pages 293–299 in A Engler, K Prantl, eds. *Die natürlichen Pflanzenfamilien*. Engelmann, Leipzig.
- White TJ, T Bruns, S Lee, J Taylor 1990 Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315–322 in M Innis, D Gelfand, J Sninsky, TJ White, eds. *PCR protocols: a guide to methods and applications*. Academic Press, San Diego, CA.
- Williams JB, GO Batzli 1982 Pollination and dispersion of five species of Lousewort (*Pedicularis*) near Atkasook, Alaska, U.S.A. *Arct Alp Res* 14:59–74.
- Williams PH 1996 Mapping variations in the strength and breadth of biogeographic transition zones using species turnover. *Proc R Soc Lond B* 263:579–588.
- Wilson EO 1992 *The diversity of life*. Belknap, Cambridge, MA.
- Yamazaki T 1988 A revision of the genus *Pedicularis* in Nepal. Pages 91–159 in H Ohba, SB Malla, eds. *The Himalayan plants*. Vol 1. University Museum, University of Tokyo, Tokyo.
- 1993 *Pedicularis* L. Pages 364–371 in K Iwatsuki, T Yamazaki, DE Boufford, H Ohba, eds. *Flora of Japan*. Vol IIIa. Angiospermae, Dicotyledoneae, Sympetalae (a). Kodansha, Tokyo.
- Yang FS, XQ Wang, DY Hong 2003 Unexpected high divergence in nrDNA ITS and extensive parallelism in floral morphology of *Pedicularis* (Orobanchaceae). *Plant Syst Evol* 240:91–105.
- Yang H, NH Holmgren, RR Mill 1998 *Pedicularis*. Pages 97–209 in CY Wu, PH Raven, eds. *Flora of China*. Vol 18. Missouri Botanical Garden, St. Louis, MO.
- Young ND, KE Steiner, CW dePamphilis 1999 The evolution of parasitism in Scrophulariaceae/Orobanchaceae: plastid gene sequences refute an evolutionary transition series. *Ann Mo Bot Gard* 86:876–893.