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Phylogeny and Classification of *Paris* (Melanthiaceae) Inferred from DNA Sequence Data

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• *Background and Aims Paris* (Melanthiaceae) is a temperate genus of about 24 perennial herbaceous species distributed from Europe to eastern Asia. The delimitation of the genus and its subdivisions are unresolved questions in the taxonomy of *Paris*. The objective of this study is to test the generic and infrageneric circumscription of *Paris* with DNA sequence data.
• *Methods* Phylogenetic analysis of 21 species of *Paris* based on nuclear ITS and plastid *psbA-trnH* and *trnL-trnF* DNA sequence data, alone and in combination, was employed to assess previous classifications.
• *Key Results Paris* is monophyletic in all analyses. Neither of the two traditionally recognized subgenera (*Paris* and *Daiswa*) are monophyletic. Sections *Axiparis*, *Kinugasa*, *Paris* and *Thibeticae* are monophyletic in only some of the analyses. Species of sections *Dunnianae*, *Fargesianae* and *Marmoratae* are consistently intercalated among species of section *Euthyra* in all analyses. Strong discordance between nuclear and plastid lineages is detected.
• *Conclusions* The data support the classification of *Paris* as a single genus rather than as three genera (*Daiswa*, *Kinugasa* and *Paris sensu stricto*). They provide justification for the transfer of section *Axiparis* from subgenus *Paris* to subgenus *Daiswa* and for the combination of sections *Dunnianae*, *Fargesianae* and *Marmoratae* into section *Euthyra*. The nuclear-plastid discordance is interpreted as the result of interspecific hybridization among sympatric species.

Key words: Classification, ITS, Melanthiaceae, nuclear-plastid incongruence, Parideae, *Paris*, phylogeny, *psbA-trnH*, *trnL-trnF*.

INTRODUCTION

Paris (Melanthiaceae: Parideae) (APG II, 2003; Zomlefer *et al.*, 2006) is a temperate genus of about 24 species of perennial herbs distributed from Europe to eastern Asia. Except for the European *P. quadrifolia* and the Caucasian *P. incompleta*, species are restricted to East Asia, chiefly in China (19 species), with the Yunnan-Guizhou Plateau as the centre of diversity (Li *et al.*, 1988; Li, 1998). *Paris* spp. typically grow in montane evergreen forests, montane cloud forests, broadleaved forests, conifer forests, mixed conifer and broadleaved forests and bamboo and scrub thickets (Li, 1998; Liang and Soukup, 2000). *Paris* is notable in China for its medicinal value. The species with a thick rhizome are traditional medicinal herbs and the major source of raw material for some medicines, e.g. 'Yunnan Baiyao', well-known for its use as an analgesic and anti-coagulant (Long *et al.*, 2002).

The classification of *Paris*, long in dispute, is still unresolved. Hara (1969), Li (1984, 1998) and Mitchell (1987, 1988) recognized it as a single genus (the broad concept of the genus is hereafter indicated by '*Paris*') based on floral and leaf merosity. *Paris* is 4- to 15-merous, whereas *Trillium*, its sister group, is 3-merous. Based on fruit type, ovary shape, seed morphology and rhizome shape, Takhtajan (1983) divided *Paris* into three genera: *Paris* in the narrow sense (hereafter indicated by '*Paris s.s.*'), *Kinugasa* and *Daiswa* (Table 1). This treatment was adopted by Dahlgren *et al.* (1985), Tamura (1998) and Farmer and Schilling (2002). Hara (1969) divided the 14 species known at the time into three sections: *Paris*, *Kinugasa* and *Euthyra*, based on fruit and seed characters. In the most recent comprehensive taxonomic revision, Li (1998) recognized subgenus *Paris* (11 species) and subgenus *Daiswa* (13 species), delimited by axile or incompletely axile placentation versus parietal placentation, respectively. Subgenus *Paris* was divided into sections *Kinugasa* (one species), *Paris* (five species) and *Axiparis* (five species), whereas subgenus *Daiswa* was divided into sections *Dunnianae* (one species), *Euthyra* (eight species), *Marmoratae* (two species), *Fargesianae* (one species) and *Thibeticae* (one species).

Recent phylogenetic studies based on DNA sequence data have proved useful for estimating the phylogeny of the tribe Parideae. Analyses based on the plastid *rbcL* and *matK* genes and the nuclear ribosomal internal transcribed spacer (ITS) region supported the monophyly of *Paris*, with *Trillium* as its sister group (Kato *et al.*, 1995*a*, *b*; Kazempour Osaloo and Kawano, 1999; Kazempour Osaloo *et al.*, 1999). Other analyses combining sequence data (ITS and *matK*) and morphological data supported the division of *Paris* into three genera (*Daiswa*, *Kinugasa* and *Paris s.s.*; Farmer and Schilling, 2002). These studies have provided valuable insights for an initial

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TABLE 1. *Comparison of morphological features among Takhtajan's (1983) three segregate genera of* Paris

	Daiswa	*Kinugasa*	*Paris s.s.*
Rhizome	Thick	Thick	Long and slender
Fruit	Capsule	Berry	Berry
Ovary	Angular	Angular	Rounded
Seed	With sarcotesta	Without sarcotesta	Without sarcotesta

molecular-based evaluation of *Paris* classification, but sampling within the genus (at most eight species) has been too low to address satisfactorily the issues of generic delimitation and infrageneric division.

Here the various classifications of *Paris* are tested with DNA sequence data by using a much larger taxon sample than has previously been available, including representatives from all described subgenera (or segregate genera) and sections. DNA sequence data are used from the ITS region and two regions of the plastid genome (*trnL-trnF* and *psbA-trnH*). These regions have proved to be of general utility for phylogenetic studies at the infrageneric level because of their relatively fast rate of sequence evolution and conserved flanking primer sites (Soltis and Soltis, 1998; Shaw *et al*., 2005).

MATERIALS AND METHODS

Taxon sampling, DNA extraction, amplification and sequencing

Twenty-one species and four varieties of *Paris* L. (Table 2) representing both subgenera and all eight sections *sensu* Li (1998) were sampled. Of the three species not included, one [*P. tetraphylla*, Japan (section *Paris*)] was unavailable, and the other two [*P. undulata*, China; *P. birmanica*, Burma (section *Euthyra*)] were not found in the areas in which they were known to be distributed and may be extinct. To assess the generic delimitation of *Paris*, nine *Trillium* spp. were included in the ingroup (Table 2), representing two of the subgenera of *Trillium* (Farmer and Schilling, 2002). *Trillium rivale* was used as outgroup in accordance with the results of Farmer and Schilling (2002).

Genomic DNA was extracted from silica gel-dried or fresh leaves by using the method of Doyle and Doyle (1987). The ITS region was amplified with primers ITS4 and ITS5 (White *et al.*, 1990). The PCR programme was as follows: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s; and 72 °C for 7 min. The *psbA-trnH* region was amplified with primers psbA and trnH (Sang *et al.*, 1997) in accordance with the protocol of Shaw *et al.* (2005). The *trnL-trnF* region was amplified with the primer pairs c–d and e–f (Taberlet *et al.*, 1991) as follows: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 56 °C (c–d) or 58 °C (e–f) for 30 s, 72 °C for 45 s; and 72 °C for 7 min.

Amplified fragments were purified by running PCR products on a 1·5 % low-melting temperature agarose gel followed by DNA recovery with a gel extraction kit (UNIQ-10, Sangon, Shanghai, China). Cycle sequence reactions of purified PCR products were performed by using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. Products were then run on a 3730XL genetic analyser (Applied Biosystems) at Sunbiotech Corp., Beijing, China. Forward and reverse strands of all ITS and *psbA-trnH* samples were sequenced. The *trnL-trnF* sequences of most samples were determined in one direction with primers c and f because the sequences were of sufficient quality to make sequence generation of the reverse strand unnecessary.

Data analysis

Sequences were compared and compiled with Sequencher 4.2 version (Gene Codes Corp., Ann Arbor, MI, USA). The alignment was performed manually in PAUP* version 4.0b10 (Swofford, 2002). The matrix is available upon request from the authors.

Phylogenetic analyses were conducted with maximum parsimony. Characters were equally weighted and unordered. Gaps were scored as missing data. Positions 510–641 in the aligned *trnL-trnF* data matrix encompassed an A/T-rich region of highly ambiguous alignment, and thus were excluded from phylogenetic analyses. The parsimony analyses were performed with the heuristic search option in PAUP*. Searches were conducted over 1000 random-taxon-addition replicates with tree bisection-reconnection branch-swapping and MulTrees enforced. All shortest trees were saved, and a strict consensus tree was computed. To estimate the support for individual clades, heuristic bootstrapping (1000 replicates) was performed. Partition homogeneity tests (Farris *et al.*, 1994) were conducted with PAUP* to determine the degree of congruence between the two plastid data sets (*trnL-trnF* versus *psbA-trnH*) and plastid versus nuclear data sets (*trnL-trnF* + *psbA-trnH* versus ITS) by using 500 replicates and an heuristic tree search with 10 random-taxon-addition replicates. Only informative characters were included in analyses.

From the phylogenetic results the evolution of ovary placentation and seed morphology, two characters regarded as critical in the classification of *Paris* were examined (Takhtajan, 1983; Li, 1984, 1998). Character states for *Paris* and *Trillium* were obtained from the literature (Freeman, 1969; Takhtajan, 1983; Zomlefer, 1996; Li, 1998; Farmer, 2000; Liang and Soukup, 2000) and personal observation by the first author. Characters were traced onto the tree generated from the combined analysis of the ITS and plastid DNA data sets with MacClade 4.0 (Maddison and Maddison, 2000).

RESULTS

Plastid DNA analysis

The length of *trnL-trnF* ranged from 938 to 1058 base pairs (bp) among species of *Paris* and from 973 to 1018 bp among species of *Trillium*. Of the 1174 aligned positions, 106 were variable, of which 31 (29·2 % of the variable positions) were potentially phylogenetically informative. Parsimony analysis of *trnL-trnF* yielded 23 839 trees of 49 steps [consistency index (CI) = 0·67,

TABLE 2. *Taxa represented in this study with voucher or source information, and GenBank accession numbers*

Taxa	Voucher or source	Accession no.		
		ITS	*psbA-trnH*	*trnL-trnF*
Paris axialis	Y. H. Ji 149 (KUN)	DQ404210	DQ404244	DQ404278
P. bashanensis	Y. H. Ji 136 (KUN)	DQ404205	DQ404239	DQ404273
P. cronquistii var. *cronquistii*	Y. H. Ji 133 (KUN)	DQ404214	DQ404248	DQ404281
P. cronquistii var. *xichouensis*	X. Gong *s. n.* (KUN)	DQ404221	DQ404255	DQ404289
P. daliensis	Q. Guo *s. n. (*KUN)	DQ404226	DQ404260	DQ404294
P. delavayi var. *delavayi*	Y. H. Ji 135 (KUN)	DQ404215	DQ404249	DQ404283
P. delavayi var. *petiolata*	S. T. Chen *s. n.* (KUN)	DQ404220	DQ404254	DQ404288
P. dulongensis	GLGS Exp. 20534 (KUN)	DQ404207	DQ404241	DQ404275
P. dunniana	Y. H. Ji 128 (KUN)	DQ404225	DQ404259	DQ404293
P. fargesii	Y. H. Ji 129 (KUN)	DQ404217	DQ404251	DQ404285
P. forrestii	Y. H. Ji 168 (KUN)	DQ404208	DQ404242	DQ404276
P. incompleta	Cult. in Royal Botanic Garden, Edinburgh (19741458B)	DQ404203	DQ404237	DQ404271
P. japonica	J. Maruta *s. n.* (KUN)	DQ404202	DQ404236	DQ404270
P. luquanensis	Y. H. Ji 206 (KUN)	DQ404219	DQ404253	DQ404287
P. mairei	W. Y. Xu *s. n.* (KUN)	DQ404213	DQ404247	DQ404282
P. marmorata	Y. H. Ji 197 (KUN)	DQ404222	DQ404256	DQ404290
P. polyphylla var. *chinensis*	Y. H. Ji 126 (KUN)	DQ404218	DQ404252	DQ404286
P. polyphylla var. *polyphylla*	Y. H. Ji 174 (KUN)	DQ404224	DQ404258	DQ404292
P. polyphylla var. *yunnanensis*	Y. H. Ji 131 (KUN)	DQ404223	DQ404257	DQ404291
P. quadrifolia	P. Bruggeman *s. n.* (KUN)	DQ404204	DQ404238	DQ404272
P. rugosa	GLGS Exp. 21597 (KUN)	DQ404211	DQ404245	DQ404279
P. thibetica	GLGS Exp. 20193 (KUN)	DQ404216	DQ404250	DQ404284
P. vaniotii	H. Li 8842 (KUN)	DQ404209	DQ404243	DQ404277
P. vietnamensis	Y. H. Ji 139 (KUN)	DQ404212	DQ404246	DQ404280
P. verticillata	L. X. Wang *s. n. (*KUN)	DQ404206	DQ404240	DQ404274
Trillium albidum	Cult. in Royal Botanic Garden, Edinburgh (19623094B)	DQ404198	DQ404232	DQ404266
T. cernuum	Cult. in Royal Botanic Garden, Edinburgh (19370524B)	DQ404193	DQ404227	DQ404261
T. cuneatum	Cult. in Royal Botanic Garden, Edinburgh (19841344)	DQ404199	DQ404233	DQ404267
T. erectum	Cult. in Royal Botanic Garden, Edinburgh (19653535A)	DQ404196	DQ404230	DQ404264
T. grandiflorum	Cult. in Royal Botanic Garden, Edinburgh (19685241)	DQ404195	DQ404229	DQ404263
T. camschatcense	Cult. in Royal Botanic Garden, Edinburgh (19741690)	DQ404197	DQ404231	DQ404265
T. luteum	Cult. in Royal Botanic Garden, Edinburgh (19841338)	DQ404200	DQ404234	DQ404268
T. ovatum	Cult. in Royal Botanic Garden, Edinburgh (19360567C)	DQ404194	DQ404228	DQ404262
T. rivale	Kazempour Osaloo and Kawano (1999), Shaw *et al.* (2005)	AB018822	AY727185	AY727232
T. tschonoskii	GLGS Exp. 20202 (KUN)	DQ404201	DQ404235	DQ404269

retention index (RI) = 0·93]. In the strict consensus tree, *Paris* was monophyletic [bootstrap percentage (bt) = 96; Fig. 1]. The basal divergence within this *Paris* clade formed two major clades ('A' and 'B', bt = 68 and 90, respectively). Clade A comprised *P. bashanensis*, *P. incompleta*, *P. japonica*, *P. quadrifolia* and *P. verticillata* corresponding to *Paris s.s.* [= subgenus *Paris sensu* Li (1998) minus section *Axiparis*]. Clade B was resolved as two subclades: one (bt = 70) comprised section *Axiparis* of subgenus *Paris* and section *Thibeticae* of subgenus *Daiswa*, and the other (bt = 82) the rest of the species of subgenus *Daiswa*. The only other clades recovered were *P. quadrifolia* + *P. verticillata* (bt = 93) and *P. forrestii* + *P. rugosa* (bt = 58).

The length of *psbA-trnH* ranged from 1078 to 1103 bp among species of *Paris* and from 645 to 1120 bp among species of *Trillium*. Of the 1221 aligned positions, 102 were variable, of which 44 (43.1 % of the variable positions) were potentially phylogenetically informative. Parsimony analysis of *psbA-trnH* resulted in 6291 trees of 104 steps (CI = 0·61, RI = 0·85). The strict consensus tree showed a topology of *Paris* completely consistent with that from the analysis of *trnL-trnF*, but with somewhat higher clade resolution (Fig. 2). *Paris* was monophyletic (bt = 97) and the two first-diverging clades A and B were recovered (bt = 83 and 79, respectively). There were five other clades with bootstrap support >50 %: section *Paris* (bt = 88); section *Thibeticae* + section *Axiparis* (bt = 61); section *Axiparis* (bt = 53); a clade of subgenus *Daiswa* excluding section *Thibeticae* (bt = 79); and a clade of capsular species with 3- to 6-whorled stamens (bt = 69).

The *trnL-trnF* and *psbA-trnH* data sets did not differ significantly in structure (P = 0·92); they were therefore combined into a single data set for phylogenetic analysis. Parsimony analysis of the combined plastid DNA data yielded 3158 trees of 270 steps (CI = 0·81, RI = 0·89). The topology of *Paris* in the combined strict consensus tree was congruent with that from the *psbA-trnH* analysis (Fig. 3). It differed from the *psbA-trnH* consensus only in the unresolved placement of *P. incompleta*. The ingroup nodes in the topology of the combined analysis received higher bootstrap support than those in the separate analyses of either plastid DNA region (cf. Figs 1–3).

ITS analysis

The length of ITS ranged from 632 to 636 bp among species of *Paris* and 635 to 638 among species of *Trillium*.

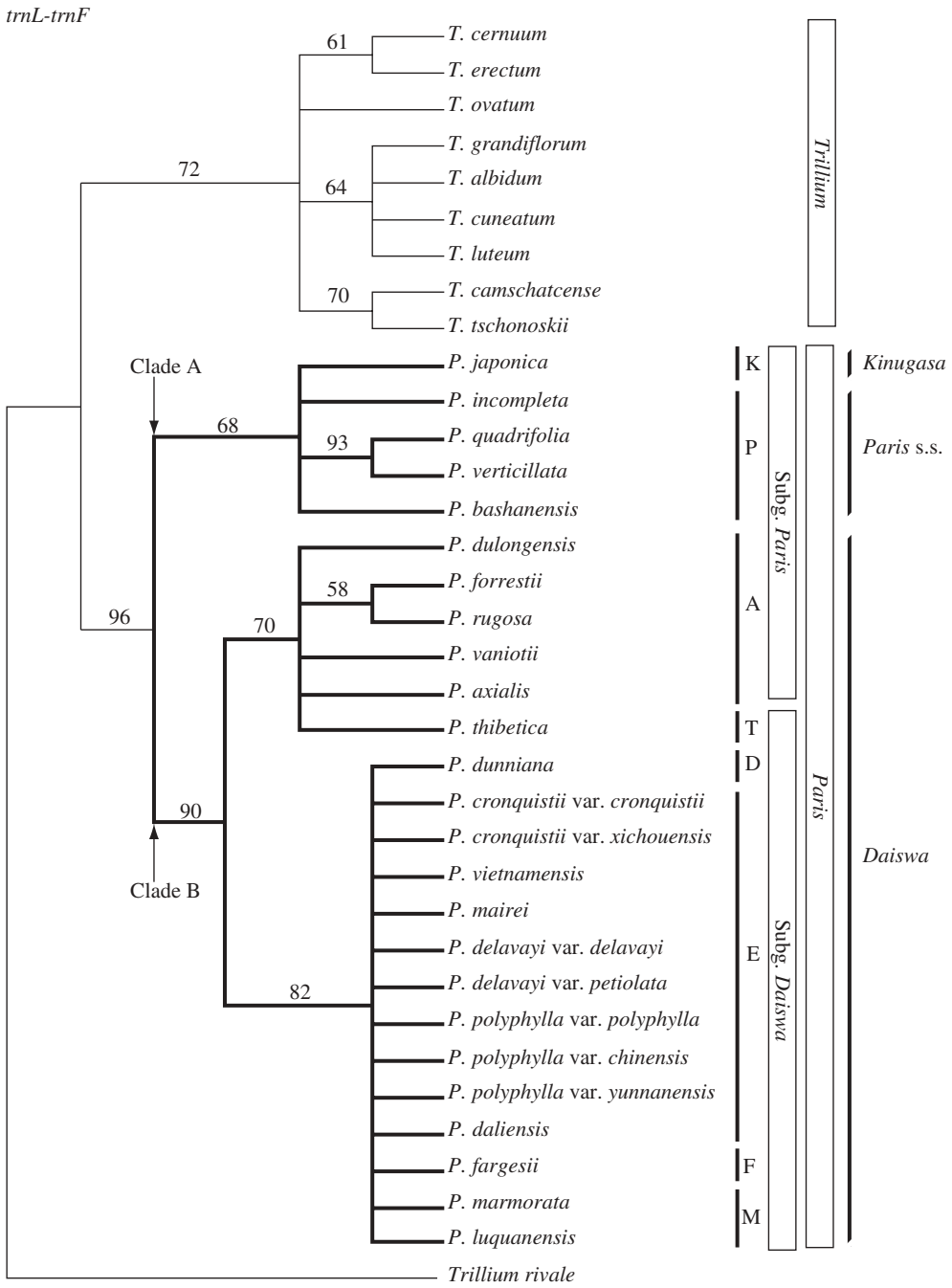

FIG. 1. The strict consensus tree of 23 839 trees from parsimony analysis of *trnL-trnF* sequences of *Paris* and *Trillium* (length = 49, CI = 0·67, RI = 0·93). Bootstrap percentages >50 % are shown above branches. Two contrasting views of generic delimitation (see text) and the subgenera and sections of Li (1998) are indicated on the right. P = sect. *Paris*, A = sect. *Axiparis*, D = sect. *Dunnianae*, E = sect. *Euthyra*, F = sect. *Fargesianae*, K = sect. *Kinugasa*, M = sect. *Marmoratae*, T = sect. *Thibeticae*.

Of the 658 aligned positions, 208 were variable, of which 89 (42·8 % of the variable positions) were potentially phylogenetically informative. Parsimony analysis of ITS yielded 132 trees of 275 steps (CI = 0·62, RI = 0·81). In the strict consensus tree, *Paris* was monophyletic (bt = 100) and resolved into the same two major clades (A and B) recovered in the plastid DNA analyses (bt = 92 and 100, respectively; Fig. 4). The topology of clade A was consistent with those from plastid DNA; *P. japonica* was sister to a clade (bt = 59) comprising the remaining taxa (section *Paris*) of clade A. Clade B, however, differed substantially from that of the plastid DNA analyses. *Paris dulongensis* (section *Axiparis*), the first diverging lineage, was sister to a clade (bt = 94) comprising all other species of section *Axiparis* and subgenus *Daiswa*. There were two clades within the latter: a clade (bt = 93) comprising

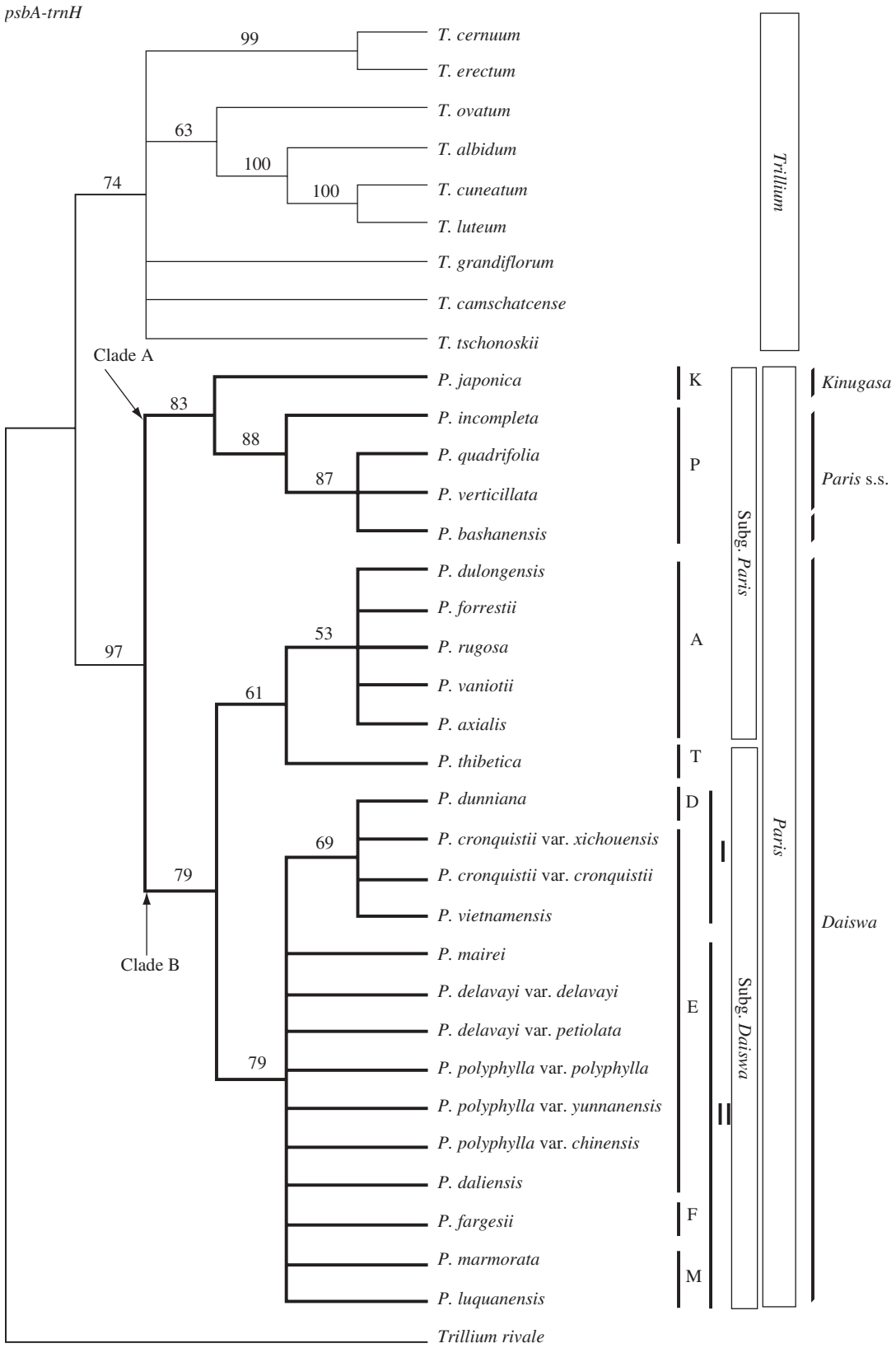

FIG. 2. The strict consensus tree of 6291 trees from parsimony analysis of *psbA-trnH* sequences of *Paris* and *Trillium* (length = 104, CI = 0·61, RI = 0·85). Bootstrap percentages >50 % are shown above branches. Two contrasting views of generic delimitation (see text) and the subgenera and sections of Li (1998) are indicated on the right. Section abbreviations are as in Fig. 1. I = capsular species with 3- to 6-whorled stamens, II = capsular species with 2-whorled stamens.

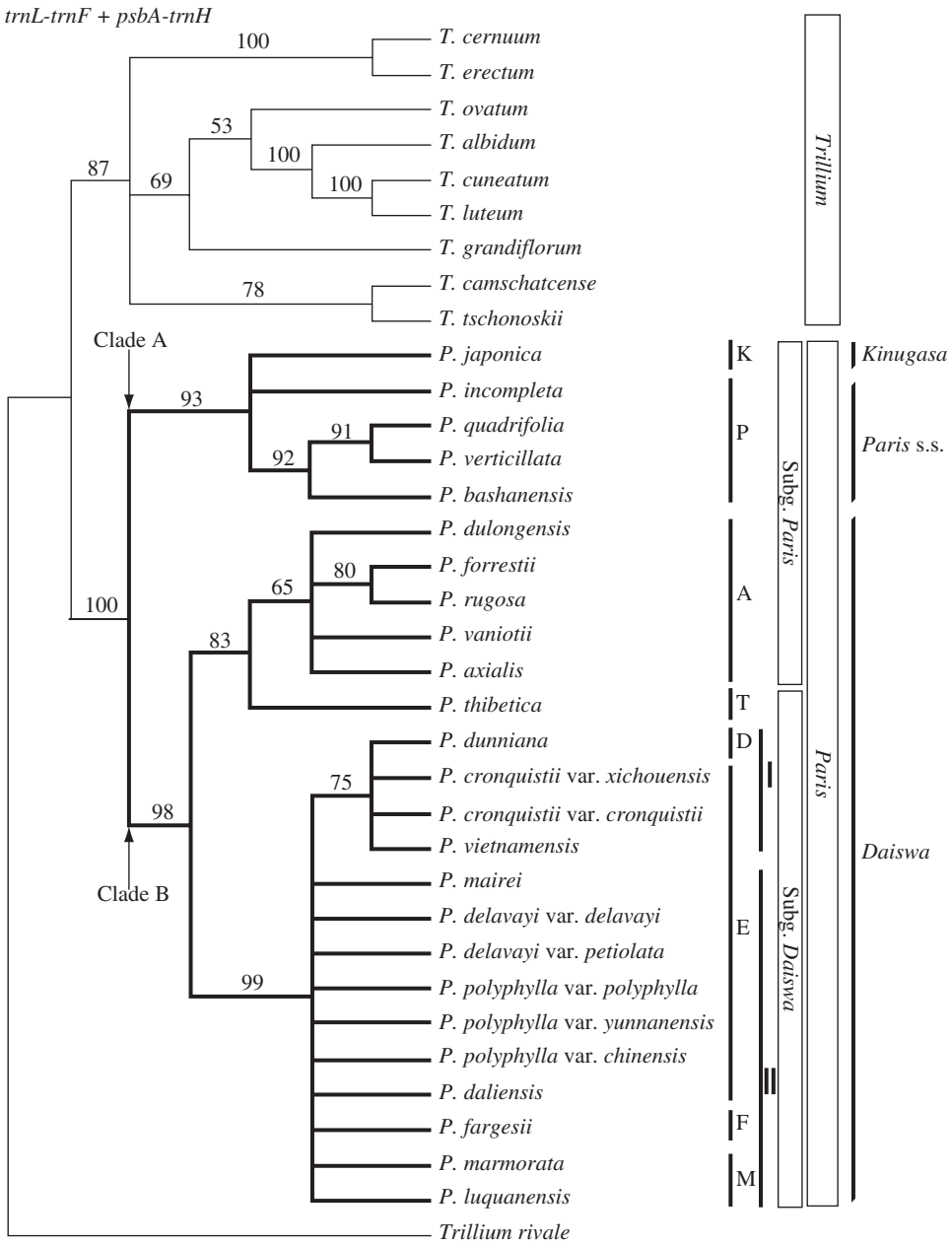

FIG. 3. The strict consensus tree of 3158 trees from parsimony analysis of combined *trnL-trnF* and *psbA-trnH* sequences of *Paris* and *Trillium* (length = 270, CI = 0·81, RI = 0·89). Bootstrap percentages >50 % are shown above branches. Two contrasting views of generic delimitation (see text) and the subgenera and sections of Li (1998) are indicated on the right. Abbreviations are as in Fig. 2.

P. axialis and a clade in which *P. forrestii* and *P. rugosa* (section *Axiparis*) formed a group (bt = 99) sister to a clade comprising all species of subgenus *Daiswa* (bt = 80). *Paris thibetica* (subgenus *Daiswa* section *Thibeticae*) was sister to a clade comprising the remaining species of the subgenus (bt = 68). Three subclades formed a trichotomy within the latter clade: a clade (bt = 53) consisting of *P. marmorata* (section *Marmoratae*) and *P. daliensis* (section *Euthyra*); a clade (bt = 78) in which *P. mairei* (section *Euthyra*) was sister to a clade formed by *P. luquanensis* (section *Marmoratae*) and *P. polyphylla* var. *yunnanensis* (section *Euthyra*); and a clade (bt = 98) in which four species of section *Euthyra* formed a group (bt = 98) sister to a clade (bt = 80) comprising species of sections *Dunnianae*, *Euthyra* and *Fargesianae*. The species with 3- to 6-whorled stamens formed a monophyletic group (bt = 60), whereas the group of species with 2-whorled stamens was paraphyletic.

Data incongruence and combined analysis

The plastid DNA and ITS data sets differed significantly in structure ($P = 0{\cdot}002$). By visual comparison of the ITS and plastid DNA consensus topologies and experimentation

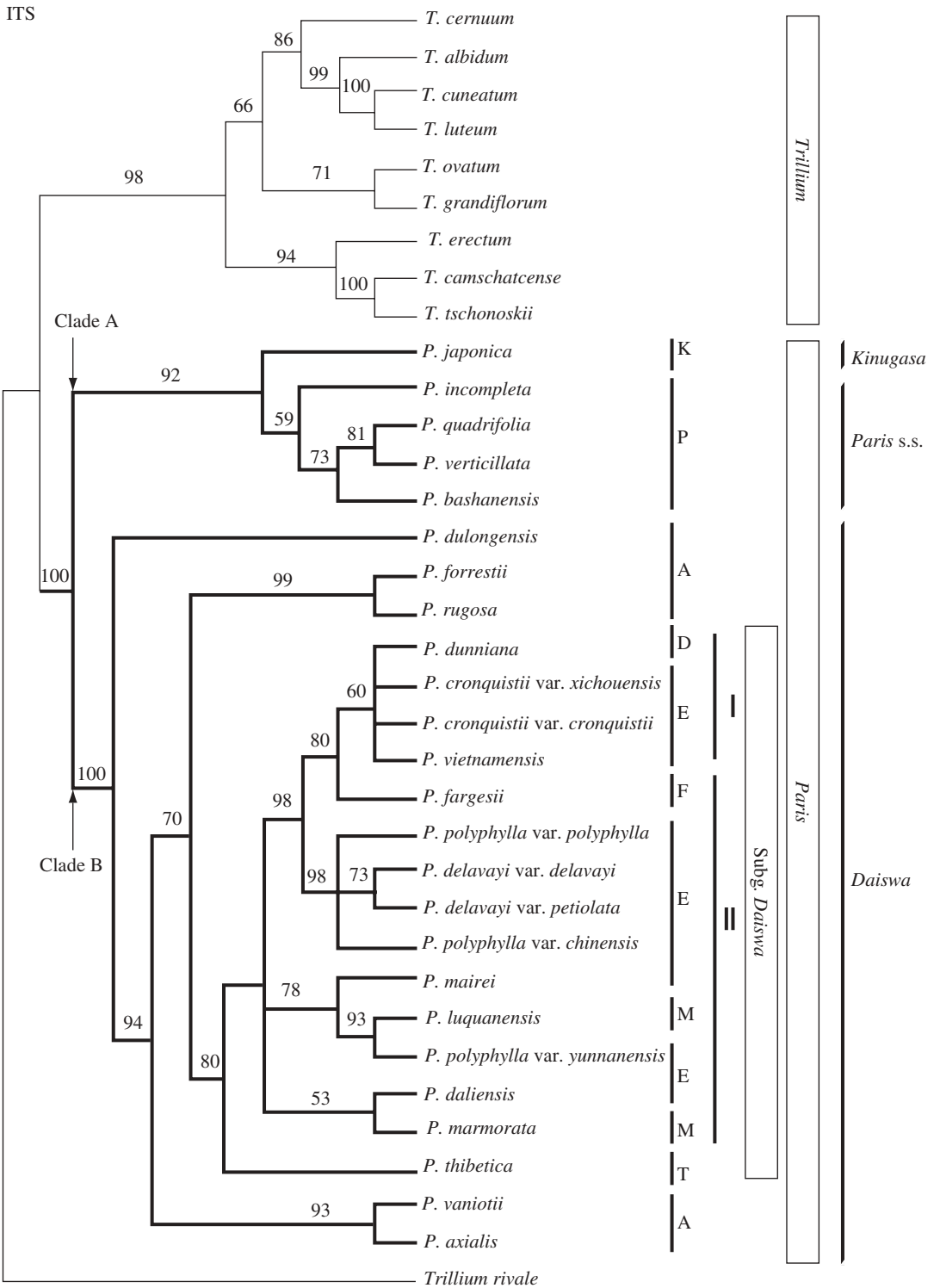

FIG. 4. The strict consensus tree of 132 trees from parsimony analysis of ITS sequences of *Paris* and *Trillium* (length = 270, CI = 0·62, RI = 0·81). Bootstrap percentages >50 % are shown above branches. Two contrasting views of generic delimitation (see text) and the subgenera and sections of Li (1998) are indicated on the right. Abbreviations are as in Fig. 2.

with various taxon exclusion sets of minimal size, a non-significant *P* value (*P* = 0·36) was recovered when *P. daliensis*, *P. dulongensis*, *P. luquanensis*, *P. mairei*, *P. marmorata*, *P. polyphylla* var. *polyphylla*, *P. polyphylla* var. *yunnanensis* and *P. thibetica* were excluded from the test. To assess previous classifications of *Paris* the ITS and plastid DNA data were combined to form a single data set for phylogenetic analysis after removing these eight taxa.

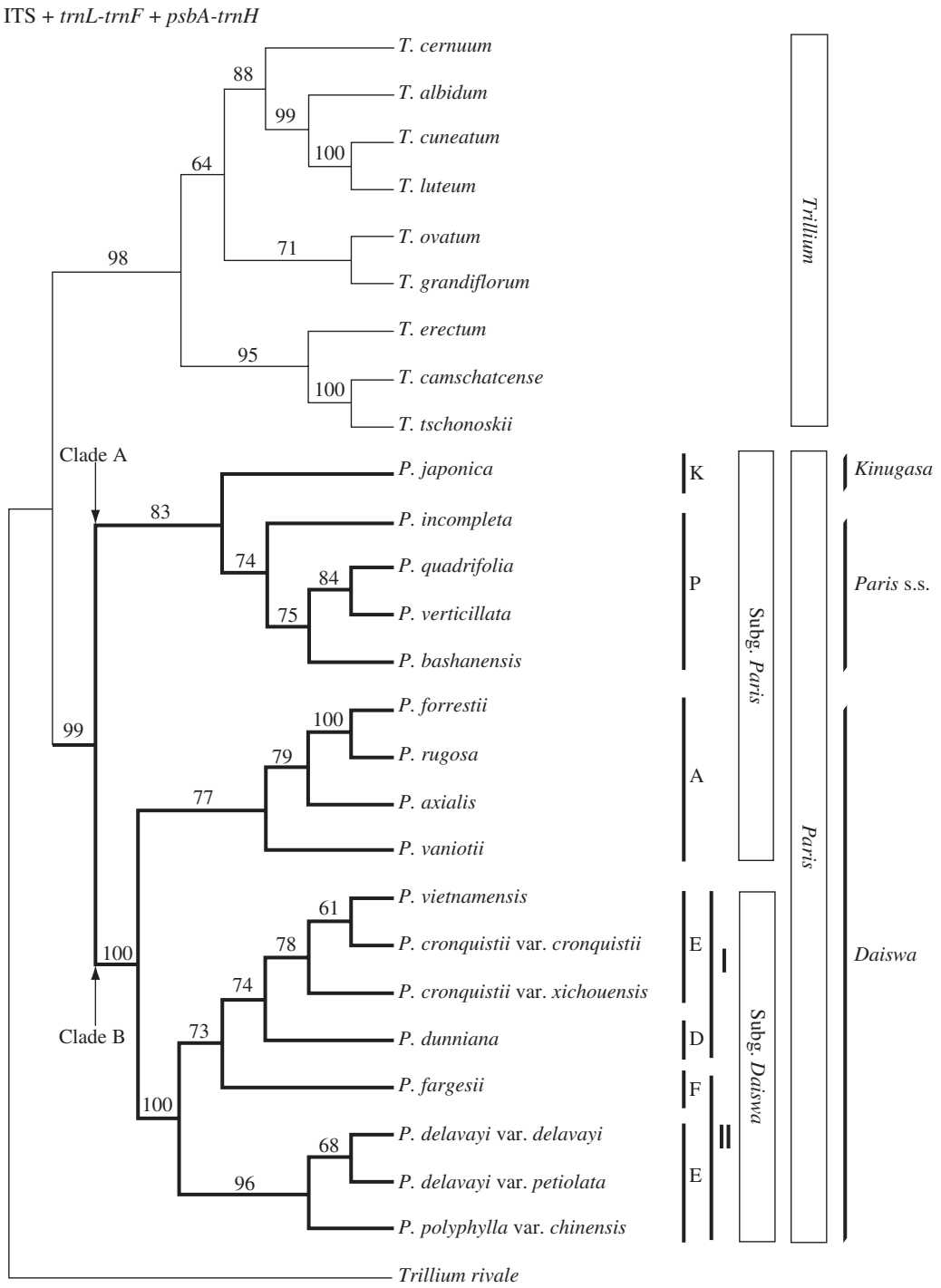

FIG. 5. The single tree from parsimony analysis of combined plastid DNA and ITS analysis with nine taxa excluded (see text; length = 347, CI = 0·61, RI = 0·85). Bootstrap percentages >50 % are shown above branches. Two contrasting views of generic delimitation (see text) and the subgenera and sections of Li (1998) are indicated on the right. Abbreviations are as in Fig. 2.

Parsimony analysis of the combined plastid DNA and ITS data set yielded one tree of 347 steps (CI = 0·61, RI = 0·85). The topology of this tree was consistent with that of the combined plastid DNA consensus but exhibited higher resolution (Fig. 5). Within clade A (bt = 83), *P. japonica* (section *Kinugasa*) was sister to a clade comprising the species of section *Paris* (bt = 74). Within clade B (bt = 100), the species of section *Axiparis* formed a clade (bt = 73) sister to a clade comprising the rest of the species of subgenus *Daiswa* (bt = 100). The latter clade consisted of all species of section *Euthyra* included, with *P. dunniana* (section *Dunnianae*) and *P. fargesii* (section

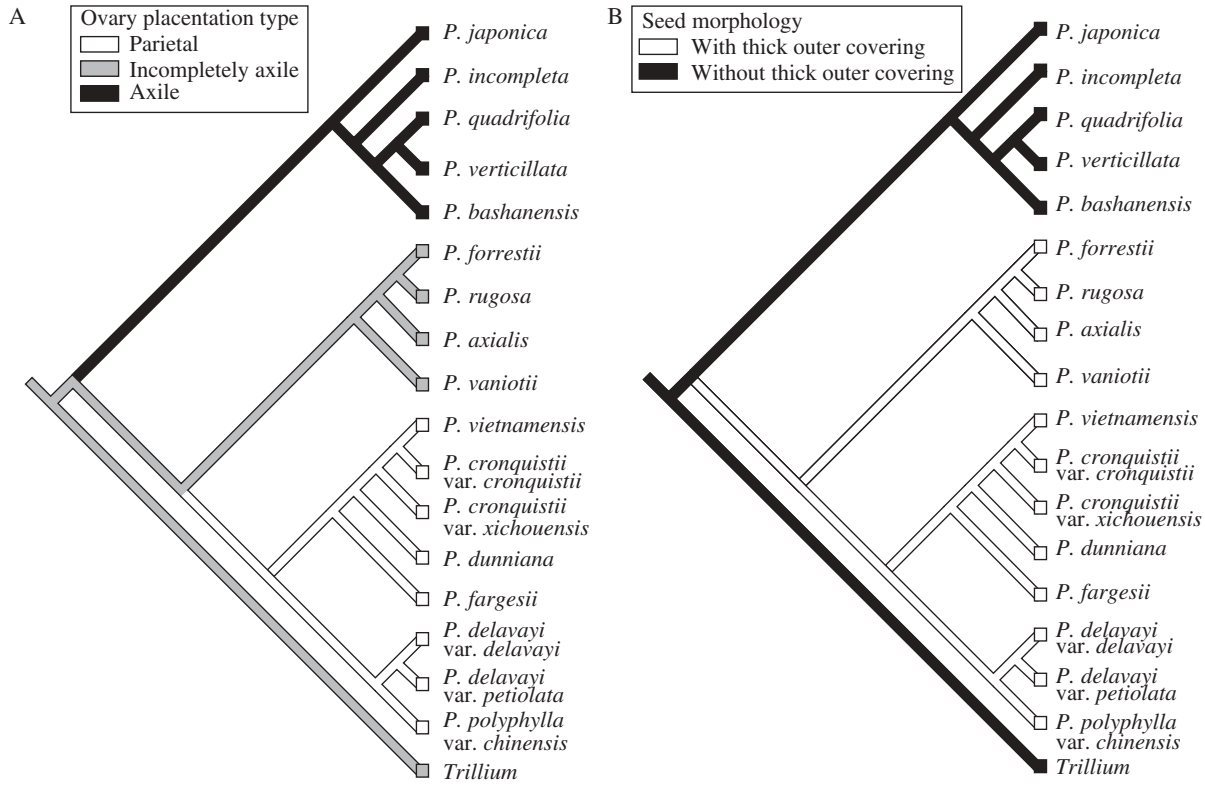

FIG. 6. The single shortest tree obtained for *Paris* from the combined ITS and plastid DNA analysis, onto which ovary placentation (A) and seed morphology (B) have been mapped.

Fargesianae) nested within it. The species with 3- to 6-whorled stamens formed a monophyletic group (bt = 74), whereas the group of species with 2-whorled stamens was paraphyletic to the clade of species with 3- to 6-whorled stamens.

Evolution of ovary placentation and seed morphology

Optimization of ovary placentation onto the shortest tree for *Paris* from the combined ITS and plastid DNA analysis (see Fig. 5) recovered two equally optimal reconstructions of two steps (Fig. 6A). Incompletely axile placentation was inferred as the plesiomorphic state, whereas axile and parietal placentation were inferred as apomorphic states in the genus. Optimization of seed morphology recovered one optimal reconstruction of one step (Fig. 6B). The presence of a thick outer covering (imperfect aril or sarcotesta) was inferred as the apomorphic state.

DISCUSSION

Discordance between ITS and plastid DNA trees

Phylogenetic incongruence between nuclear and cytoplasmic genes has been reported in a number of previous studies (e.g. Soltis and Kuzoff, 1995; Wendel *et al.*, 1995; Soltis *et al.*, 1996; Hardig *et al.*, 2000; Setoguchi and Watanabe, 2000; Yoo *et al.*, 2002). The phenomenon has been attributed to lineage sorting (Neigel and Avise, 1986; Harrison, 1991; Hardig *et al.*, 2000) or introgression of the cytoplasmic genome from one species onto the nuclear background of another (or vice versa) by interspecific hybridization (Soltis and Kuzoff, 1995; Soltis *et al.*, 1996; Wendel and Doyle, 1998). Cytoplasmic gene flow with or without nuclear introgression is frequently observed (summarized by Rieseberg and Soltis, 1991; Rieseberg and Wendel, 1993). In contrast, nuclear introgression occurring without cytoplasmic gene flow appears to be rare, and only relatively few examples have been reported (e.g. Wagner *et al.*, 1987; Arnold and Robinson, 1991; Setoguchi and Watanabe, 2000).

Although no natural interspecific hybridization has been reported in *Paris*, experimental outcrossing by manual pollination has been effected between most Chinese species with thick rhizomes (X. Gong, Kunming Institute of Botany, China, unpubl. res.). This indicates that natural hybridization between sympatric species is possible if other factors (e.g. pollination mechanisms) are compatible. The eight taxa removed prior to the recovery of a non-significant *P* value in the incongruence length difference tests between the nuclear and combined plastid data sets all share a distribution from the Hengduan Mountains to the Eastern Himalaya and are endemic to these areas, and some of them are sympatric. Some morphological intermediates have been reported, e.g. between *P. marmorata* and *P. mairei*, *P. mairei* and *P. polyphylla* var. *yunnanensis*, and *P. polyphylla* var. *yunnanensis* and *P. polyphylla* var. *polyphylla* (Li, 1998). Characters

TABLE 3. *Comparison of pollen ornamentation, ovary placentation and seed morphology among* Paris thibetica*, section* Axiparis*, and other capsular species*

	Paris thibetica	Section *Axiparis*	Other capsular species
Pollen ornamentation	Reticulate	Reticulate	Foveolate
Seeds	With imperfect aril	With imperfect aril	With sarcotesta
Ovary placentation	Parietal	Incompletely axile	Parietal

intermediate between those of *P. luquanensis* and *P. polyphylla* var. *yunnanensis*, *P. mairei* and *P. thibetica*, and *P. mairei* and *P. daliensis* have also been observed in the herbarium and in the field (Y. Ji, Kunming Institute of Botany, China, unpubl. res.). This suggests that natural hybridization occurs among at least some of these species.

Comparison of the morphology of the eight putative hybrids with nuclear and plastid topologies suggests that the ITS topology is consistent with the morphology of *P. thibetica*, whereas the plastid topology is consistent with that of the other taxa. *Paris thibetica* is unique in the genus in having characters that are intermediate between the species of section *Axiparis* and other capsular species (Table 3). Moreover, the species is sister to the clade of section *Axiparis* in plastid DNA topologies (Figs 2 and 3) but occupies the first diverging lineage within the clade consisting of capsular species in the ITS topology (Fig. 4). As such, *P. thibetica* is possibly a cross-bred derivative between an ancestor from section *Axiparis* and a capsular species, and 'chloroplast capture' (Soltis and Kuzoff, 1995) in an introgressant or maternal inheritance in the hybrid species could be the cause for the incongruence. Because the plastid topology best reflects phylogenetic predictions based on morphology for the other seven taxa, it is hypothesized that the discordance between nuclear and plastid lineages results from nuclear introgression without gene flow from the plastid genome. As Wagner *et al.* (1987) suggested, nuclear genes might be able to cross some species boundaries that plastid DNA is unable to cross. This hypothesis can be further tested in *Paris* through phylogenetic analysis of low-copy nuclear genes (e.g. *waxy*) and intensive sampling from putative hybrid populations.

Generic circumscription

The treatment of a single genus or three genera is an unresolved question in the taxonomy of *Paris* (Hara, 1969; Takhtajan, 1983; Li, 1984, 1998; Mitchell, 1987, 1988; Farmer and Schilling, 2002). According to the general principles of classification outlined by Backlund and Bremer (1998), a genus should be not only monophyletic with strong statistical support, but should also be recognizable from morphological characters. In the present study, *Paris* as circumscribed by Hara (1969), Li (1984, 1998) and Mitchell (1987, 1988) is consistently monophyletic in all separate and combined analyses of ITS, *trnL-trnF* and *psbA-trnH* data sets with strong bootstrap support. Furthermore, in all species of *Paris*, pollen is ellipsoidal (versus spherical in *Trillium*), and the pollen aperture is monosulcate (versus inaperturate in *Trillium*) (Takahashi, 1982, 1984; Wei, 1988, 1998; Wei and Wang, 2001). Likewise, the three segregated genera *Kinugasa*, *Paris s.s.*, and *Daiswa* recognized by Takhtajan (1983) are each supported as monophyletic in all analyses except those of *trnL-trnF* and combined plastid DNA (in both of which relationships are partly unresolved). *Paris* (i.e. *sensu lato*) is supported by the probable morphological synapomorphy of 4- to 15-merous flowers and leaves (Hara, 1969; Li, 1984, 1998; Mitchell, 1987, 1988), as compared with the 3-merous condition of *Trillium*. Moreover, there are putative morphological synapomorphies for each of the segregate genera of Takhtajan (1983) (Table 1). On the basis of both monophyly and recognition by morphological characters, either classification is justifiable. The issue is nonetheless resolvable if one recognizes the strongly supported sister clade of *Paris* as a single genus, as have recent workers (as *Trillium*; Kato *et al.*, 1995*a*, *b*; Kazempour Osaloo and Kawano, 1999; Kazempour Osaloo *et al.*, 1999; Farmer and Schilling, 2002). If this clade is to be recognized at the genus level, then it follows logically that its sister clade (*Paris s. l.*) should be recognized at the level of genus.

Infrageneric division

Subgenera. The two subgenera recognized by Li (1984, 1998) do not accord with the topologies of *Paris* from all separate and combined analyses (Table 4). They become monophyletic only upon transfer of the species of section *Axiparis* subgenus *Paris* to subgenus *Daiswa*. In all other respects the present data support the subgeneric treatment of Li (1984, 1998). Thus, the previous use of both axile and incompletely axile placentation together to define subgenus *Paris* (Li, 1984, 1998) must be reassessed. Section *Axiparis* is unique in the genus *Paris* in its incompletely axile placentation (Li, 1984, 1998; Table 3). All 21 species of *Trillium* that have been examined for placentation type have incompletely axile placentation (Y. Ji, Kunming Institute of Botany, China, unpubl. res.). As concluded from Fig. 6A, incompletely axile placentation is most likely to be the plesiomorphic state in the genus *Paris*, and axile and parietal placentation thus define subgenus *Paris* and the rest of the species in subgenus *Daiswa*, respectively.

The clade comprising the species of section *Axiparis* and those of subgenus *Daiswa* is supported by several characters. The plant height of the species of section *Axiparis* is >40 cm, similar to the species of subgenus *Daiswa*,

TABLE 4. *Bootstrap support for major clades in each of the analyses of* Paris

	trnL-trnF	*psbA-trnH*	ITS	*trnL-trnF* + *psbA-trnH*	*trnL-trnF* + *psbA-trnH* + ITS
Clade A [sect. *Kinugasa* (= *Kinugasa*) + sect. *Paris* (= *Paris. s.s.*)]	68 %	83 %	92 %	93 %	83 %
Sect. *Paris* (= *Paris s.s.*)	Polytomy	88 %	59 %	Separate	74 %
Clade B [(subg. *Daiswa* + sect. *Axiparis*) = *Daiswa*]	98 %	79 %	100 %	98 %	100 %
Sect. *Axiparis* + *P. thibetica*	70 %	61 %	Separate	83 %	*P. thibetica* excluded
Sect. *Axiparis*	Polytomy	53 %	Separate	65 %	77 %[1]
Sect. *Dunnianae* + sect. *Fargesianae* + sect. *Marmoratae* + sect. *Euthyra*	82 %	79 %	68 %	99 %	100 %[2]

[1] with *P. dulongensis* excluded.
[2] with *P. daliensis*, *P. dulongensis*, *P. luquanensis*, *P. mairei*, *P. marmorata*, *P. polyphylla* var. *polyphylla* and *P. polyphylla* var. *yunnanensis* excluded.

TABLE 5. *Comparison of morphology among the five recircumscribed sections*

	Subgenus *Daiswa*			Subgenus *Paris*	
	Sect. *Euthyra*	Sect. *Thibeticae*	Sect. *Axiparis*	Sect. *Paris*	Sect. *Kinugasa*
Rhizome	Thick	Thick	Thick	Long and slender	Thick
Stamens	2- to 6-whorled	2-whorled	2-whorled	2-whorled	2-whorled
Ovary shape	Angular	Angular	Angular	Rounded	Angular
Placentation type	Parietal	Parietal	Incompletely axile	Axile	Axile
Fruit	Capsule	Capsule	Berry	Berry	Berry
Seeds	With sarcotesta	With imperfect aril	With imperfect aril	Without sarcotesta or aril	Without sarcotesta or aril
Pollen ornamentation	Foveolate	Reticulate	Reticulate	Reticulate	Gemmate

whereas that of the species of subgenus *Paris* is <40 cm (Li, 1998; Liang and Soukup, 2000), as are all *Trillium* species. The seeds have an imperfect aril in section *Axiparis* and a sarcotesta in subgenus *Daiswa*. The presence of a thick outer covering in these two sections is probably a synapomorphy that unites these two groups (Fig. 6B). The basic karyotype of section *Axiparis* is $k(2n) = 6m + 4t$, which is similar to that of subgenus *Daiswa*. In contrast, other species of subgenus *Paris* are $k(2n) = 6m + 2t + 2st$ (Li *et al.*, 1988, 1998). Finally, both section *Axiparis* and subgenus *Daiswa* are distributed in tropical and subtropical areas, from northern Vietnam to southern, central and eastern China and the Hengduan Mountains and eastern Himalaya. The other species of subgenus *Paris*, in contrast, are concentrated in north-temperate regions of Asia and Europe (Li *et al.*, 1988, 1998).

Sections. From the result of the combined plastid DNA and ITS analysis (Fig. 5), sections *Kinugasa*, *Paris* and *Axiparis* are monophyletic. Sections *Kinugasa* and *Paris* are also monophyletic in both the *psbA-trnH* and ITS analyses, and section *Axiparis* is also monophyletic in both the *psbA-trnH* and combined plastid DNA analyses (Table 4). The monophyly of these three sections and their distinctive characters (Table 5) justify their delimitation by Li (1998). The species of sections *Dunnianae*, *Fargesianae*, *Marmoratae* and *Euthyra* form a clade in all separate and combined analyses (Figs 1–5 and Table 4), which suggests that the previous delimitation of these sections (Li, 1998) must be reassessed. These sections share a capsular fruit, seeds with sarcotesta and pollen with foveolate ornamentation. Hence, section *Euthyra* should be expanded to accommodate all the species of the three sections. *Paris thibetica* is sister to the clade of Section *Axiparis* in the *psbA-trnH* and combined plastid DNA analyses, whereas it is sister to the clade of the other capsular species (the revised section *Euthyra*) in the ITS analysis. Because of its problematic morphology (Table 3) and the possible hybridization events involving this species as previously discussed, this species is retained here in its own section (*Thibeticae*).

Based on the present study, the revised infrageneric system of *Paris* includes two subgenera: subgenus *Paris* and subgenus *Daiswa*. The former comprises sections *Kinugasa* and *Paris*, the latter sections *Axiparis*, *Thibeticae* and *Euthyra*. All five sections are distinctive on the basis of morphology (Table 5).

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