A molecular phylogeny and a new classification of *Pyrola* (Pyroleae, Ericaceae)

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Abstract The northern temperate genus *Pyrola* L. is the largest and arguably the most taxonomically complex element in the tribe Pyroleae (Ericaceae). Here we present a molecular phylogenetic study with extensive sampling comprising 26 ingroup and 7 closely related taxa. The results, based on parsimony and Bayesian analyses of nuclear (ITS) and chloroplast (*atpB-rbcL*, *trnS-trnG*, *trnL-trnF*) DNA sequences, add substantially to our understanding of relationships within this diverse group and call for taxonomic changes. *Pyrola* is confirmed as a monophyletic group with two redefined sections and six series: *P.* sect. *Pyrola* (*P.* ser. *Pyrola*, ser. *Ellipticae* and ser. *Rugosae*) and *P.* sect. *Scotophylla* (*P.* ser. *Japonicae*, ser. *Scotophyllae* and ser. *Chloranthae*). Members of each respective section and series share similar morphological traits and/or geographical distributions. For the potential hybrids *P. media* and *P. faurieana*, the maternal donor was identified by their close affinity to *P. minor* in the chloroplast trees whereas the paternal donor remained unclear.

Keywords Ericaceae; hybridization; molecular systematics; morphology; *Pyrola*; Pyroleae; taxonomic treatment

■ INTRODUCTION

Pyroleae are a small and well-defined tribe of evergreen herbs and subshrubs in the Monotropoideae (Ericaceae), comprising *Chimaphila* Pursh (ca. 5 species), *Pyrola* L. (ca. 30 species) as well as the two monotypic genera *Moneses* Salisb. ex Gray and *Orthilia* Raf. (Haber & Cruise, 1974; Takahashi, 1988; Qin & Stevens 2005). They are found in patches in the understories of temperate coniferous or sometimes deciduous forests in the Northern Hemisphere. Being mixotrophic, i.e., gaining carbon nutrition via a combination of mycoheterotrophy and photosynthesis, Pyroleae may have an influence on the dynamics and composition of northern temperate forest communities (Tedersoo & al., 2007).

Pyrola has long been recognized as a natural group, in contrast to the continued controversies concerning its phylogenetic position within Pyroleae (e.g., Andres, 1914; Copeland, 1947; Křísa, 1971; Takahashi, 1988; Freudenstein, 1999). In our recent phylogenetic study, *Pyrola* was hypothesized to be sister to the *Chimaphila-Moneses* clade, with these three genera in turn being sister to *Orthilia* within Pyroleae (Liu & al., unpub.), but the infrageneric classification of *Pyrola* continues to be a matter of great debate and confusion. The major infrageneric classifications proposed for *Pyrola* are summarized in Table 1. Andres (1914) outlined the first infrageneric taxonomic system of *Pyrola* and recognized two subgenera: the monotypic *P.* subg. *Amelia* (Alef.) Andres (*P. minor* L.) and *P.* subg. *Thelaia* (Alef.) Andres. *Pyrola* subg. *Thelaia* was subdivided into two sections: *P.* sect. *Ampliosepala* Andres with short triangular sepals and *P.* sect. *Euthelaia* Andres with elongate sepals. Copeland (1947) simplified the system of Andres to three sections: *P.* sect. *Amelia* (Alef.) Benth. & Hook. f., *Scotophila* (Nutt.) Copeland and *Thelaia* (Alef.) Benth. & Hook. f. Křísa (1971) recognized two subgenera: *P.* subg. *Amelia* and subg. *Pyrola*, which were the same as those of Andres. *Pyrola* subg. *Pyrola* was further divided into three sections: *P.* sect. *Pyrola*, *Chlorantha* Křísa, and *Scotophylla* (Nutt.) Křísa, with six newly described series in section *Pyrola* and two in section *Chlorantha*. Generally, *P.* sect. *Pyrola* Křísa corresponds to *P.* sect. *Euthelaia* Andres, and sections *Chlorantha* Křísa

Table 1. A synopsis of the previous classifications of *Pyrola* by Andres (1914), Copeland (1947) and Křísa (1971). Number of species for each rank in parentheses.

Andres (1914)	Copeland (1947)	Křísa (1971)
Subg. *Amelia* (1)	Sect. *Amelia* (2)	Subg. *Amelia* (1)
Subg. *Thelaia*	Sect. *Scotophila* (5)	Subg. *Pyrola*
Sect. *Ampliosepala*	Sect. *Thelaia* (4)	Sect. *Pyrola*
Subsect. *Elliptica* (2)		Ser. *Pyrola* (2)
Subsect. *Obscura* (6)		Ser. *Incarnatae* (4)
Subsect. *Scotophylla* (2)		Ser. *Japonicae* (6)
Subsect. *Rotundoides* (2)		Ser. *Sororiae* (3)
Sect. *Euthelaia*		Ser. *Amoenae* (2)
Subsect. *Erxlebenia* (7)		Ser. *Asarifoliae* (4)
Subsect. *Alefeldiana*		Sect. *Chlorantha*
§1 *Genuina* (7)		Ser. *Chloranthae* (2)
§2 *Amoena* (2)		Ser. *Ellipticae* (3)
§3 *Pictoides* (3)		Sect. *Scotophylla* (3)

and *Scotophylla* Křísa correspond to *P.* sect. *Ampliosepala* Andres. Although Křísa's system was the most comprehensive and widely followed, the naturalness of the grouping has been shown to be questionable by various evidence. For instance, *P.* ser. *Japonicae* Křísa should be separated from *P.* sect. *Pyrola* due to its longer and wider seeds with longer endosperms (Takahashi, 1987b); *P. faurieana* Andres must be segregated from *P.* sect. *Chlorantha* and associated with *P. media* Sw. based on their larger pollen tetrads with verrucate sculpture (Takahashi, 1986, 1993); and *P. minor*, which is characterized by having small pollen tetrads with verrucate sculpture, must best be placed near *P.* sect. *Pyrola* (Takahashi, 1986). With its pitted inner periclinal walls, seed morphology still supported *P. minor* as forming a subgenus on its own (Takahashi, 1993). With the obvious contradictions among these taxonomic classifications of *Pyrola*, there is an evident and urgent need for a revision of the whole genus, including information from molecular as well as morphological characters.

As a first attempt to address infrageneric relationships within *Pyrola* in a phylogenetic context, Freudenstein (1999) examined the nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) sequences of eight species from North America. Although his results provided valuable insight into the classification and evolution of *Pyrola*, his restricted taxon sampling, especially the lack of European and eastern Asian species and his use of a single molecular marker, were insufficient to achieve a robust molecular phylogeny for the genus. In the present study we expand sampling to maximize coverage of geographical diversity within *Pyrola*, and used DNA sequence data from the ITS region and three chloroplast DNA (cpDNA) regions (*atpB-rbcL*, *trnS-trnG*, *trnL-trnF*). We aim to (1) re-evaluate phylogenetic relationships within *Pyrola* and test previous hypotheses of infrageneric relationships proposed by Křísa (1971) and others; (2) identify morphological synapomorphies that characterize the various clades detected by the molecular analyses; and (3) construct a new phylogenetic classification of the genus.

■ MATERIALS AND METHODS

Plant material and taxon sampling. — Plants sampled for this study comprise 31 accessions of *Pyrola* representing 26 species. Four species (*Orthilia secunda* (L.) House, *Moneses uniflora* A. Gray, *Chimaphila japonica* Miq., *C. umbellata* (L.) W.P.C. Barton) from the remaining genera of Pyroleae were also included. *Enkianthus* (*E. chinensis* Franch. and *E. quinqueflorus* Lour.) and *Actinidia chinensis* Planch. were selected as outgroup in accordance with recent results inferred from different DNA regions by Freudenstein (1999) and Kron & al. (2002). When selecting taxa for inclusion in our analyses their geographic occurrence was also considered. In most cases, at least two populations per species were analyzed. Taxon names and vouchers for all sequences are listed in the Appendix. Voucher specimens are deposited at KUN, MICH and HAST.

Experimental strategy. — Genomic DNA was extracted from 15 mg silica-gel–dried leaves using a modified CTAB procedure of Doyle & 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 & al., 1990). Universal primer pairs "Oligo 2" and "Oligo 5" (Manen & al., 1994), trnS (GCU) and trnG (UCC) (Hamilton, 1999), and c/f (Taberlet & al., 1991) were used to amplify *atpB-rbcL*, *trnS-trnG*, and *trnL-trnF*, 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 aligned using Clustal X (Thompson & al., 1997) and adjusted manually by Bioedit v.7.0.5.2 (Hall, 1999). In the alignment process, both sequence similarity and mechanisms of molecular evolution were considered (Kelchner, 2000). All chloroplast sequences were concatenated to make the cpDNA dataset. Although several attempts were made, we were not able to amplify some taxa for plastid sequence data and *A. chinensis* for ITS (Appendix). Therefore, these taxa were scored as missing data ("?") for these regions and included in the combined cpDNA and ITS/cpDNA matrices. Sequences acquired in this study are deposited in GenBank (Appendix).

The cpDNA and ITS data matrices were each analyzed separately and combined using both maximum parsimony (MP) and Bayesian inference (BI). Parsimony analysis was performed using PAUP* v.4.0b10 (Swofford, 2003). All characters were treated as unordered and were equally weighted. Each analysis consisted of a heuristic search with 1000 random sequence addition replicates (saving 100 trees per replicate), stepwise addition, MULTREES, and tree-bisection-reconnection (TBR) branch swapping. Bootstrap values were calculated from 1000 bootstrap replicates, each comprising 100 random sequence addition replicates, saving ten trees per replicate. The Bayesian analysis was conducted using the program MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003). Prior to analysis, MrModeltest v.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 & Buckley, 2004). The best-fit models selected were GTR+G and SYM+G for the cpDNA 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

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 vs. 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 ≥95% were considered statistically significant. Runs were repeated twice to confirm results.

Incongruence tests. — To examine the extent of conflict between the cpDNA and ITS datasets for a comparable set of taxa, the incongruence length difference (ILD) test (Farris & al., 1994, 1995) was implemented using the partition homogeneity test of PAUP*. This test was carried out with 1000 replicated analyses, using the heuristic search option with simple addition of taxa and tree-bisection-reconnection (TBR) branch swapping. Incongruence also was determined visually for trees with incongruent topologies between different datasets (nrDNA vs. cpDNA). When 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.

■ RESULTS

Chloroplast DNA sequence comparisons and phylogenetic analyses. — The results of the ILD test indicate that these three cpDNA regions are not significantly different from one another. The contribution of each region to the length of the matrix was as follows: *atpB-rbcL* 1015 bp, *trnS-trnG* 898 bp and *trnL-trnF* 998 bp. The combined matrix of cpDNA data for 42 accessions contained 2925 aligned positions, of which 201 characters (383–396 for *atpB-rbcL*; 696–851, 625–628, 356–360, 256–261, 178–184, 104–107 for *trnS-trnG*; 927–931, 893–896, 688–692 for *trnL-trnF*) were removed from subsequent analyses because of alignment ambiguities. Of the remaining 2719 characters, 408 (15.0%) were potentially informative. All plastid regions had similar levels of potentially informative characters, with *trnS-trnG* being the most informative (20.14%), followed by *trnL-trnF* (14.33%) and *atpB-rbcL* (11.99%). Parsimony analysis of the plastid regions yielded 768 equally most-parsimonious trees of 809 steps (consistency index [CI] = 0.8300; retention index [RI] = 0.9347). The majority-rule consensus tree from the Bayesian searches is presented in Fig. 1.

The phylogenies estimated using MP and Bayesian analyses of cpDNA data are highly congruent, with the differences between them denoted by dashed lines. Both analyses strongly support the monophyly of *Pyrola* (BS = 100; PP = 1.00) and its placement sister to a clade comprising *Moneses* and *Chimaphila* (BS = 90; PP = 1.00). A major dichotomy is evident within *Pyrola* and is supportive of two infrageneric clades, designated as "clade I" and "clade II". Each of these clades is strongly supported (BS = 100; PP = 1.00). Clade I comprises three highly supported subclades (BS = 100; PP = 1.00), designated as A, B and C, with subclade C sister to a clade grouping subclade A and B (with weakly supported PP values, 0.75). This relationship, however, is collapsed in the MP strict consensus tree. Clade II contains three strongly supported subclades (BS = 100; PP = 1.00), designated as D, E and F. Subclades D and E are strongly supported as sister taxa (BS = 92; PP = 0.99), which are, in turn, sister to subclade F.

Nuclear rDNA ITS sequence comparisons and phylogenetic analyses. — ITS analysis included 41 terminals, 31 of which were ingroup taxa. The aligned ITS data matrix contained 664 positions, of which 173 (27.3%) were potentially parsimony-informative. MP analysis of these 664 positions resulted in 836 minimal length trees, each of 340 steps (CI = 0.7985; RI = 0.8915). In Fig. 2 the results of the MP analysis are superimposed on the Bayesian inference tree, with the differences denoted by dashed lines.

Both reconstructions support the monophyly of *Pyrola* (BS = 90; PP = 1.00), but its phylogenetic position within Pyroleae is not resolved well. Within *Pyrola*, many of the major clades (subclades) identified using cpDNA sequences are not maintained, except for clade II (BS = 51; PP = 1.00), subclades C (BS = 92; PP = 1.00), D (BS = 98; PP = 1.00), and E (BS = 56; PP = 0.98).

Comparion of cpDNA and nuclear rDNA ITS phylogenies and a total evidence analysis. — Overall, the ITS-derived tree is much less resolved than the tree inferred using three cpDNA markers. The ILD tests indicate significant differences between the nrDNA ITS and cpDNA data partitions ($P = 0.01$). A visual comparison of plastid and ITS-derived trees indicates that there are some discrepancies between them. Analyses of cpDNA data strongly support *P. media* and *P. faurieana* in the subclade B with two accessions of *P. minor*, *P. elliptica* Nutt. and *P. alpina* Andres (BS = 100; PP = 1.00). Analyses of ITS data, however, place *P. media* and *P. faurieana* in a weakly supported clade with *P. americana* Sweet (PP = 0.72). Two kinds of combined analyses with and without *P. media* and *P. faurieana* were conducted.

The complete set of taxa alignment contained 3383 characters, of which 589 (17.4%) were informative. Parsimony analysis of the combined data produced 144 equally most-parsimonious trees, each of 1163 steps (CI = 0.8074; RI = 0.9175). The majority-rule consensus tree from the Bayesian searches, with accompanying PP and BS values, is presented in Fig. 3. With *P. media* and *P. faurieana* removed, parsimony analysis resulted in 288 equally parsimonious trees of 1154 steps (CI = 0.8570; RI = 0.9194). This analysis produces a resolved consensus tree (not shown) that is almost identical in topology to one with all taxa included.

The Bayesian and MP strict consensus trees are highly congruent, with the exception of clade I, which resolved as a trichotomy in the latter. In both analyses nearly all major clades

and subclades identified using cpDNA data are maintained. However, some minor differences are evident within the subclade B. Analyses of the cpDNA sequences suggest that two accessions of *P. minor* have a close affinity with *P. media* and *P. faurieana* (BS = 97; PP = 1.00), whereas in the combined topologies accessions of *P. minor* form a moderately supported clade with *P. elliptica* and *P. alpina* (BS = 57; PP = 1.00). Similarly, ITS data suggest close relationships between *P. minor*, *P. elliptica* and *P. alpina* (BS = 84; PP = 1.00).

■ DISCUSSION

Our individual and combined ITS and cpDNA phylogenies continue to support our earlier finding that *Pyrola* is monophyletic and sister to a clade of *Chimaphila-Moneses*. Morphological synapomorphies for *Pyrola* include curved style orientation and chromosome number of $2n = 46$ (except *P. minor* which is characterized by a short and erect style, and *P. media* with chromosome number of $2n = 92$; Liu & al., unpub.). *Pyrola* is a very uniform group possessing only subtle variations in such features as leaf shape and size, and form of the scape bracts, as well as sepal shape and flower color (Křísa, 1971; Haber & Cruise, 1974; Haber, 1983). These minor morphological variations have posed great difficulties in species delimitation for systematic studies. Upon expanded sampling, our results significantly improve the current understanding of the infrageneric relationships within *Pyrola*. In cpDNA and combined analyses, several major clades were revealed for the first time and there seems to be either morphological or

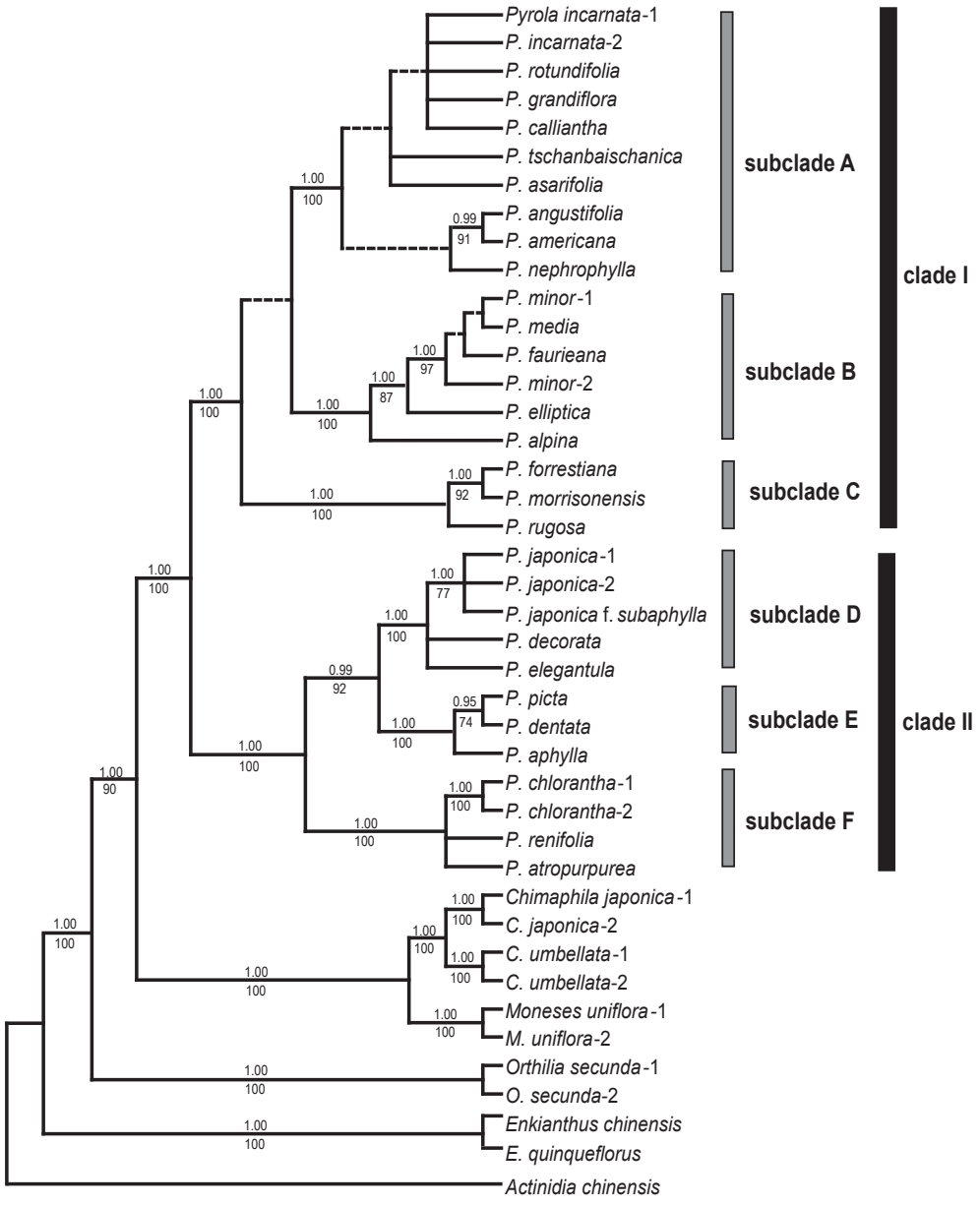

Fig. 1. Phylogenetic relationships in *Pyrola* as indicated by a majority-rule consensus tree of 8500 trees (after discarding burn-in = 15%) from Bayesian analysis based on cpDNA (*atpB-rbcL*, *trnS-trnG*, and *trnL-trnF*) datasets. Numbers above branches refer to posterior probabilities, and numbers below branches are bootstrap support values from parsimony analyses. Branches represented by dashed lines are not found in the strict consensus trees from the parsimony analysis of the same dataset. Only bootstrap values >50 and post probability values >0.95 are shown.

geographical evidence for uniting the members of each group. Although ITS analysis does not resolve the major relationships within the genus, some small terminal clades receive moderate to high support (e.g., subclades C, D and E) (Fig. 2). Compared to our molecular results, most of the groups within *Pyrola* recognized by Andres (1914), Copeland (1947) and Křísa (1971) are not monophyletic, with the sole exception of Křísa's *P.* sect. *Scotophylla*. Next, a formal and modern infrageneric classification of *Pyrola* is proposed on the basis of our phylogenetic analyses, potential morphological synapomorphies and diagnostic characters. Unless otherwise stated, our discussion is mainly based on the Křísa's system.

The chloroplast and combined phylogenies recover two strongly supported clades (clade I and clade II). Clade I consists of *P.* ser. *Ellipticae* (Andres) Křísa (included by Křísa in *P.* subg. *Amelia*), most representatives of *P.* sect. *Pyrola* (excluding the members of *P.* ser. *Japonicae* and *Amoenae* (Andres) Křísa), and an East Asian group (*P. forrestiana* Andres, *P. morrisonensis* Hayata, *P. rugosa* Andres). This clade includes the type of *Pyrola*, *P. rotundifolia* L., so it automatically becomes *Pyrola* sect. *Pyrola* by the rules of the *ICBN*. The remaining species, i.e., the members of clade II constitute *P.* sect. *Ampliosepala*.

1. [clade I] ***Pyrola*** sect. ***Pyrola*** ≡ *Thelaia* Alef. in Linnaea 28: 8, 33. 1856 ≡ *Pyrola* sect. *Thelaia* (Alef.) Benth. & Hook. f., Gen. Pl. 2: 603. 1876 ≡ *Pyrola* subsect. *Alefeldiana* Andres in Verh. Bot. Vereins Prov. Brandenburg 56: 48. 1914, p.p. ≡ *Pyrola* sect. *Euthelaia* Andres in Verh. Bot. Vereins Prov. Brandenburg 56: 44. 1914, p.p. – Type: *P. rotundifolia* L.

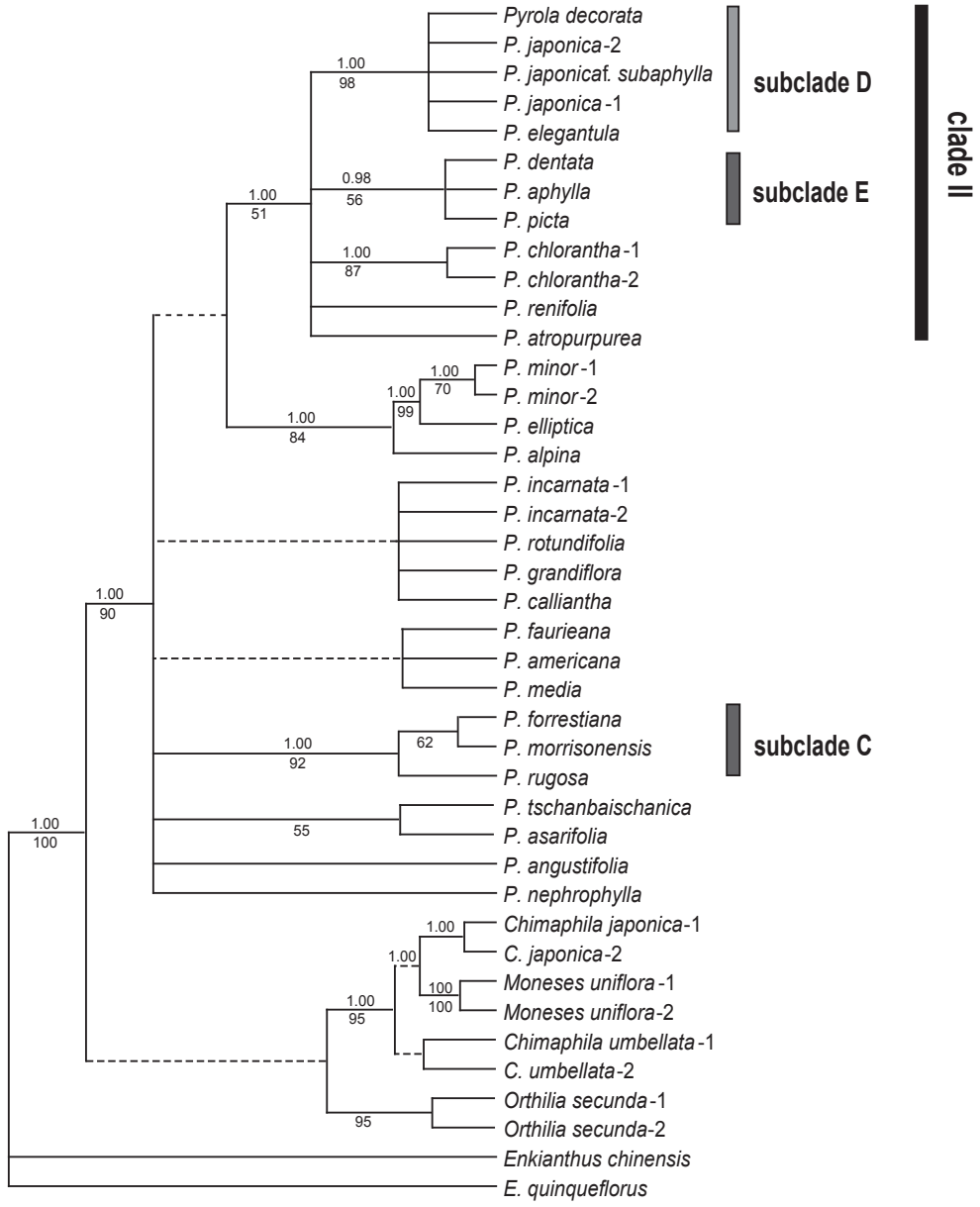

Fig. 2. Phylogenetic relationships in *Pyrola* as indicated by a majority-rule consensus tree of 8500 trees (after discarding burn-in = 15%) from Bayesian analysis based on ITS datasets. For further explanation see Fig. 1.

= *Amelia* Alef. in Linnaea 28: 8, 25. 1856 ≡ *Pyrola* sect. *Amelia* (Alef.) Benth. & Hook. f., Gen. Pl. 2: 603. 1876 – Type: *P. minor* L.

Our cpDNA and combined analyses resolve *P.* sect. *Pyrola* as a monophyletic group (BS = 100, PP = 1.00) containing three subclades (A, B and C; Figs. 1, 3). Takahashi (1986, 1987a) found that *P.* ser. *Ellipticae* differs from *P.* ser. *Chloranthae* in having winter flower buds protected by scales. In this study, we notice that whether the winter flower buds are protected by scales or not can be regarded as the most important character in the infrageneric classification of *Pyrola*. One morphological synapomorphy for *P.* sect. *Pyrola* is the winter flower buds perfectly protected by several scales. The other synapomorphy is their leaf blades without palisade tissue (Copeland, 1947; Pyykkö, 1968; pers. obs.) (Fig. 4). Bayesian analysis reveals that subclades A and B comprise a weakly supported group (PP = 0.73–0.75), which in turn is sister to subclade C; in the MP tree, however, subclades A, B and C comprise a trichotomy. Although relationships within *P.* sect. *Pyrola* are not fully resolved, our analyses reveal that these three subclades are each strongly supported as monophyletic. Here, we delimit subclade A, B and C as *P.* ser. *Pyrola*, ser. *Ellipticae* and ser. *Rugosae*, respectively.

1a [subclade A] ***Pyrola*** ser. ***Pyrola*** ≡ *Pyrola* [unranked] *Genuina* Andres in Verh. Bot. Vereins Prov. Brandenburg 56: 52. 1914 (‘§’) p.p. ≡ *Pyrola* ser. *Genuinae* (Andres) Křísa in Novit. Bot. Inst. Bot. Univ. Carol. Prag, 1965: 32. 1965 – Type: *P. rotundifolia* L.

= *Pyrola* ser. *Incarnatae* Křísa in Novit. Bot. Inst. Bot. Univ. Carol. Prag, 1965: 33. 1965 – Type: *P. incarnata* (DC.) Fisch. ex Kom.

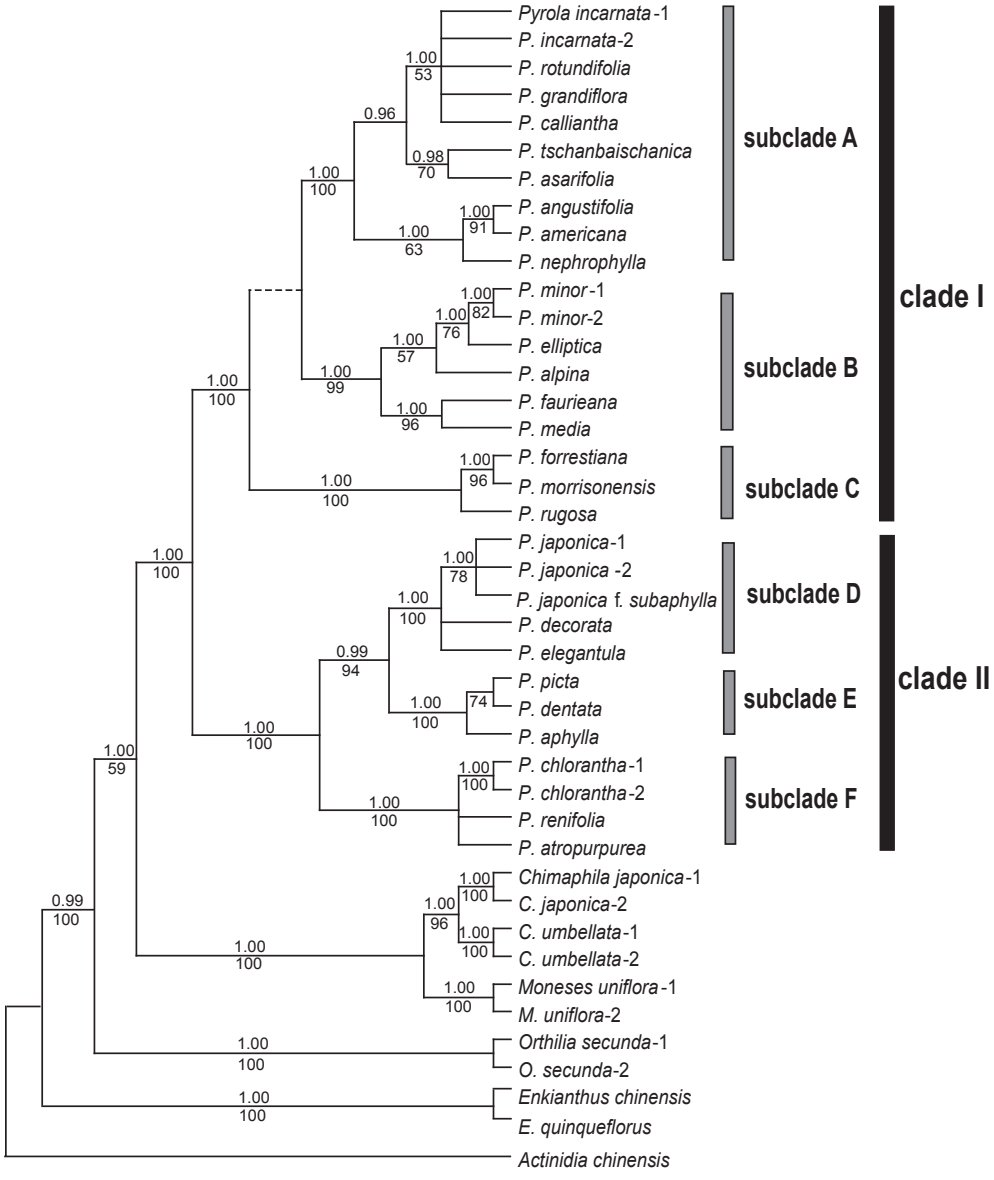

Fig. 3. Phylogenetic relationships in *Pyrola* as indicated by a majority-rule consensus tree of 8500 trees (after discarding burn-in = 15%) from Bayesian analysis based on combined ITS, *atpB-rbcL*, *trnS-trnG*, and *trnL-trnF* datasets. For further explanation see Fig. 1

= *Pyrola* ser. *Asarifoliae* Křísa in Novit. Bot. Inst. Bot. Univ. Carol. Prag, 1965: 33. 1965 – Type: *P. asarifolia* Michx.

This clade mainly comprises representatives from different series in Křísa's *P.* sect. *Pyrola* (see Table 1; Figs. 1, 3), i.e., *P. rotundifolia* and *P. grandiflora* Radius (ser. *Pyrola*), *P. incarnata* (DC.) Fisch. ex Kom. (ser. *Incarnatae*), *P. angustifolia* (Alef.) Hemsl., *P. asarifolia* Michx. and *P. americana* Sweet (ser. *Asarifolia* Křísa) and *P. nephrophylla* Andres (ser. *Sororiae* Křísa). *Pyrola calliantha* Andres and *P. tschanbaischanica* Y.L. Chou & Y.L. Chang, which were not treated in Křísa's system, are also included. Geographically, species of this clade are widely distributed in the Northern Hemisphere, with some representatives extending as far south as Himalayan areas (*P. calliantha*) of East Asia and Mexico (*P. angustifolia*) of the New World and as far north as the subarctic zone (e.g., *P. rotundifolia*) (Křísa, 1967). Although strongly supported as monophyletic, it is difficult to find a morphological synapomorphy for this series. One possible is the independently evolved lanceolate sepals (Fig. 4), which also occurs in the subclade D of clade II. Leaf and floral characters are continuous, making species within this series difficult to identify and delimit. A considerable diversity of taxonomic opinion as well as nomenclatural complexity has arisen (Křísa, 1966, 1967, 1969, 1971; Haber, 1972, 1983, 1985; Haber & Takahashi, 1988). Such taxonomic puzzles are also reflected on our individual MP phylogenetic trees, in which phylogenetic relationships are unresolved as a polytomy (Figs. 1, 2). Although two sister lineages are identified in our combined analysis (Fig. 3), there are no obvious morphological characteristics or distributional information for uniting the members of each group. Pairwise sequence divergence values across this series ranged from identity to 0.00799 in ITS data and from identity to 0.00494 in cpDNA data. An alternative explanation for the lack of phylogenetic signal in portions of the trees may be a rapid diversification and/or geographical dispersal. Thus, species delimitation within this series needs more taxa sampling from different distributional areas.

1b [subclade B] ***Pyrola*** ser. ***Ellipticae*** (Andres) Křísa in Bot. Jahrb. Syst. 90: 504. 1971 ≡ *Pyrola* subsect. *Elliptica* Andres in Verh. Bot. Vereins Prov. Brandenburg 56: 45. 1914 – Type: *P. elliptica* Nutt.

= *Pyrola* subsect. *Erxlebenia* Andres in Verh. Bot. Vereins Prov. Brandenburg 56: 48. 1914, p.p. ≡ *Pyrola* ser. *Sororiae* Křísa in Novit. Bot. Inst. Bot. Univ. Carol. Prag, 1965: 34. 1965 (excl. *P. nephrophylla* Andres) – Type: *P. sororia* Andres.

In our analyses, *P.* subg. *Amelia* (*P. minor*), ser. *Ellipticae* (*P. elliptica*, *P. alpina*, *P. faurieana*), and ser. *Sororiae* (*P. media*) are included in this clade, all of which share an independently evolved morphological synapomorphy of the short triangular sepals (Fig. 4), which also occurs in subclades E and F (see below). With its actinomorphic flowers, short and straight styles and anthers without tubes, *P. minor* has been recognized as a distinct genus, *Amelia* Alef., *Erxlebenia* Opiz, and *Braxilia* Raf. (Alefeld, 1856; Rydberg, 1914; House, 1921), or as a monotypic subgenus of *Pyrola*, *P.* subg. *Amelia* (Andres, 1914; Copeland, 1947; Křísa, 1971; Takahashi, 1986, 1993). Copeland (1947) regarded it as a transitional taxon between *Orthilia* and *Pyrola*. Based on evidence from palynology and seed morphology, Takahashi (1986, 1993) suggested that *P. minor* is the most primitive species of the genus and closely related to Křísa's *P.* sect. *Pyrola*. While its phylogenetic position was not resolved by a limited sampling ITS analysis, Freudenstein

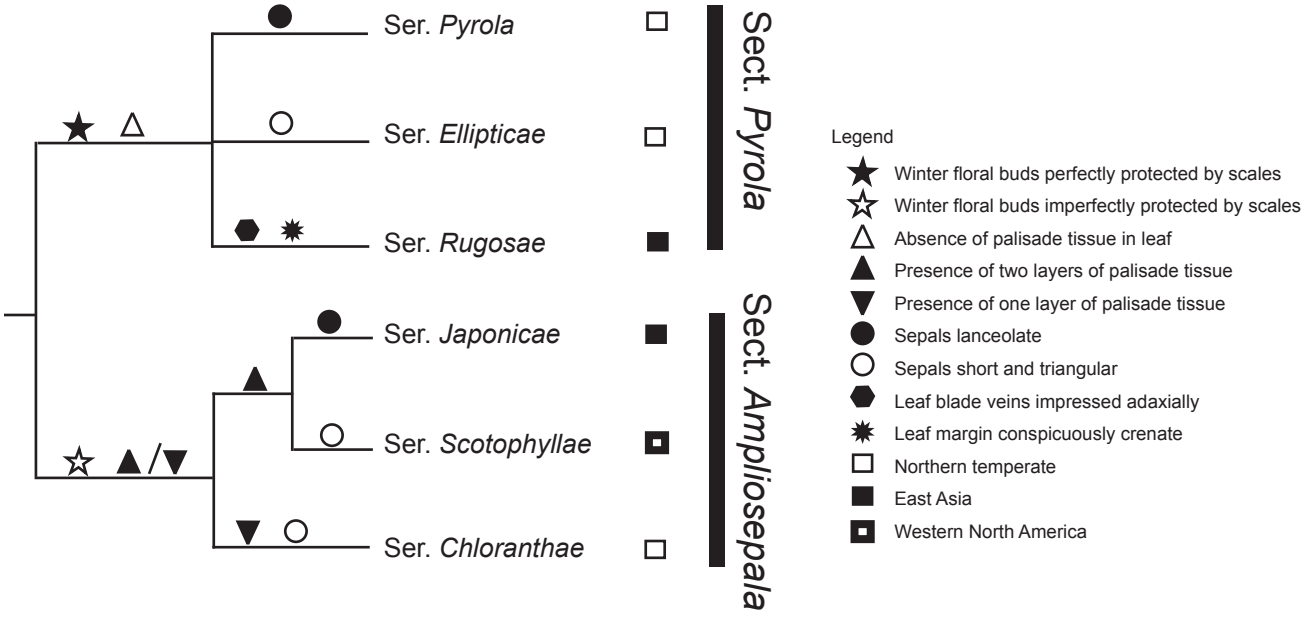

Fig. 4. Potential synapomorphic characters for sections and series of the *Pyrola* mapped on to a simplified cladogram inferred from a MP strict consensus tree based on combined ITS and cpDNA sequences analyses.

(1999) observed that style elongation in *P. minor* terminates before the stage at which it might become curved. In our analyses, *P. minor* is not placed in an isolated position, but instead forms a well-supported group with *P. alpina* and *P. elliptica* (Fig. 2; BS = 84; PP = 1.00) and has a close relationship with *P. alpina* and *P. elliptica*, and with the potential hybrids *P. media* and *P. faurieana* (Figs. 1, 3; BS = 99–100; PP = 1.00). Thus, we support the Freudenstein's viewpoint (1999) that a short style is an apomorphy in the genus. Additionally, it is reported that *P. minor* has the ability to hybridize with the following species: *P. rotundifolia*, *P. grandiflora* and *P. asarifolia* (Knaben, 1944; Böcher, 1961; Knaben & Engelskjøn, 1968; Haber, 1984, 1985; Haber & Takahashi, 1993), which are all included in our series *Pyrola*. This means that no reproductive barrier exists between *P. minor* and the species of *P.* ser. *Pyrola*. Consequently, *P. minor* might best be placed in our new *P.* ser. *Ellipticae*.

When Haber (1984) reported the first documented hybrid species *P. minor* × *P. asarifolia* in North America, he stressed speciation via hybridization as an important evolutionary mechanism within the genus. *Pyrola media*, found from Europe to central Siberia and Central Asia, is generally recognized as being a tetraploid hybrid between *P. minor* and *P. rontundifolia* s.l. (Hagerup, 1941; Knaben, 1944; Böcher, 1961). *Pyrola faurieana*, a species found in Sakhalin, northern Japan and the southern Kuriles, is also regarded as of hybrid origin with *P. minor* and *P. incarnata* being its parents (Haber & Takahashi, 1993). Although ancient hybridization events are difficult to determine using current phylogenetic methodologies (Linder & Rieseberg, 2004), more recent hybridization events can be assessed by comparing phylogenetic hypotheses derived from nuclear and plastid genomes and observing marked incongruence between the resulting tree topologies (Rieseberg, 1991; Wendel & al., 1995). In the present study, *P. media* and *P. faurieana* have a chloroplast affinity to *P. minor* with strong support (BS = 97; PP = 1.00), whereas Bayesian analysis of the ITS data indicates that *P. media* and *P. faurieana* are weakly allied with *P. americana* (PP = 0.72). Assuming *P. media* and *P. faurieana* are both of hybrid origin, we can confidently identify *P. minor* as their maternal (cpDNA) donor, but their paternal (nrDNA) donor needs further study to identify.

According to Křísa (1971), *P.* sect. *Chlorantha* consists of two series: ser. *Ellipticae* (*P. elliptica*, *P. alpina*, *P. faurieana*) and ser. *Chloranthae* (*P. renifolia* Maxim., *P. chlorantha* Sw.), showing similar floral features including short wide sepals, anthers with appendages, and long, clearly curved styles. In our phylogenetic trees (Figs. 1–3), these two series are distinct and distantly related, suggesting that some or all of the characters used to define *P.* sect. *Chlorantha* are homoplasious and *P.* ser. *Ellipticae* should be separated from Křísa's *P.* sect. *Chlorantha*. This treatment is congruent with the observations of the pollen morphology and floral winter buds (Takahashi, 1986, 1987a). Pollen of *P.* ser. *Ellipticae* is characterized by having mainly rugulate sculpture unlike those with psilate to psilate-rugulate sculpture in *P.* ser. *Chloranthae*. The floral winter buds of the species *P. elliptica*, *P. alpina* and *P. faurieana* are protected by several scales, a clear difference from the naked floral winter buds in *P.* ser. *Chloranthae*.

1c [subclade C] ***Pyrola*** ser. ***Rugosae*** (Andres) Zhen W. Liu & H. Peng, **ser. nov.** – Type: *P. rugosa* Andres.

Lamina ovovata vel suborbiculata, crassi-coriacea, aspera, marge perspicue crenatus vel serrulatus, supra viridis, subtus pallide viridis; Sepala ovato-triangularia vel lanceolata. China austro-occidentalis (Yunnan, Szetschuan) et Insula Taiwan.

This series only contains three species with an exclusively East Asian distribution. *Pyrola morrisonensis* is endemic to Taiwan, and *P. rugosa* and *P. forrestiana* are restricted to high-altitude forest (2800–3200 m) in southwest China (Qin & Stevens, 2005). *Pyrola morrisonensis* and *P. forrestiana* have been placed in different groups, the former with short triangular sepals in *P.* subsect. *Obscura* of *P.* sect. *Ampliosepala* and the latter with elongate sepals in *P.* § *Genuina*, an unranked taxon subordinate to *P.* sect. *Euthelaia* subsect. *Alefeldiana* (Andres, 1914). In our analyses, *P. morrisonensis*, *P. forrestiana*, and *P. rugosa* constitute a distinct and strongly supported branch (BS = 92–100; PP = 1.00; Figs. 1–3) in our *P.* sect. *Pyrola*. In addition to the molecular data, impressed veins on the upper leaf surface and conspicuously crenate margin are morphological synapomorphies for the new series, *P.* ser. *Rugosae* (Fig. 4).

2. [clade II] *Pyrola* sect. ***Ampliosepala*** Andres in Verh. Bot. Vereins Prov. Brandenburg 56: 44. 1914, p.p. ≡ *Scotophila* Nutt. in Trans. Amer. Philos. Soc., n.s., 8: 271. 1843 ≡ *Pyrola* subsect. *Scotophylla* (Nutt.) Andres in Verh. Bot. Vereins Prov. Brandenburg 56: 46. 1914 ≡ *Pyrola* sect. *Scotophila* (Nutt.) Copeland in Madroño 9: 99. 1947 – Lectotype (designated here): *P. aphylla* Sm.

There are strong support values (BS = 51–100; PP = 1.00) for the monophyly of our *P.* sect. *Ampliosepala* in all analyses, containing representative members of Křísa's *P.* sect. *Chlorantha* (*P.* ser. *Chloranthae*), *P.* sect. *Pyrola* (*P.* ser. *Japonicae*, *Amoneae*) and *P.* sect. *Scotophylla* (Figs. 1–3). Morphological synapomorphies for this clade include the naked winter flower buds and the presence of one or two palisade layers in the leaf blades (Fig. 4). Other morphological features that characterize this clade, but are probably not synapomorphic for it, include coriaceous dark green leaves often with veins, whitish above and paler green or purplish-red beneath. Plastid and combined sequences show that subclade F is sister to a strongly supported group made up of subclades D and E (BS = 100; PP = 1.00; Figs. 1, 3). Here, each of the subclades D, E and F corresponds to newly delimited *P.* ser. *Japonicae*, *Scotophyllae* and *Chloranthae*.

2a [subclade D] ***Pyrola*** ser. ***Japonicae*** Křísa in Novit. Bot. Inst. Bot. Univ. Carol. Prag, 1965: 33. 1965 – Type: *P. japonica* Klenze ex Alefeld.

= *Pyrola* [unranked] *Amoena* Andres in Verh. Bot. Vereins Prov. Brandenburg 56: 52. 1914 ('§') ≡ *Pyrola* ser. *Amoenae* (Andres) Křísa in Novit. Bot. Inst. Bot. Univ. Carol. Prag, 1965: 33. 1965 – Type: *P. decorata* Andres.

Included within *P.* ser. *Japonicae* are representatives of Křísa's *P.* ser. *Japonicae* (*P. japonica* Klenze ex Alef.) and ser. *Amoenae* (*P. decorata* Andres) as well as *P. elegantula* Andres from Guangdong province in China, all of which are restricted

to East Asia. The poorly resolved phylogenetic relationships among constituents of this series (Figs. 1–3) is consistent with their morphological similarities. Pairwise divergence estimates within these species are from 0.00159 to 0.00636 in ITS data and from 0.00138 to 0.00475 in cpDNA data. Speciation in this group, therefore, may be recent and rapid. *Pyrola albo-reticulata* Hayata, a species native to Taiwan recorded by Hayata (1913), was treated as a synonym of *P. decorata* in a Taiwanese flora (Hsieh, 1978). *Pyrola sumatrana* Andres from north Sumatra (Atjeh Gjalanden, G. Losir, 2700–2880 m, leg. van Steenis 1937, Sing No 26317) according to present knowledge is the most southerly occurring taxon of the genus *Pyrola* in the Eurasian continent (Křísa, 1967). Hara (1970) suggested that *P. sumatrana* and *P. albo-reticulata* are very closely allied to *P. decorata* and *P. japonica*. Although *P. albo-reticulata* and *P. sumatrana* are not included in this study, we consider that they may well belong to our newly recognized *P.* ser. *Japonicae* because of their similar morphological features and adjacent distributional areas. Being characterized by the lanceolate sepals, species within this series have been placed in Křísa's *P.* sect. *Pyrola*. Our results, however, suggest that lanceolate sepals, although occurring in our *P.* ser. *Pyrola*, is an independently evolved synapomorphy for this series (Fig. 4).

2b [subclade E] ***Pyrola*** ser. ***Scotophyllae*** (Nutt.) Zhen W. Liu & H. Peng, **stat. nov.** – Type: *P. aphylla* Sm.

This strongly supported subclade (BS = 56–100; PP = 0.98–1.00; Figs. 1–3) includes three western North American endemics—*P. picta* Sm., *P. dentata* Sm., *P. aphylla* Sm.—which totally correspond to Křísa's *P.* sect. *Scotophylla*. This series is united by a separately evolved synapomorphy of short and triangular sepals (Fig. 4). Species within this subclade have been recognized as distinct in most treatments of the genus since they were originally described by Smith (1814) from collections made by Menzies on West Redonda Island off the coast of British Columbia (Don, 1824; Andres, 1914; Copeland, 1947; Křísa, 1971; Takahashi, 1987a, 1993; Freudenstein, 1999). However, Munz & Keck (1959) and Haber (1987) recognized them as a single, highly variable species *P. picta*. Based on the similarity of their pollen and seed morphology, Takahashi (1986, 1993) also recognized the close affinity of these three species. In this study, the phylogenetic relationships between them are not certain. The plastid and combined analyses reveal that *P. aphylla* is basal to a moderately supported sister group between *P. picta* and *P. dentata* (BS = 74; PP = 0.95), whereas ITS-derived trees resolve them as a trichotomy. Notably, despite our eastern Asian *P.* ser. *Japonicae* species and western North American *P.* ser. *Scotophyllae* species being widely separated geographically, molecular results indicate that they are clustered as sister groups with strong support (BS = 92–94; PP = 0.99; Figs. 1, 3). One morphological synapomorphy of leaf blade with two layers of palisade tissue unites them. In addition, Takahashi (1993) suggested that they have larger endosperm than other *Pyrola* species.

2c [subclade F] ***Pyrola*** ser. ***Chloranthae*** Křísa in Bot. Jahrb. Syst. 90: 504. 1971 ≡ *Pyrola* subsect. *Obscura* Andres in Verh. Bot. Vereins Prov. Brandenburg 56: 46. 1914 (excl. *P. morrisonensis* Hayata) ≡ *Pyrola* sect. *Chlorantha* Křísa in Bot. Jahrb. Syst. 90: 503. 1971 – Type: *P. chlorantha* Sw.

As mentioned above, Křísa's *P.* sect. *Chlorantha* is not monophyletic when *P. alpina*, *P. elliptica* and *P. faurieana* are included. Our molecular analyses indicate that the remaining species of *P. renifolia* and *P. chlorantha* together with an unranked species *P. atropurpurea* Franch. constitute a well-supported (BS = 100; PP = 1.00) yet unresolved group (Figs. 1, 3). Morphological synapomorphies for this group include the presence of one layer of palisade tissue in the leaf blade (Copeland, 1947) and the independently evolved triangular and short sepals (Fig. 4). *Pyrola chlorantha* is characterized by light green flowers, unlike the pure white flowers in the other two species. *Pyrola renifolia*, with kidney-like leaves, occurs in northeast China and Japan, whereas *P. atropurpurea*, with cordiform leaves, has a more southern distribution in southwest China. Considering the limited molecular resolution recovered within this subclade, further study of this group is needed.

■ CONLUSION

In this study, we present results of the first comprehensive molecular phylogeny of *Pyrola* by wide-ranging taxonomic, morphological and geographical sampling. Phylogenetic analyses using different approaches consistently supported the monophyly of the genus. Two sections were recognized and redefined within *Pyrola*: *P.* sect. *Pyrola* and *P.* sect. *Ampliosepala*, corresponding to the two major clades detected. The first section is characterized by winter floral buds protected by scales and the absence of palisade tissue in the leaves, whereas the second section is redefined to include all species with naked winter flower buds and with palisade tissue. Within each section, three series are recognized: *P.* ser. *Pyrola*, ser. *Ellipticae* and ser. *Rugosae* within *P.* sect. *Pyrola* and *P.* ser. *Japonicae*, ser. *Scotophyllae* and ser. *Chloranthae* within *P.* sect. *Ampliosepala*. Members of each of these newly defined sections and series share similar geographical distributions and/or morphological or ecological traits. Our molecular phylogenetic results provide a framework within which to examine the diversification of morphological traits. Compared with the phylogenetic treatment in this study, most infrageneric ranks recognized by Andres (1914), Copeland (1947) and Křísa (1971) are found not to be monophyletic and many of the morphological characters used previously vary continuously and have multiple origins. Moreover, species relationships at deeper nodes (e.g., in *P.* ser. *Pyrola*, ser. *Chloranthae*, ser. *Japonicae* and ser. *Scotophyllae*) were unresolved, indicating that much more study at the level of series is needed in future.

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Appendix. Voucher information and GenBank accession numbers for taxa used in this study. Voucher specimens are deposited in the following herbaria: KUN = Kunming Institute of Botany, China; MICH = The University of Michigan Herbarium, U.S.A. HAST = Herbarium, Biodiversity Research Center, Academia Sinica, Taipei. Voucher information is presented for those sequences newly obtained for this investigation. Regions not sampled are indicated by an en-dash (–).

Taxon: GenBank accession nos.: *atpB-rbcL*, *trnS-trnG*, *trnL-trnF*, ITS; voucher specimen and /or locality, herbarium.

Pyrola americana Sweet: FJ378555, FJ378640, FJ378614, AF133737; *JVF 2485*, U.S.A., MICH; ***P. alpina*** Andres: FJ378561, FJ378645, FJ378620, FJ378592; *Yukie Yamaguchi 069*, Japan, KUN; ***P. angustifolia*** (Alef.) Hemsl.: FJ378537, FJ378634, FJ378596, AF133738; *JVF 2871*, Mexico, MICH; ***P. aphylla*** Sm.: FJ378564, FJ378648, FJ378622, AF133743; *JVF 2502*, WA, U.S.A., MICH; ***P. atropurpurea*** Franch.: FJ378541, FJ378627, FJ378600, FJ378579; *Z.W. Liu 008*, Yunnan, China, KUN; ***P. asarifolia*** Michx.: FJ378552, FJ378637, FJ378611, AF133736; *JVF 2512*, MI, U.S.A., MICH; ***P. calliantha*** Andres: FJ378544, FJ378630, FJ378603, FJ378582; *Z.W. Liu 018*, Shaanxi, China, KUN; ***P. chlorantha*** -1 Sw.: FJ357275, FJ357258, FJ357289, FJ378574; *Magnus Liden 047*, Sweden, KUN; ***P. chlorantha*** -2 Sw.: FJ378556, FJ378641, FJ378615, AF133742; *JVF 2466*, MI, U.S.A., MICH; ***P. decorata*** Andres: FJ378542, FJ378628, 378601, FJ378580; *Z.W. Liu 011*, Yunnan, China, KUN; ***P. dentata*** Sm.: FJ378553, FJ378638, FJ378612, FJ378587; *JVF 2495*, WA, U.S.A., MICH; ***P. elegantula*** Andres: FJ378547, FJ378633M FJ378606, FJ378585; *Z.W. Liu 032*, Guangdong, China, KUN; ***P. elliptica*** Nutt.: FJ378550, FJ378635, FJ378609, AF133740; *JVF 2527*, U.S.A., MICH; ***P. faurieana*** Andres: –, FJ378623, FJ378595, FJ378575; HT 33712, Japan, KUN; ***P. forrestiana*** Andres: FJ378543, FJ378629, FJ378602, FJ378581; *Z.W. Liu 016*, Yunnan, China, KUN; ***P. grandiflora*** Radius: FJ378554, FJ378639, FJ378613, HM021772; *JVF 2602*, U.S.A., MICH; ***P. inacarnata*** (DC.) Fisch. ex Kom. -1: FJ378540, –, FJ378599, FJ378578; *Z.W. Liu 007*, Jilin, China, KUN; ***P. inacarnata*** (DC.) Fisch. ex Kom. -2: FJ378559, FJ378643, FJ378618, FJ378590; *Yukie Yamaguchi 067*, Japan, KUN; ***P. japonica*** Klenze ex Alef. -1: FJ378558, –, FJ378617, FJ378589; *Jun sung Bin 21*, Korea, KUN; ***P. japonica*** Klenze ex Alef. -2: FJ378562, FJ378646, –, FJ378593; HT 33686, Japan, KUN; ***P. japonica*** f. ***subaphylla*** (Maxim.) Ohwi: FJ378560, FJ378644, FJ378619, FJ378591; *Yukie Yamaguchi 068*, Japan, KUN; ***P. media*** Sw.: FJ378548, FJ378634, FJ378607, FJ378586; *Magnus. Liden 040*, Sweden, KUN; ***P. minor*** L. -1: FJ357276, FJ357259, FJ357290, FJ378573; *Z.W. Liu 033*, Changbai Mt., Jilin, China, KUN; ***P. minor*** L. -2: JF378551, JF378636, JF378610, AF133745; *JVF 2519*, WA, U.S.A., MICH; ***P. morrisonensis*** Hayata: FJ378546, FJ378632, FJ378605, FJ378584; *Chien-I Huang 2314*, Taiwan, HAST. ***P. picta*** Sm.: FJ378549, –, FJ378608; AF133741; *JVF 2488*, WA, U.S.A., MICH; ***P. renifolia*** Maxim.: FJ378539, FJ378626, FJ378598, FJ378577; *Z.W. Liu 006*, Jilin, China, KUN; ***P. rotundifolia*** L.: FJ378557, FJ378642, FJ378616, 378588; *Z.W. Liu 065*, Xingjiang, China, KUN; ***P. rugosa*** Andres: FJ378538, FJ378625, FJ378597, FJ378576; *Z.W. Liu 004*, Yunnan, China, KUN; ***P. tschanbaischanica*** Y.L. Chou & Y.L. Chang: FJ378545, FJ378631, FJ378604, FJ378583; *Z.W. Liu 019*, Jilin, China, KUN; ***Chimaphila japonica*** Miq. -1: FJ357266, –, FJ357280, FJ378565; *Z.W. Liu 024*, WuDing Lion Mt., Yunnan, China, KUN; ***C. japonica*** Miq. -2: FJ357267, FJ357250, FJ357281, FJ378566; *H. Takahashi et. al #33975*, Hokkaido, Japan, KUN; ***C. umbellata*** (L.) W.P.C. Barton -1: FJ357271, FJ357254, FJ357285, FJ378567; *Magnus Liden 044*, Sweden, KUN; ***C. umbellata*** (L.) W.P.C. Barton -2: FJ357272, FJ357255, FJ357286, AF133748; *JVF 2493*, WA, U.S.A., MICH; ***Moneses uniflora*** A. Gray -1: FJ357273, FJ357256, FJ357287, FJ378568; *Z.W. Liu 029*, Kangding, Sichuan, China, KUN; ***M. uniflora*** A. Gray -2: FJ357274, FJ357257, FJ357288, AF133750; *JVF 2483*, MI, U.S.A., MICH; ***Orthilia secunda*** (L.) House -1: FJ357277, FJ357260, FJ357291, FJ378569; *Z.W. Liu 015*, Changbai Mt., Jilin, China, KUN; ***O. secunda*** (L.) House -2: FJ357278, FJ357261, FJ357292, AF133746; *JVF 2481*, MI, U.S.A., MICH; ***Enkianthus chinensis*** Franch.: –, FJ357263, FJ357294, FJ378571; *S.D. Zhang*, *H-199*, Anhui, China, KUN; ***E. quinqueflorus*** Lour.: –, FJ357264, FJ357295, FJ378572; *S.D. Zhang*, *H-107*, Yunnan, China, KUN; ***Actinidia chinensis*** Planch.: FJ357279, FJ357265, FJ357296; *Z.W. Liu 090*, Yunnan, China, KUN.