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REGULAR PAPER

Phylogeny of Pyroleae (Ericaceae): implications for character evolution

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Abstract Pyroleae (Ericaceae) consist of four genera, all of which are distributed widely in temperate coniferous or sometimes deciduous forests of the Northern Hemisphere. To investigate the phylogenetic relationships among these genera and to explore the evolution of the characteristics of the subfamily, we conducted maximum parsimony and Bayesian analyses with nrDNA ITS and three cpDNA intergenic spacers (*atp*B-*rbc*L, *trn*S-*trn*G and *trn*L-*trn*F). The results from cpDNA and combined cpDNA + ITS data sets strongly support the monophyly of Pyroleae as well as a sister relationship between *Pyrola* and *Moneses–Chimaphila*, with *Orthilia* as the basal lineage. The sister-group relationship between *Moneses* and *Chimaphila* is supported by a set of synapomorphies, e.g., single flower, colpate pollen, five bundles in the style, straight fruiting pedicel orientation, complete capsule dehiscence, and the basic chromosome number, $x = 13$. The *Moneses–Chimaphila–Pyrola* clade is supported by at least one homologous character of pollen in tetrads. Conflicts associated with the phylogenetic position of *Orthilia* may imply a hybrid origin for it, and therefore further study is needed.

Keywords Character evolution · Ericaceae · Molecular phylogeny · Morphology · Pyroleae

Introduction

Pyroleae are a small and well-defined tribe of evergreen herbs and subshrubs in the Monotropoideae (Ericaceae), comprising *Chimaphila* (ca. 5 species), *Pyrola* (ca. 30 species) as well as the two monotypic genera *Moneses* and *Orthilia* (Haber and Cruise 1974; Takahashi 1988; Qin and Stevens 2005). They are found in patches in the understories of temperate coniferous forests in the Northern Hemisphere—most frequently in coniferous forest, but sometimes in deciduous ones. Being mixtrophic—gaining carbon nutrition via a combination of mycoheterotrophy and photosynthesis, pyroloids man have an influence on the dynamics and composition of northern temperate forest communities (Singh and Carew 1990; Landhäusser et al. 1997; Tedersoo et al. 2007).

Pyroleae have long been recognized as a natural group, in contrast to the continued controversies about their phylogenetic position relative to Ericaceae. In most previous classifications of the Ericaceae (e.g., Henderson 1919; Copeland 1941, 1947; Wood 1961; Stevens 1971; Wallace 1975; Takhtajan 1980; Thorne 1983, 1992), the pyroloids are regarded as an element within Ericaceae. However, based on a suite of easily observed and well-known characters, i.e., subherbaceous habit, barely united

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petals, and partially mycotrophic life style, they have been recognized as a separate family Pyrolaceae by Drude (1889) and Cronquist (1981). Recent morphological and molecular cladistic analyses at higher levels in the family, however, suggest that pyroloids are best recognized as members of Ericaceae or that Ericaceae are paraphyletic (Anderberg 1993; Judd and Kron 1993; Kron 1996).

Based on variation in floral and vegetative structures and in chromosome number, a surprising number of alternative hypotheses have been proposed for the relationships between these few genera. Andres (1914) provided a graphical taxonomic scheme, in which *Orthilia* is sister to the rest of the tribe. Henderson (1919) and Copeland (1947) arranged the genera in a linear sequence from *Orthilia* or *Chimaphila* through *Pyrola* to *Moneses*, mainly based on the presence or absence of nectary disks in the flowers and morphological and anatomical characters correlated with increasing mycotrophism. Knaben and Engelskjøn (1968) inferred that the pattern of chromosome number evolution within Pyroleae successively decreases from *Pyrola* ($2n = 46$) through *Orthilia* ($2n = 38$) to *Moneses* ($2n = 26$) and *Chimaphila* ($2n = 26$). On the basis of palynological evidence Warner and Chinnappa (1986) and Takahashi (1988) concluded that the characteristics of the monad pollen within *Orthilia* is a plesiomorphy and proposed an evolutionary trend from *Orthilia* through *Pyrola* and *Moneses* to *Chimaphila*, in which pollen exine sculpture changes from verrucate through reticulate to psilate. Mainly based on a series of morphological characters, two groups of *Moneses–Chimaphila* and *Orthilia–Pyrola* were identified by Křísa (1971), Haber and Cruise (1974) and Freudenstein (1990). Most recently Freudenstein (1999) also recognized the same two groups based on analyses of the nrDNA ITS sequence data. His sampling, however, was incomplete and the relationship between *Chimaphila* and *Moneses* was not well resolved.

Conflicting phylogenetic relationships among Pyroleae may reflect the fact that these taxa exhibit a considerable superficial resemblance to one another. Integration of morphological and molecular characters in phylogenetic analyses is likely to be informative. Therefore, in this study we investigate the phylogeny of the Pyroleae by widening taxon sampling and using data from the chloroplast (*atp*B-*rbc*L, *trn*S-*trn*G and *trn*L-*trn*F) and nuclear (ITS) regions. We aim to (1) develop a classification that better reflects phylogenetic relationships among the four genera; (2) reconstruct the evolutionary history of selected morphological characters in Pyroleae; (3) identify those morphological characters that are most useful in supporting phylogenetic relationships, estimated on the basis of molecular data.

Materials and methods

Accessions examined

Herbarium vouchers and GenBank accession numbers for all taxa considered in this study are listed in Table S2. Voucher specimens are deposited at KUN and MICH. Recently, a family-wide phylogenetic analysis of Ericaceae indicated that the first split in the phylogeny is between the Enkianthoideae and the rest of the family, followed by the Monotropoideae clade and Arbutoideae clade (Kron et al. 2002). In this study, in addition to our ingroup of Pyroleae, some closely related representatives from other Monotropoideae, Arbutoideae and Enkianthoideae were included. In the nrDNA ITS phylogenetic analysis, 32 accessions, representing 14 genera and 27 species, were considered, of which 15 accession were new and the remaining accessions obtained from GenBank. The nrDNA ITS and cpDNA (*atp*B-*rbc*L, *trn*L-*trn*F and *trn*S-*trn*G) regions were not sequenced correspondingly for all included taxa; molecular materials of *Arbutus*, Monotropeae and Pterosporeae were not available for us. In the combined analysis, only the species having both ITS and at least two chloroplast marks were included. *Enkianthus*, with several plesiomorphic morphological characters (e.g., anthers possessing an endothecium, inverting late in development, opening by elongate slits, and releasing pollen in monads) were used to root all trees and to identify the morphological character polarity (Judd and Kron 1993; Anderberg 1994).

Experimental methods

To eliminate fungal contamination, our genomic DNA was directly extracted from 15 mg silica-gel-dried leaves using a modified CTAB procedure of Doyle and Doyle (1987). Double-stranded DNAs of the complete ITS region (including ITS1, 5.8S and ITS2) were PCR-amplified using primers ITS4 and ITS5 (White et al. 1990). Universal primer pairs ‘Oligo 2’ and ‘Oligo 5’ (Manen et al. 1994), *trn*S (GCU) and *trn*G (UCC) (Hamilton 1999), and c/f (Taberlet et al. 1991) were used to amplify *atp*B-*rbc*L, *trn*S-*trn*G, and *trn*L-*trn*F, respectively. These PCR reactions contained 2.0 µl of 10× Taq DNA polymerase reaction buffer (TaKaRa Biotechnology Dalian Co., Ltd.), 2.5 mM/l of each dNTP (TaKaRa), 1.5 mM/l of $MgCl_2$, 1.0 µl of 5% dimethyl sulfoxide, 0.2 mM/l of each primer (Shanghai Sangon Biological Engineering Technology and Service Co., Ltd.), 1.5 units of AmpliTaq DNA polymerase (TaKaRa), 1.5 µl of unquantified genomic template DNA and sterile water to a final volume of 20 µl. The PCR parameters were as follows: initial denaturation for 3 min at 94°C, followed by 30 cycles of denaturation (94°C, 45 s), annealing (55°C, 1 min) and extension (72°C,

3 min), and a final extension for 7 min at 72°C. PCR products were isolated and purified using a Gel Extraction Mini Kit (Watson Biotechnologies, Inc.) following the manufacturer's instructions. Sequencing reactions were performed with the dideoxy chain termination method running on an ABI PRISM 3730 automated sequencer.

Sequence comparisons and phylogenetic analyses

Sequences were assembled initially using SeqMan of the DNASTAR 5.01 software package (DNASTAR, Inc., Madison, USA) and using BLAST to confirm our ITS sequences are from ericaceous plants. Sequences were aligned using Clustal X (Thompson et al. 1997) and then manually adjusted as necessary using the BioEdit sequence alignment editor (Hall 1999). In the alignment process, both sequence similarity and mechanisms of molecular evolution were taken into account (Kelchner 2000). Gaps were positioned to minimize nucleotide mismatches and total number of indels. Regions of questionable alignment were excluded from subsequent phylogenetic analyses. The reliable indel information from the nrITS alignments was incorporated into the phylogenetic analyses using the program SeqState (Müller 2005), using simple indel coding (SIC; Simmons and Ochoterena 2000). All chloroplast sequences were concatenated to make the cpDNA data set, and missing data were incorporated for those few accessions where *atp*B-*rbc*L, *trn*S-*trn*G or *trn*L-*trn*F sequence data were not available.

The cpDNA and ITS data matrices were each analyzed separately and combined using both maximum parsimony (MP) and Bayesian inference (BI). Parsimony analyses were conducted using PAUP* version 4.0b10 (Swofford 2003). All characters were treated as unordered and were equally weighted. Each analysis consists of a heuristic search with 1,000 random sequence addition replicates (saving 100 trees per replicate), stepwise addition, MULTREES, and tree-bisection-reconnection (TBR) branch swapping. Maximum parsimony bootstrap percentages were calculated from 1,000 bootstrap replicates, each comprising 100 random sequence addition replicates, saving 10 trees per replicate. Bayesian inference was conducted using the program MrBayes version 3.1.1 (Huelsenbeck and Ronquist 2001). Prior to the analysis, MrModeltest vers. 2.2 (Nylander 2004) was used to select an evolutionary model of nucleotide substitution that best fits these data, as selected by the Akaike Information Criterion (AIC) (Posada and Buckley 2004). The selected best-fit models were GTR + I, GTR + G, HKY + G and SYM + I + G for *atp*B-*rbc*L, *trn*S-*trn*G and *trn*L-*trn*F and ITS matrices, respectively. The default priors of MrBayes were used. For each analysis, four simultaneous runs were done (starting from random trees), having six heated and two cold chains with a default temperature (0.2). A Metropolis-coupled Markov chain Monte Carlo (MCMCMC) algorithm was employed for 1×10^6 generations, sampling trees every 100 generations. Analyses were run until the average standard deviation of the split frequencies approached 0.01, indicating that four runs converged on a stationary distribution. Additionally, the plot of generation versus log probability was inspected after the run to ensure that stationarity was reached. A burn-in of 15% of the resulting trees for each run was discarded to ensure summary of trees after convergence of the log-likelihood score. The remaining 17,000 trees were imported into PAUP* and condensed into a majority rule consensus tree to obtain posterior probabilities (PP) for each node. Internodes with posterior probabilities $\geq$95% were considered statistically significant. Runs were repeated twice to confirm results.

Incongruence tests

Prior to combining the ITS and cpDNA data partitions for simultaneous phylogenetic analyses, the incongruence length difference (ILD) test (Farris et al. 1995) was carried out using the partition-homogeneity test of PAUP* to examine the extent of conflict between data sets. For each test, 100 replicates were analyzed with heuristic search, each with 10 random sequence additions. If incongruence was detected, the conflicting branches were evaluated individually for relative support given parsimony bootstrap and Bayesian posterior probabilities. Eventually, the data were combined regardless of the outcome of the ILD test (see Discussion). Templeton tests were performed using PAUP* to assess the contribution of specific nodes to the conflict between trees. A "test tree," the strict consensus of the most parsimonious trees inferred from a given data set, was compared to two types of "rival trees": (1) the strict consensus of the most parsimonious trees inferred from another data set and (2) modified "test trees" with constrained nodes where topological conflict was observed ("test" and "rival" are used here in the sense of Mason-Gamer and Kellogg (1996)). For example, where a particular conflict in tree topology existed between the strict consensus trees from nrITS and the cpDNA data sets, we specifically modified the nrITS tree to reflect each conflicting relationship suggested by the cpDNA tree and then compared the nrITS strict consensus tree (test tree) to each modified tree (rival tree) for the nrITS data set.

Evaluation of major morphological transitions

In this study, we did not carry out an exhaustive search for morphological characters that might be synapomorphies within Pyroleae. Instead, we wanted to better understand the pattern of evolution of those characters traditionally considered important in the classification of the tribe.

Therefore, states for thirteen morphological characters were scored based on observations from herbarium specimens, a reading of the relevant literature (e.g., Henderson 1919; Copeland 1947; Křísa 1971; Haber and Cruise 1974; Takahashi 1988; Anderberg 1994) and our experiences working with this group. The thirteen discrete morphological characters and their respective character states are summarized in Table 1. These characters represent vegetative morphology (1) and reproductive morphology (floral structures 2–11, fruit morphology and anatomy 12–13). All characters were treated as unordered; twelve characters were binary, and one was multistate. The data matrix is presented in Table S1. Characters were polarized using outgroup analysis: plesiomorphic (scored as 0) and apomorphic (scored as 1). Nearly all characters were readily divisible into discrete states, thus avoiding arbitrary decisions relating to state delimitation. We focused on these traits not only because they have traditionally been considered important in the classification of the group, but also to hypothesize the evolution of these characters in the tribe. Parsimony ancestral reconstructions of all thirteen morphological characters were undertaken using the strict consensus tree recovered from the parsimony analysis of the combined ITS + cpDNA data sets in the program Mesquite version 2.01 (Maddison and Maddison 2005).

Results

Phylogenetic analysis of the nrITS data set

The ITS data matrix for 32 accessions contained 672 aligned positions plus 82 indels, of which 332 (44.0%) were potentially parsimony informative. MP analysis of this data resulted in 14 minimal length trees, each of 887 steps [consistency index (CI) = 0.6877; retention index (RI) = 0.8261]. The strict consensus tree resulting from the most parsimony analysis with bootstrap support (BS) and posterior probabilities (PP) is shown in Fig. 1.

In both MP and BI analyses of the ITS data set, the Pyroleae are well-supported as monophyletic (BS = 100, PP = 1.00); *Moneses* (BS = 100, PP = 1.00) and *Chimaphila* (BS = 62, PP = 0.65) are strongly supported as sister groups (BS = 94, PP = 1.00); only parsimony analysis resolve *Pyrola* as a sister group to *Orthilia* (BS = 74). Although *Pyrola* is a well-supported natural group (BS = 100, PP = 1.00), ITS sequences failed to resolve the internal relationships.

Table 1 Morphological characters and states examined in this study

1. Underground structure: root = 0, rhizome = 1
2. Inflorescence type: elongate raceme = 0, corymb = 1, single flower = 2
3. Corolla type: sympetalous = 0, choripetalous = 1
4. Anthers with well developed fibrous endothecium: present = 0, absent = 1
5. Filament vestiture: glabrous = 0, pubescent = 1
6. Pollen aperture: colporate = 0, colpate = 1
7. Pollen cohesion: monads = 0, tetrads = 1
8. Style orientation: erect = 0, curved = 1
9. Style length: long (ca. 10 mm) = 0, short = 1 (ca. <2 mm)
10. Style vasculature: 10 bundles = 0, 5 bundles = 1
11. Nectary disk: present = 0, absent = 1
12. Fruiting pedicel orientation: curved = 0, straight = 1
13. Capsule dehiscence: complete (valves joined without fibers) = 0, incomplete (valves joined by fibers) = 1

The numbers of each character and its character states correspond to those presented in Table S1

Phylogenetic analysis of the combined cpDNA data set

The results of the ILD test indicated that the three cpDNA regions were not significantly different from one another. The contribution of each region to the length of the matrix was as follows: *atp*B-*rbc*L 972 bp, *trn*S-*trn*G 869 bp and *trn*L-*trn*F 944 bp. The combined matrix of cpDNA data for the 23 accessions contained 2,785 aligned positions, of which 175 were removed from subsequent analyses because of alignment ambiguities. Of the remaining 2,610 characters, 415 (15.9%) were potentially informative. All plastid regions had similar levels of potentially informative characters, with *trn*S-*trn*G being the most informative (6.2%), followed by *trn*L-*trn*F (5.6%) and *atp*B-*rbc*L (4.1%). MP analysis of these 2,610 positions resulted in 12 minimal length trees with 670 steps (CI = 0.8910, RI = 0.9369).

The phylogenies estimated using MP and Bayesian analyses of cpDNA data are well-resolved and highly consistent with one another (Fig. 2). The monophyly of Pyroleae and its constituent genera is well supported; *Orthilia* is sister to the rest of the tribe (BS = 100, PP = 1.00); and a sister relationship between *Pyrola* and *Chimaphila–Moneses* is strongly supported (BS = 96, PP = 1.00). Contrary to ITS analyses, the cpDNA data sets produce considerably greater resolution within *Pyrola*, forming a major dichotomy.

Comparison of cpDNA and nuclear rDNA ITS phylogenies and a total evidence analysis

Overall, the ITS-derived tree is less resolved than the tree derived using three cpDNA markers. Our Templeton test using cpDNA data constrained by the ITS analysis of sister relationships between *Orthilia* and *Pyrola* indicate that the difference in the placement of *Orthilia* was statistically significant ($P < 0.05$). Our ITS data, on the other hand, do not directly reject the null hypothesis that *Moneses*,

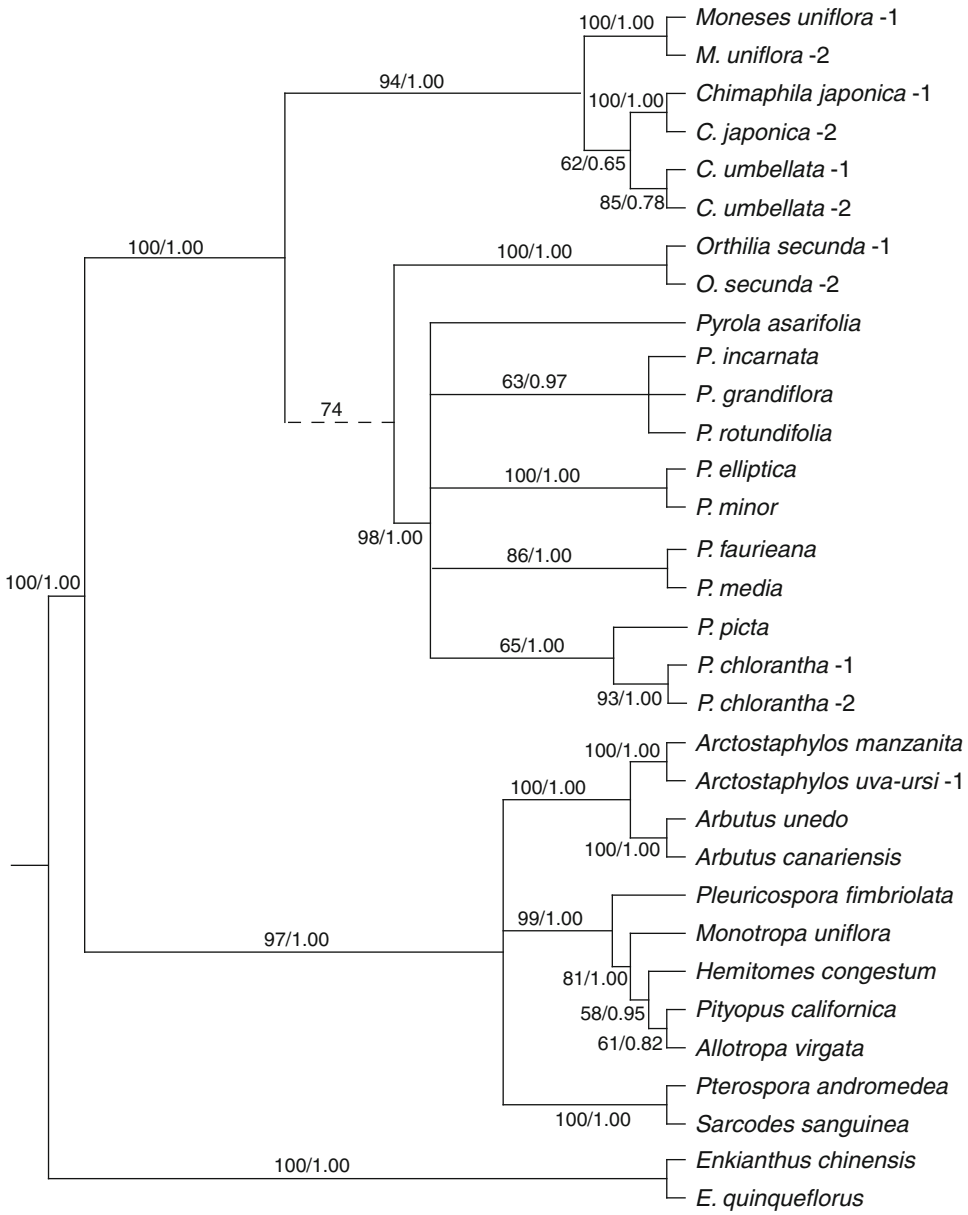


Fig. 1 Phylogenetic relationships in Pyroleae as indicated by the strict consensus tree from MP analysis of ITS sequence data. *Numbers above branches* are maximum parsimony bootstrap support followed by Bayesian posterior probabilities. Branches represented by *dashed lines* are not found in the 50% majority rule consensus tree from the Bayesian analysis of the same data set

Chimaphila and *Pyrola* form a well supported clade based on the cpDNA analysis ($P > 0.05$). ILD test found significant incongruence between the nrITS and cpDNA data partitions (ITS v cpDNA, $P = 0.01$). Variable evolutionary rates among data sets can be problematic when combining data, but Bayesian analyses with case appropriate evolutionary models fitted to individual partition of data can help alleviate many of these problems (Nylander et al. 2004). A compromise is not needed to decide whether to combine data based on different models of evolution among partitions (Bull et al. 1993; Chippindale and Wiens 1994). Thus, we proceeded with a combined ITS and chloroplast data set.

For the parsimony analysis, the total number of characters of the coalesced nrITS and cpDNA data was 3,270 aligned positions plus 52 indels from ITS data, 599 (18.0%) of which were informative. Twenty-two terminals were included, 19 of which were ingroup taxa. MP analysis of the combined data produced two trees (Fig. 3) of 1,127 steps (CI = 0.8687, RI = 0.9067). The topologies of the cpDNA tree (Fig. 2) and combined tree (Fig. 3) were highly congruent, but the support for the *Moneses–Chimaphila–Pyrola* clade was lower in the MP combined analysis (BS = 79).

Synapomorphic morphological characters in Pyroleae

The molecular phylogeny presented here provides a framework for evaluating character transformation within the Pyroleae, thus allowing us to identify traits that have

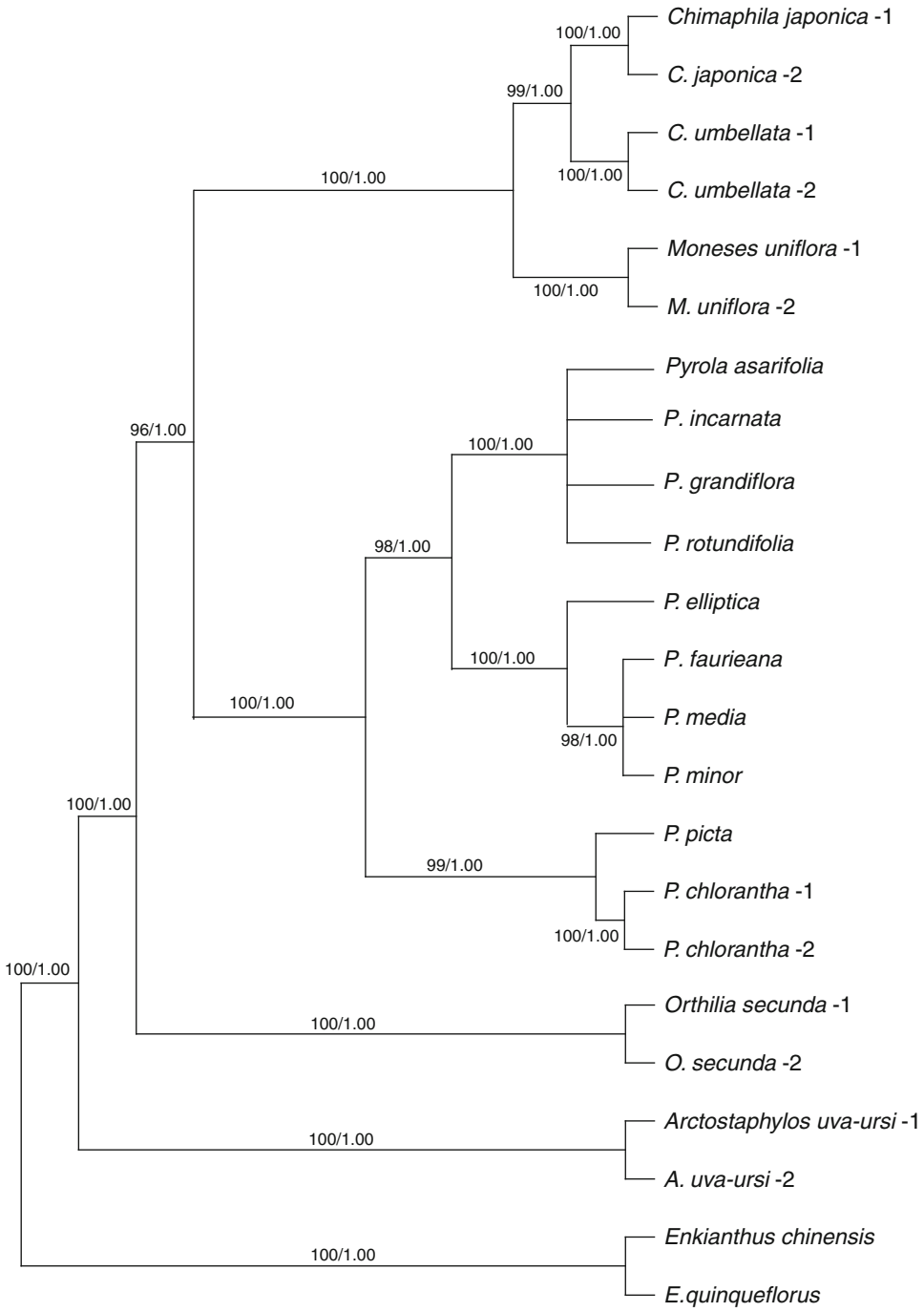


Fig. 2 Phylogenetic relationships in Pyroleae as indicated by the strict consensus tree from MP analysis of cpDNA (*atp*B-*rbc*L, *trn*S-*trn*G, and *trn*L-*trn*F) data. *Numbers above branches* are maximum parsimony bootstrap support followed by Bayesian posterior probabilities

potential predictive phylogenetic value. The morphological characters which support the monophyly of the groups inferred by phylogenetic analyses of molecular data are summarized in Fig. 4. A subshrub or herbaceous habit (1:1; character: state, respectively) and choripetalous flowers (3:1) are synapomorphic conditions for Pyroleae. The *Moneses–Chimaphila* clade is supported by five characters: single flowers (2:2), colpate pollen (6:1), five bundles in the style (10:1), straight fruiting pedicel orientation (12:1), and complete capsule dehiscence (13:0). The *Moneses–Chimaphila–Pyrola* clade is united by one synapomorphy of tetrad pollen (7:1). Other characters previously thought to be synapomorphic for the *Pyrola* and *Orthilia* clade, e.g., ten bundles in the style (10:0) and curved fruiting pedicel (12:0) are plesiomorphic. Characters of anthers with well developed fibrous endothecium (character 4),

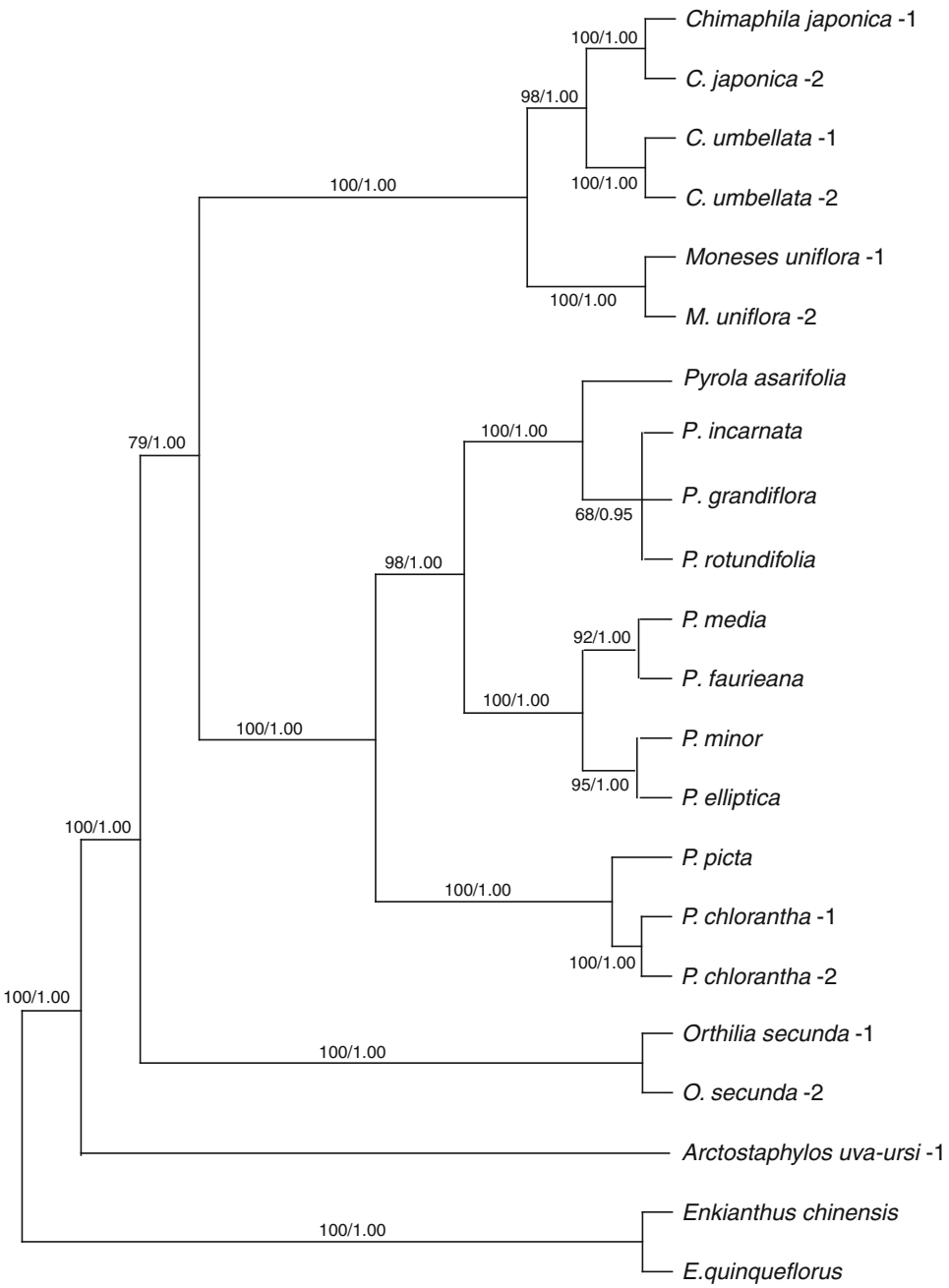


Fig. 3 Phylogenetic relationships in Pyroleae as indicated by the strict consensus tree from MP analysis of the combined ITS and cpDNA (*atp*B-*rbc*L, *trn*S-*trn*G, and *trn*L-*trn*F) data. *Numbers above branches* are maximum parsimony bootstrap support followed by Bayesian posterior probabilities

nectary disk (character 11) and type of capsule dehiscence (character 13) are ambiguously distributed between the genera of Pyroleae.

Discussion

Monophyly of Pyroleae

The Monotropoideae comprise the three tribes Pyroleae, Pterosporeae and Monotropeae, the two latter of which lack chlorophyll and represent one end of a continuum from autotrophism to mycotrophic parasitism in the subfamily (Björkman, 1960; Kron et al. 2002). The present ITS analyses indicate that in the early evolution of the Monotropoideae two evolutionary lineages emerged; one of them evolved into the Pyroleae, of which four genera are the only known representatives today; the other evolved into the rest of the tribe plus some members of Arbutoideae (e.g., *Arbutus*, *Arctostaphylos*). Although the data strongly indicate that Arbutoideae are derived from within Monotropoideae, the exact relationships in respect to Monotropeae and

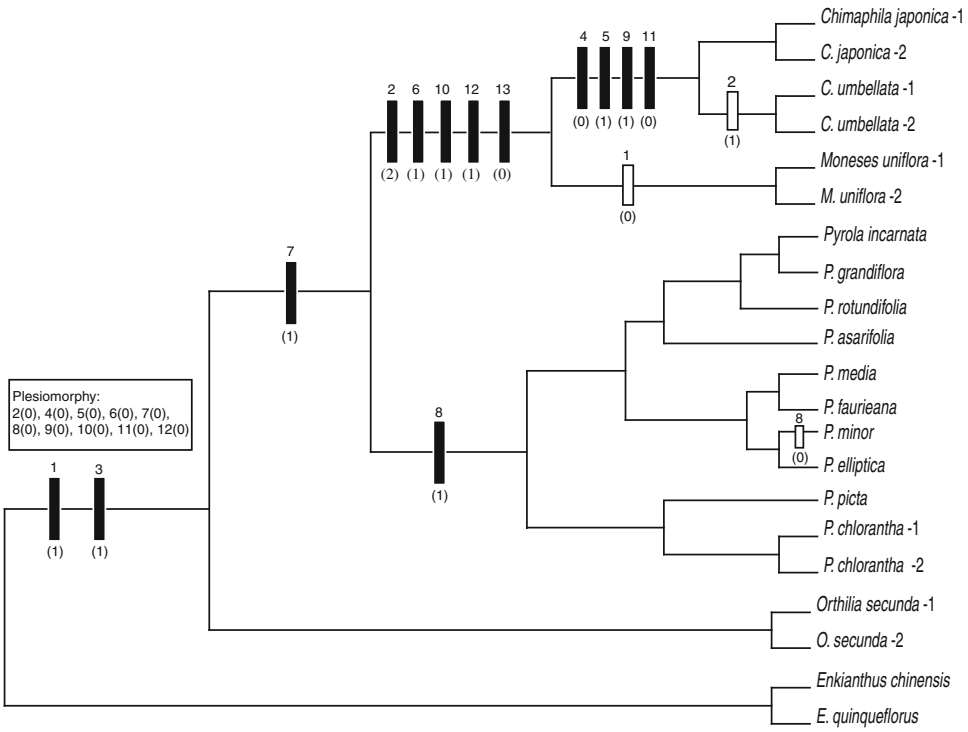


Fig. 4 Examined synapomorphic characters of Pyroleae and outgroups mapped on to a tree inferred from the strict consensus tree derived from combined ITS and cpDNA data sets. *Numbers* correspond to those characters listed in Table 1, with states in *parentheses*

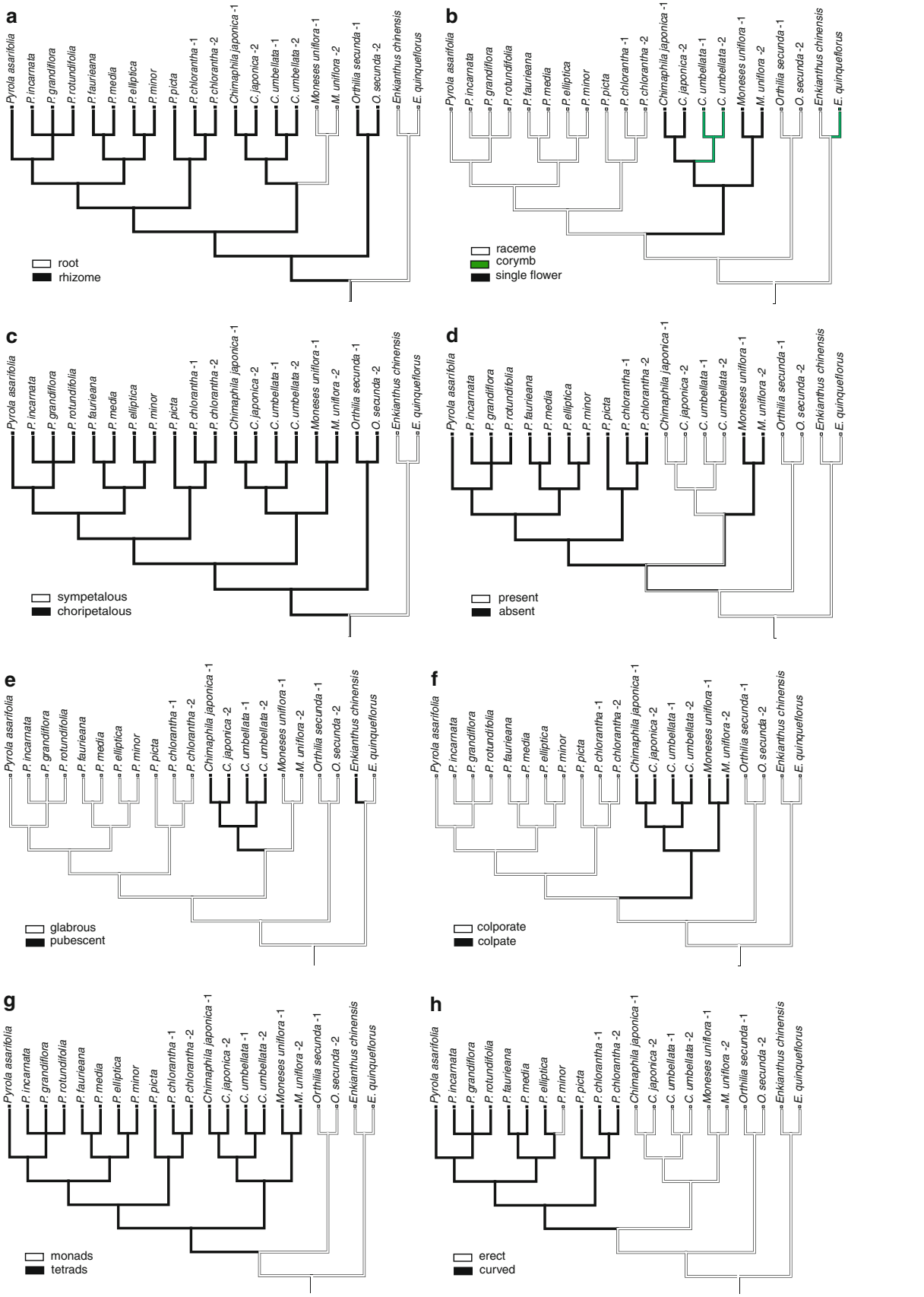
Pterosporeae are not completely clear (Fig. 1) and further studies are needed. Based on current plastid and combined analyses, Pyroleae are monophyletic (Figs. 1, 2, 3), which is supported by two synapomorphic characters traditionally used to diagnose the family Pyrolaceae: a subshrub or herbaceous habit (1:1) and choripetalous flowers (3:1) (Fig. 4).

Relationships among the genera of Pyroleae

The analyses presented here continue to support the close relationship between *Moneses* and *Chimaphila* (Figs. 1, 2, 3), and is consistent with the judgments from most previous authors (e.g., Copeland 1947; Křísa 1971; Haber and Cruise 1974; Freudenstein 1990). According to our ancestral character reconstruction, there are at least four distinctive synapomorphies for this clade: three-colpate pollen (6:1), five bundles in the style vasculature (10:1), straight fruiting pedicel orientation (12:1), and capsule dehiscence without fibers (13:0) (Fig. 4). Although not coded here, this clade may also be united by the same chromosome number ($2n = 26$). The single flowered inflorescence type (2:2) may be another synapomorphy for the *Moneses–Chimaphila* clade, which further evolved into a corymb type in *Chimaphila*, except in *C. japonica*. *Chimaphila* is strongly supported as monophyletic by chloroplast and combined analyses (BS = 100, PP = 1.00), whereas the clade receives only weak support in ITS analyses (BS = 62, PP = 0.65). Notably, when indels were not coded as additional characters for the phylogenetic analysis, the placement of *Moneses* within *Chimaphila* makes the genus *Chimaphila* paraphyletic (tree not shown), a result also found by Freudenstein (1999). Morphological synapomorphies of *Chimaphila* include anthers with a well developed fibrous endothecium (4:0), a pubescent filament vestiture (5:1), a short, peltate style (9:1) and a nectary disk (11:0). A comprehensive phylogenetic study based on morphological and molecular evidence is in progress.

Pyrola is the most species-rich genus in the tribe and accounts for approximately 80% of the species diversity (ca. 30 species). The status of *Pyrola* as a separate genus has never been in question, and our current molecular data strongly support its monophyly (BS = 100, PP = 1.00; Figs. 1, 2, 3). Curved style orientation (8:1; Fig. 4) and chromosome number of $2n = 46$ are synapomorphic for

Fig. 5 Overlay of selected morphological characters on the strict consensus tree from MP analysis of the combined ITS and cpDNA (*atp*B-*rbc*L, *trn*S-*trn*G, and *trn*L-*trn*F) data. Morphological transition was inferred using unordered parsimony in the program Mesquite version 2.01 (Maddison and Maddison 2005). **a** Underground structure, **b** inflorescence type, **c** corolla type, **d** anthers with well developed fibrous endothecium, **e** filament vestiture, **f** pollen aperture, **g** pollen cohesion, **h** style orientation, **i** style length, **j** style vasculature, **k** nectary disk, **l** fruiting pedicel orientation, **m** capsule dehiscence. Species examined on the phylogenetic tree are *Pyrola asarifolia*, *P. incarnata*, *P. grandiflora*, *P. rotundifolia*, *P. faurieana*, *P. media*, *P. elliptica*, *P. minor*, *P. picta*, *P. chlorantha*, *Chimaphila japonica*, *C. umbellata*, *Moneses uniflora*, *Orthilia secunda*, *Enkianthus chinensis* and *E. quinqueflorus* in an order from *left* to *right*


a
Pyrola asarifolia
P. incarnata
P. grandiflora
P. rotundifolia
P. faurieana
P. media
P. elliptica
P. minor
P. picta
P. chlorantha -1
P. chlorantha -2
Chimaphila japonica -1
C. japonica -2
C. umbellata -1
C. umbellata -2
Moneses uniflora -1
M. uniflora -2
Orthilia secunda -1
O. secunda -2
Enkianthus chinensis
E. quinqueflorus
root
rhizome
b
raceme
corymb
single flower
c
sympetalous
choripetalous
d
present
absent
e
glabrous
pubescent
f
colporate
colpate
g
monads
tetrads
h
erect
curved

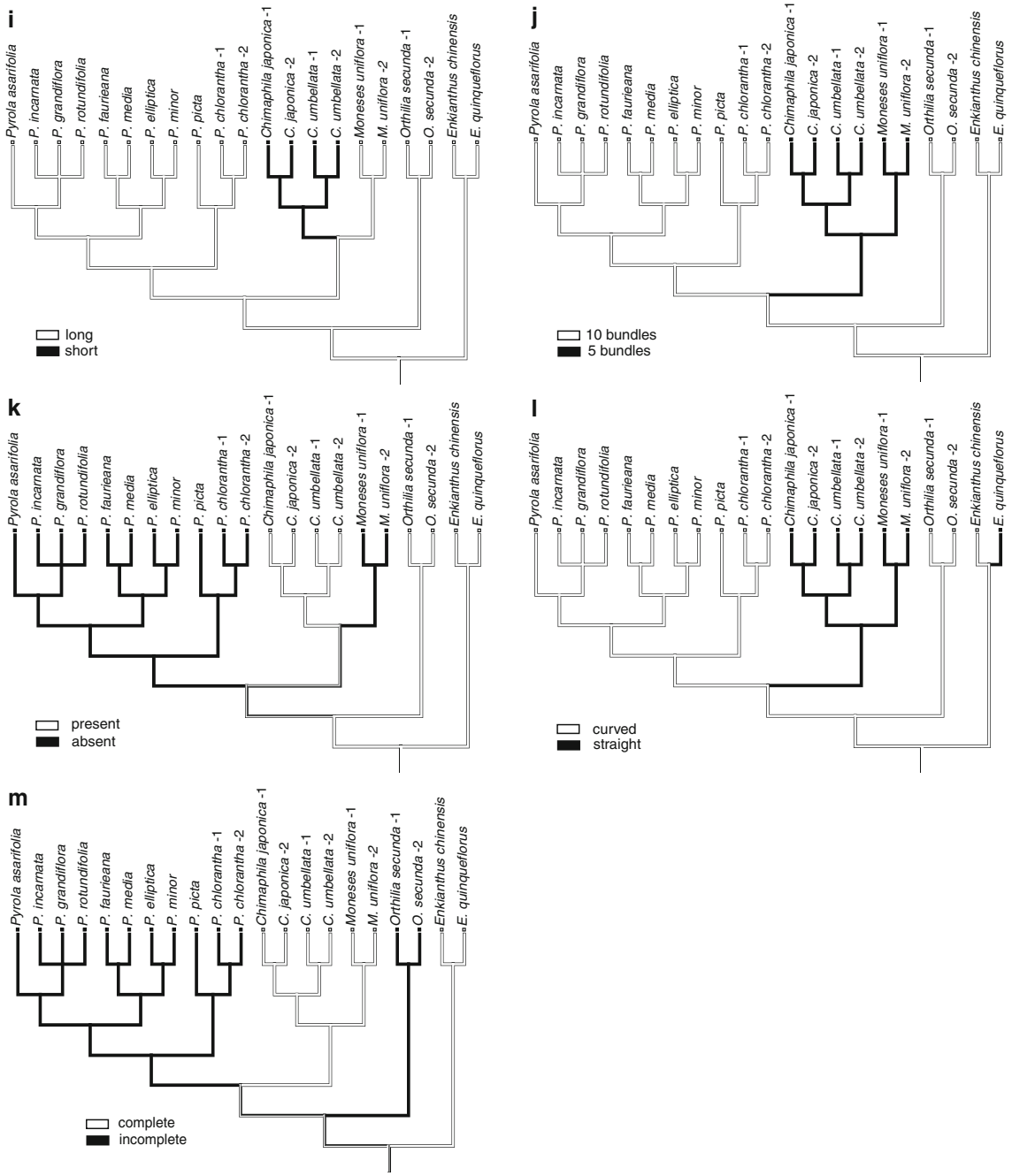


Fig. 5 continued

this genus. The classification and delimitation of species within *Pyrola*, however, has always been problematic due to subtle variation in such features as leaf shape, size and form of scape bracts, and various floral modifications including sepal shape, anther form and color, and flower color (Haber and Cruise 1974). To date, on the basis of morphological and anatomical features, various infrageneric taxonomic treatments have been proposed (Andres 1914; Copeland 1947; Křísa 1971; Haber and Cruise 1974). A worldwide phylogenetic study using nrDNA and chloroplast sequences is in progress. Initially, *Orthilia secunda* was described under *Pyrola* as *P. secunda*. Studies from some authors (e.g., House 1921; Jensen 1961; Nowicke 1966), however, indicated that *Orthilia* should be regarded as an independent genus.

The phylogenetic position of *Pyrola* and *Orthilia* has long been controversial. Cladistic studies by Křísa (1971) and Freudenstein (1990) reported that *Orthilia* and *Pyrola*

are sister-groups, sharing such features as incomplete capsule dehiscence, downward fruiting pedicel orientation and style vasculature with ten bundles. In spite of the similarities between *Pyrola* and *Orthilia*, they can be easily distinguished from one another by some characters. *Orthilia* is characterized by a one-sided raceme, chromosome number of $2n = 38$ and monad pollen. In this study, MP analysis of ITS sequences recovers a moderately supported sister group relationship between *Pyrola* and *Orthilia* (BS = 74) (Fig. 1). In contrast, chloroplast and combined analyses strongly suggest that *Orthilia* is sister to a clade with all other Pyroleae and that the genus *Pyrola* is sister to the clade of *Chimaphila* and *Moneses* (Figs. 2, 3), which is united by a synapomorphy of tetrad pollen (7:1) (Fig. 4). Templeton tests indicate that conflicts associated with the relationship between *Orthilia* and *Pyrola* were rejected by cpDNA tree using nrITS constraint, but were not rejected by the nrITS tree using cpDNA constrain. This means that the observed conflict is a nonstochastic process and hybridization and/or lineage sorting events may have played a role in the early evolution of *Orthilia*. Böcher (1961) pointed out that *O. secunda* is of hybrid origin between species with $n = 23$ and $n = 13$, with the number $2n = 38$ arising as a result of non-separation of a tetravalent from $2n = 36$. A more comprehensive understanding of *Orthilia* will require other approaches, such as the use of low copy nuclear genes.

Evolution of selected characters

Our uncertainty on how to place an individual genus in the correct phylogenetic position within Pyroleae may be due to a mosaic pattern of variation within these genera, with each possessing ancestral as well as derived features. The well-resolved phylogeny obtained in this study provides an opportunity for an analysis of character state transformations and when and how often such changes have occurred. We tentatively optimized thirteen selected morphological characters on to the strict consensus tree from the combined data sets (Fig. 5a–m).

Underground structure (character 1)

In *Moneses*, there is a root system similar in appearance to the rhizome of other Pyroleae but lacking scales. The character reconstruction indicates that the rhizome is a synapomorphy for Pyroleae, with the state reversing to root in the ancestor of *Moneses* (Fig. 5a).

Inflorescence (character 2) and corolla type (character 3)

Features of the inflorescence are generally considered important for the intergeneric classification of Pyroleae. A raceme identifies *Pyrola* and *Orthilia* (the inflorescence of *Orthilia* is characterized by being secund), a corymb or umbel occurs in *Chimaphila*, while the inflorescence is further reduced to a single flower in *Moneses*. Optimization of this character onto the phylogenies indicates that a raceme is plesiomorphic in the tribe, while both corymbose and solitary flowers are derived from racemose ancestors (Fig. 5b). Besides Monotropoideae, choripetalous petals occur independently in Bejarieae, Empetreae and members of the Phyllodoceae (e.g., *Elliottia bracteata*, *Kalmia buxifolia*) within Ericaceae (Kron et al. 2002). Our present study indicates that this feature is synapomorphic for Pyroleae and reversed from a sympetalous ancestral group (Fig. 5c).

Anthers with well developed fibrous endothecium (character 4) and filament vestiture (character 5)

The character of anthers with well developed fibrous endothecium is clearly plesiomorphic in *Orthilia*, but its evolutionary history within the remaining genera is ambiguous (Fig. 5d). Either this character state has evolved once in the common ancestor of *Pyrola*, *Chimaphila*, and *Moneses* followed by a reversal in *Chimaphila* or it evolved separately in *Pyrola* and *Moneses*. Pubescent filament vestiture is a derived condition, and occurs in only *Chimaphila* (Fig. 5e).

Pollen aperture (character 6) and pollen cohesion (character 7)

Orthilia is characterized by monad pollen (i.e., pollen grains are separated from each other), unlike the tetrad pollen found in *Pyrola* and *Moneses* and polyad pollen (i.e., adjacent tetrads are loosely connected to each other) found in *Chimaphila* (Erdtman 1952; Nowicke 1966; Takahashi 1988). Warner and Chinnappa (1986) and Freudenstein (1999) suggested that the monad pollen of *Orthilia* is apomorphic rather than plesiomorphic and the monads result from the breakup of tetrads. Our results, however, indicate that the monad pollen type in *Orthilia* is a plesiomorphic character shared with the outgroup *Enkianthus* and that tetrad pollen found in the remaining genera is derived (character 7, Fig. 5g). Additionally, the colporate pollen aperture is plesiomorphic in *Orthilia* and *Pyrola*, and the colpate pollen aperture is a synapomorphy for *Moneses* and *Chimaphila* (Fig. 5f).

Style orientation (character 8), style length (character 9) and style vasculature (character 10)

A very short style (ca. <2 mm) is characteristic of genus *Chimaphila*, in contrast to the rest of the tribe where the style is relatively long (ca. 10 mm). Our results indicate

that this characteristic feature of *Chimaphila* is a synapomorphy derived from a long style (Fig. 5i). Another important feature is style orientation. Our data indicate that an erect style is maintained in *Orthilia*, *Moneses* and *Chimaphila*, while the style shifts to be curved in *Pyrola* as an apomorphy (Fig. 5h). Haber and Cruise (1974) considered that five bundles in the style vasculature in *Chimaphila* and *Moneses* represent a more primitive state than the ten bundles found in *Pyrola* and *Orthilia*, where vasculature might be considered to be derived. Our results, however, do not support this view, but instead indicate that ten bundles is plesiomorphic in Pyroleae and the five bundles in the *Moneses–Chimaphila* clade are derived (Fig. 5j).

Nectary disk (character 11)

Knudsen and Olesen (1993) reported that *Chimaphila* produces nectar and is visited by nectar-gathering insects; neither *Moneses* nor *Pyrola* produce nectar, but instead are buzz-pollinated; *Orthilia*, which does produce nectar, is visited by both nectar-gathering and buzz-pollinating insects. They suggested that nectar secretion is an ancient attractant and that buzz-pollination is an advanced system. Freudenstein (1999) further inferred that buzz-pollination in *Moneses*, *Orthilia* and *Pyrola* has arisen separately based on a bifurcated phylogeny of Pyroleae (*Chimaphila–Moneses*, *Orthilia–Pyrola*). In this study, we again corroborate the plesiomorphic state of the presence of a nectary disk in the Pyroleae (Fig. 5k). Unfortunately, reconstructing the pattern of evolution of the pollination system cannot be undertaken until there has been a parallel study of the outgroup *Enkianthus*.

Fruiting pedicel orientation (character 12) and capsule dehiscence (character 13)

Within Pyroleae, the curved fruiting pedicel and incomplete capsule dehiscence (valves joined by fibers) characterize *Pyrola* and *Orthilia* and indicate a close affinity between them (Haber and Cruise 1974; Freudenstein 1990). Copeland (1947) suggested that the complete capsule dehiscence (valves joined without fibers) in *Moneses* and *Chimaphila* is a derived character, in contrast to the proposal proposed by Freudenstein (1990) that the incomplete capsule dehiscence of *Pyrola* and *Orthilia* is derived. Our results indicate that the curved fruiting pedicel is plesiomorphic in Pyroleae, and the erect type is a synapomorphy for *Moneses–Chimaphila* derived from the curved type (Fig. 5l). Reconstruction of the character capsule dehiscence under parsimony optimization is ambiguous regarding the origin of this feature (Fig. 5m). One scenario is that the incomplete capsule dehiscence is a synapomorphy for the *Moneses–Chimaphila–Pyrola* clade and reverses to complete dehiscence for the ancestor of *Moneses–Chimaphila*. The other scenario is that complete capsule dehiscence is a plesiomorphy retained in *Moneses* and *Chimaphila* and that incomplete capsule dehiscence appeared independently in *Pyrola* and *Orthilia*.

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