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Reassessing the relationships between *Gordonia* and *Polyspora* (Theaceae) based on the combined analyses of molecular data from the nuclear, plastid and mitochondrial genomes

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Abstract. The combined analyses, based on ITS, *trn*L-F and *mat*R DNA sequence data respectively from the nuclear, plastid and mitochondrial genomes, reveal that *Gordonia* is not a monophyletic group, and on the contrary, distributed in two major lineages in Theaceae. The only North American species, *G. lasianthus*, is located in Gordonieae together with *Schima* and *Franklinia,* whereas the Chinese *Gordonia* species are positioned in Theeae together with *Camellia, Pyrenaria* s.l. and *Apterosperma*. This result, to great extent, supports the viewpoints of separating the North American and Asiatic *Gordonia* species into two different genera, *Gordonia* s.str. and *Polyspora,* respectively.

Key words: ITS, *trn*L-F, *mat*R, *Gordonia*, *Polyspora*, phylogenetic relationship, Theaceae.

Introduction

Gordonia and *Polyspora* belong to the family Theaceae. The name *Gordonia* was first given to a plant from southeastern North America, *Gordonia lasianthus* (L.) Ellis (1771, cited by Keng 1984). This species has long pedicels, three or four caducous bracteoles near the apex of the pedicel, five rounded sepals that are distinct from the bracteoles, forming a campanulate calyx persistent at postanthesis, five petals distinct from the sepals, irregularly united stamens, a conical tomentose ovary, and a stout style with a five-lobed stigma (Sealy 1958; Keng 1980, 1984).

It is interesting that later described species in *Gordonia* were all from Asia, and several Asian genera have thereafter been merged with *Gordonia,* of which *Polyspora* Sweet (1826, cited by Keng 1984) is the earliest synonym. *Polyspora* was named after a plant originally described under the name *Camellia axillaris* Roxb. ex Ker Gawl. This species was described on cultivated plants from India. Its native home as later studies revealed is, however, S. China and Hong Kong, This plant has an extremely short pedicel which is completely hidden at first by the bracteoles; these are not distinct from the sepals, but with them form a gradual series of about 10 perules which protect the flower in bud. The lowermost perules, however, are deciduous, while the uppermost are persistent in fruit and presumably represent the sepals. The style is stout and pentafid at the apex (Sealy 1958; Keng 1980, 1984).

Pitard (1902) was the first to present the idea that *Gordonia* should be divided into two genera: *Gordonia* s.str., containing the sole North American species, *G. lasianthus*, and *Nabiasodendron*, containing the other species, all Asiatic. A key point was his discovery and emphasis of the subepidermal origin of periderm in *Gordonia* s.str., *Schima* and *Franklinia*, a condition common in Ternstroemioideae. *Nahiasodendron*, on the contrary, have a "pericyclic" origin of the periderm, the condition common to the remaining members of Camellioideae (or Theoideae). Based on non-molecular data, some authors (Airy-Shaw 1936; Ohwi 1941; Melchior 1964; Gregor 1978a, b; Kvacek and Walther 1984; Mai and Walther 1985; Ye 1990) supported a separation of the North American and Asian species into different genera, and they selected *Polyspora*, the earliest synonym for the Asiatic *Gordonia* species, as the generic name including the Asian species.

Recently, based on chloroplast *rbc*L and *mat*K sequence data, Prince and Parks (2001) rejected the monophyly of *Gordonia*. Nowadays, combined multigene analysis from different genomes is becoming an important tendency in phylogenetic study and a combined data set may give a more robust result than analyses of each genome separately (Anderberg et al. 2002). The addition of nuclear and mitochondrial data may provide more compelling evidence than the plastid genome alone especially if lineage sorting or hybridization is suspected. Therefore, in this paper, we employed three DNA sequences, ITS, *trn*L-F and *mat*R, from the nuclear, plastid and mitochondrial genomes, respectively, to reassess the phylogenetic relationship between *Gordonia* and *Polyspora*.

Some authors (e.g. Sealy 1958; Keng 1980, 1984) suggested inclusion of *Laplacea*, a genus distributed in Central and South America and Malesia, into *Gordonia*. Because no materials of *Laplacea* were available, the present study does not deal with this genus. Recent studies based on molecular data did not find Theoideae (or Camellioideae) and Ternstroemioideae, the two core members of Theaceae, to be sister to each other, and suggested to treat the two subfamilies as separate families, respectively (Morton et al. 1996, 1997; Savolainen et al. 2000; Soltis et al. 2000; Prince and Parks 2001; Anderberg et al. 2002). In the following Theaceae will be used in the strict sense (=Theoideae sensu Cronquist 1981).

Materials and methods

Taxa. Thirty-two taxa were included in the present three-gene study. All genera of Theaceae and most genera of Ternstroemiaceae (= Ternstroemioideae sensu Cronquist 1981) were well represented, Because recent molecular data analyses (Morton et al. 1996, 1997; Soltis et al. 2000; Prince and Parks 2001; Anderberg et al. 2002) have not provided statistical evidence for determining which taxon is the closest relative of Theaceae, Ternstroemiaceae, the traditional relative, is selected as outgroup in present study. The sample of taxa is somewhat different in the three individual data sets because it was not always possible to obtain polymerase chain reaction (PCR) products for all genes. The taxa used in this study, and voucher information for newly determined sequences, and their GenBank accession numbers are listed in Table 1. All voucher specimens for the newly determined sequences were deposited in the Herbarium of Kunming Institute of Botany (KUN) of the Chinese Academy of Sciences, Kunming, Yunnan, P. R. China.

Molecular methods. Total DNA was extracted from fresh or silica-gel-dried leaves using the CTAB method (Doyle and Doyle 1987). Polymerase chain reaction (PCR) was conducted in GeneAmp 9600 (PE Applied Biosystems, Foster City, CA, USA) to amplify the entire ITS region (including ITS1, 5.8S gene, and ITS2), the entire *trn*L-F region (including the *trn*L intron, *trn*L3′ exon and *trn*L-F spacer), and the *mat*R gene.

Table 1. Voucher information for taxa included in the investigation on the relationships between *Gordonia* and *Polyspora*

Taxa	Voucher	Locality**	Accession number		
			ITS	*trn*L-F	*mat*R
THEACEAE					
Apterosperma oblata H.T. Chang	S.X. Yang s.n.	Guangdong, China	AY070324*	AY214934	AY163755
Camellia fascicularis H.T. Chang			AF3 15485*		
Camellia henryana Coh. St. var. *trichocarpa* (H.T. Chang) Ming	S.X. Yang 95684	Yunnan, China		AY214935	AY163729
Camellia sinensis (L.) Kuntze			AF3 15492*		
Camellia yunnanensis (Pitard ex Diels) Cohen Stuart var. *camellioides* (Hu) Ming	S.X. Yang 93408	Yunnan, China	AF456256	AF534659	AY163744
Franklinia alatamaha Marshall	J.B. Yang s.s.	AA, US	AY096016*	AF534671	AY163731
Gordonia lasianthus L.	S.X. Yang 991006	Florida, US	AF456254	AY214936	AY163735
Hartia sinensis Dunn	S.X. Yang 98913	Yunnan, China	AF456261	AF534672	AY163738
Hartia villosa (Merr.) Merr.	S.X. Yang 98924	Guangxi, China	AF456262		
Parapyrenaria multisepala (Merr. et Chun) H.T. Chang	S.X. Yang 97792	Hainan, China	AF456263	AY2 16568	AY163742
Polyspora axillaris (Roxb.) Dietr.	S.X. Yang 97785	Hainan, China	AY214930	AY214937	
Polyspora chrysandra Cowan	S.X. Yang s.n.	Yunnan, China	AY214931	AF534678	AY163741
Polyspora hainanensis H.T. Chang	S.X. Yang 97789	Hainan, China	AY214932	AY216566	
Polyspora longicarpa (Chang) C.X. Ye	S.X. Yang 98911	Yunnan, China	AF456264	AY214938	
Polyspora tonkinensis Pitard	S.X. Yang 98970	Hunan, China	AY214933	AY216563	AY163728
Pyrenaria yunnanensis Hu	S.X. Yang 97797	Yunnan, China	AF456270		AY163730
Schima khasiana Dyer	S.X. Yang *et al.* 102	Yunnan, China	AF456269	AF534680	AY163740
Schima superba Gardner et Champ.			AF354641*		
Stewartia gemmata Chien et Cheng	S.X. Yang 98947	Hunan, China		AY216565	AY163732
Stewartia ovata (Cavanilles) Weatherby f. *grandiflora* (Bean) Kobuski	J. B. Yang s.n.	AA, US	AF339861*	AY216564	
Stewartia pseudocamellia Maxim.			AF339863*		
Stewartia rostrata Spongb.	S.X. Yang 991005	Jiangxi, China	AF456271		
Stewartia serrata Maxim.	S.X. Yang s.n.	Yunnan, China			AY163736
Tutcheria spectabilis (Champ.) Dunn	S.X. Yang 97749	Guangxi, China	AF456280	AY216569	AY163743
TERNSTROEMIACEAE					
Adinandra hainanensis Hayata	S.X. Yang 97786	Hainan, China	AF456255		
Adinandra hirta Gagnep.	S.X. Yang 98799.	Yunnan, China		AF534657	AY163739

Table 1 (continued)

Taxa	Voucher	Locality**	Accession number		
			ITS	*trn*L-F	*mat*R
Anneslea fragrans Wall.	S.X. Yang 94554	Yunnan. China	AY096024*	AP534658	AY163734
Cleyera pachyphylla Chun et H.T.Chang	S.X. Yang s.n.	Yunnan, China	AY096025*	AF534664	AY163737
Eurya alata Kobuski	S.X. Yang 97772	Guangdong, China	AF456259		
Eurya handel-mazzettii H.T.Chang	S.X. Yang 991013	Yunnan, China		AF534667	AY163748
Euryodendron excelsum H.T. Chang	S.X. Yang 97774	Guangdong, China	AF456260	AP534668	AY163733
Ternstroemia gymnanthera (Wright et Arn.) Beddome	S.X. Yang 991001	Yunnan, China	AF456272	AF534683	AY163754

*sequences from GenBank; **AA: Arnold Arboretum.

Primers for PCR amplification or DNA sequencing are listed in Table 2. PCR products were separated with 1.5% agarose TAE gel and purified using Wizard PCR preps DNA Purification System (Promega Madison, WI, USA). Purified PCR products were sequenced using the Dideoxy Chain Termination method (Sanger et al. 1997) and an ABI PRISM^{TM} Bigdye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase FS (PE Applied Biosystems, Foster City, CA, USA). All protocols of DNA sequencing followed the manufacture's manual. Sequencing was performed using an ABI 310 DNA Sequencing System (PE Applied Biosystems, Foster City, CA, USA).

Phylogenetic analyses. The DNA sequences of the three data sets were aligned using the software Clustal X (Thompson et al. 1997) and Mega2b3 (Kumar et al. 2000) using default settings and then adjusted manually when necessary. The aligned sequences were analyzed using PAUP* (version 4.0, Swofford 1998). Phylogenetic analyses were performed using maximum parsimony (MP) and maximum likelihood (ML) with gaps treated as missing data. The MP analyses were performed by heuristic searches using Fitch parsimony. All searches consisted of 100 random taxon additions with TBR branch swapping, and MULPARS and ACCTRAN options were in effect. In the ML analysis, we used the following options: heuristic search, as-is addition sequence, the HKY85 base substitution model, statistical base frequencies, equal distribution of rates at variable sites and unenforced molecular clock. Starting branch lengths were obtained by using Rogers-Swofford approximation method. Bootstrap analyses (Felsenstein 1985) were performed in order to access the degree of support of each node revealed in the MP and ML trees with 1000 replicates. Characters were weighted equally in all phylogenetic analyses.

Results

Because the tree topologies from MP analyses and ML analyses are very similar or almost the same, the following descriptions will focus on the MP analyses. Subtle variations observed in ML analyses are marked in MP trees. Some phylogenetic information generated by the three data sets was compared in Table 3.

Table 2. Primers for PCR amplification and DNA sequencing

ITS	ITS4	5′ TCCTCCGCTTATTGATATGC3′
	ITS5	5′ GGAAGTAAAAGTCGTAACAAGG3′
*trn*L-F	trn c	5′ CGAAATCGGTAGACGCTACG3′
	trn f	5′ ATTTGAACTGGTGACACGAG3′
*mat*R	26F	5′ GACCGCTNACAGTAGTTCT 3′
	1858R	5′ TGCTTGTGGGCYRGGGTGAA 3′
	879F	5′ ACTAGTTATCAGGTCAGAGA 3′
	1002R	5′ CACCCACGATTCCCAGTAGT 3′

ITS data set. This data set included 27 taxa and 756 characters, of which 273 were parsimony-informative. Four most parsimonious trees of 732 steps were obtained with a consistency index (CI) of 0.6776 and a retention index (RI) of 0.8523. The strict consensus tree (Fig. 1) further confirmed the three lineages (Stewartieae, Gordonieae and Theeae) recognized by Prince and Parks (2001) in Theaceae. *Stewartia* and *Hartia* formed the first clade (Stewartieae-clade), with 100% bootstrap support (bs), which was sister to the rest comprising the Gordonieae and Theeae-clade (bs = 100%). The Gordonieae-clade was well-supported (bs = 100%) and was a trichotomy formed by *Gordonia* s.str., *Schima* and *Franklinia*. The Theeae-clade was also strongly supported (bs = 100%), and was composed of four well-supported monophyletic groups represented by *Apterosperma, Polyspora* (bs = 93%), *Camellia* (bs = 98%), and *Pyrenaria* s.l. (bs = 99%) (including *Pyrenaria, Tutcheria* and *Parapyrenaria*) respectively, in which *Apterosperma* was the basal branch, and *Polyspora* grouped with *Camellia* (bs = 84%), and then sister to *Pyrenaria* s.l. (bs = 58%). Only a minor change happened inside the Gordonieae-clade in the ML analysis (Fig. 1).

***Trn*L-F data set.** This data set included 22 taxa and 982 characters, of which 79 were informative. Sixty-three most parsimonious trees of 129 steps were obtained with CI = 0.9015 and RI = 0.9607. In the strict consensus tree (Fig. 2), the three lineages (Stewartieae, Gordonieae and Theeae) of Theaceae were strongly supported, but the relationships between the three clades were unresolved. *Gordonia* s.str. and *Polyspora* were distributed in different clades. *Gordonia* s.str., *Schima*, and *Franklinia* formed the Gordonieae-clade (bs = 99%). *Polyspora,* as a monophyletic group (bs = 60%), was positioned together with *Apterosperma, Camellia,* and *Pyrenaria* s.l. in the Theeae-clade (bs = 100%). In the ML analysis, some branches with low support value in the Theeae showed a little different topology, and Gordonieae and Stewartieae formed a sister group but with poor support (bs < 50%) (Fig. 2).

***Mat*R data set.** This data set included 20 taxa and 1727 characters, of which 24 were

Table 3. Comparison of phylogenetic information generated ITS, *trn*L-F and *mat*R data sets

Parameter	ITS data set	*trn*L-F data set	*mat*R data set
Sequences length range (bp)	603~658	896~930	1727
Characters of data matrix	756	982	1727
Invariant characters	416	872	1694
Variable characters	340	110	33
Parsimony-informative characters	273	79	24
Consistency index (CI)	0.6776	0.9015	0.9706
Retention index (RI)	0.8523	0.9607	0.9863

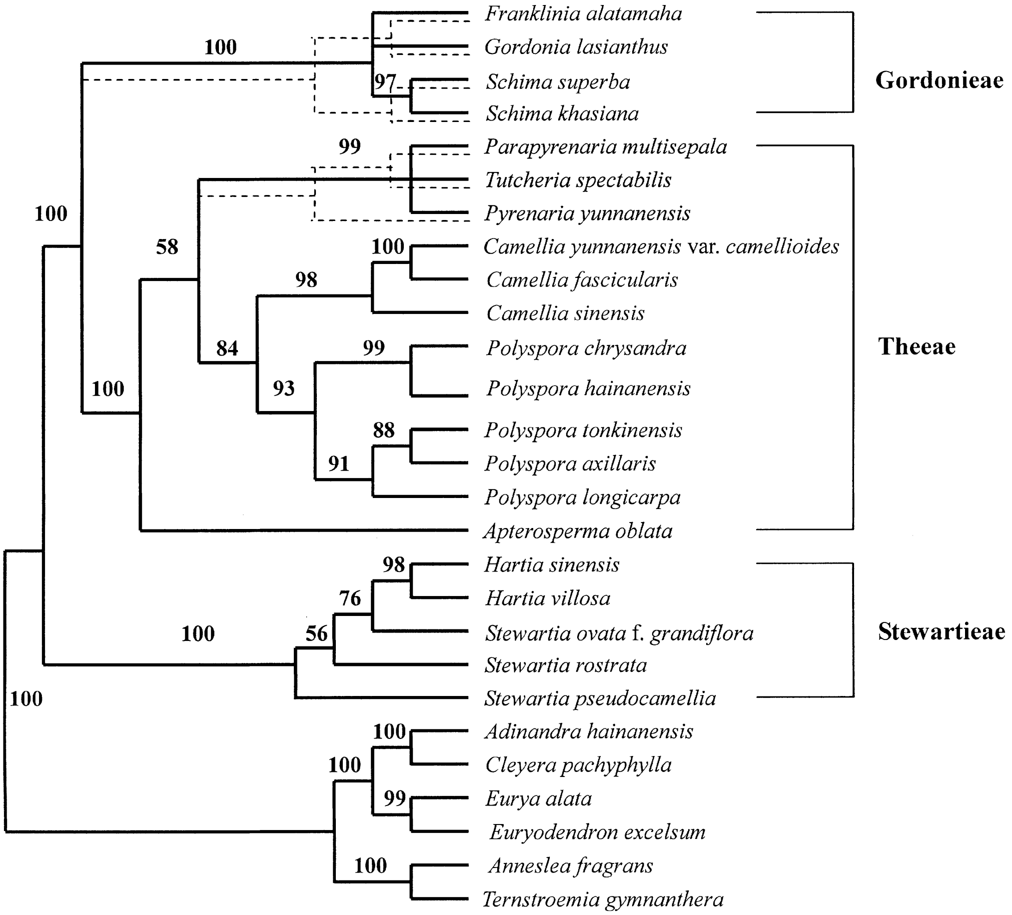

Fig. 1. The strict consensus tree of the two most parsimonious trees based on the ITS sequences treating gaps as missing data (732 steps, CI = 0.6776, RI = 0.8523). Numbers above the lines represent the bootstrap values in 1000 replicates. Dashed lines show the different topology from maximum likelihood analysis

informative. Only one most parsimonious tree of 34 steps was obtained with CI = 0.9706 and RI = 0.9863. The topology (Fig. 3) retained the three well-supported lineages in Theaceae (Stewartieae, Gorcionieae and Theeae) as well as the relationships between them being similar to the ITS data set (Fig. 1). *Gordonia* s.str. and *Polyspora* were located in Gordonieae-clade (bs = 89%) and Theeae-clade (bs = 92%), respectively.

Combined analysis. Fifteen taxa were included in this analysis and only one most parsimonious trees of 820 steps was obtained with Cl = 0.7584 and RI = 0.8120. The topology (Fig. 4) most closely mirrored the results from the separate analyses (Figs. 1-3). *Stewartia* and *Hartia* formed the basal Stewartieae-clade with bs = 100%. *Gordonia* s.str. and *Polyspora* were located in Gordonieae-clade (bs = 100%) and Theeae – clade (bs = 100%), respectively. The former genus was accompanied by *Schima* and *Franklinia,* and the latter was by *Apterosperma, Camellia* and *Pyrenaria* s.l.

Discussion

The combined and separate analyses based on the three DNA sequences data sets reveal similar topologies in which *Gordonia* s.str. and *Polyspora* do not form a monophyletic group, and on the contrary, are respectively distributed in two major lineages of Theaceae. The results suggest that the genus *Gordonia* is

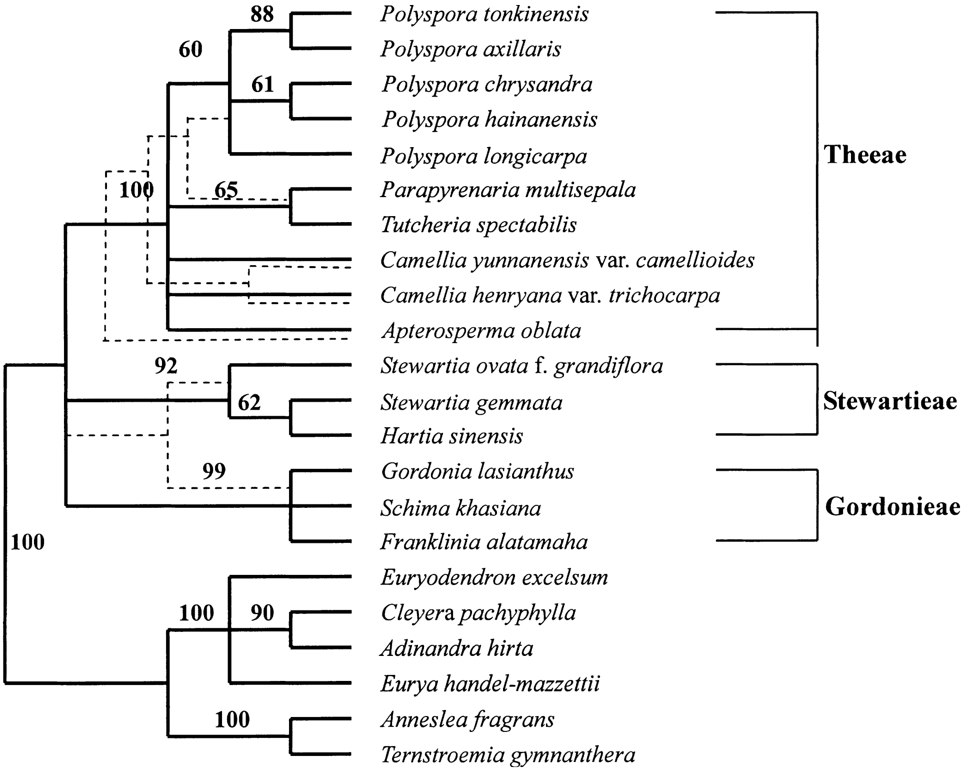

Fig. 2. The strict consensus tree of the 63 most parsimonious trees based on the *trn*L-F sequences treating gaps as missing data (129 steps, CI = 0.9015, RI = 0, 9607). Numbers above the lines represent the bootstrap values in 1000 replicates. Dashed lines show different topology from maximum likelihood analysis

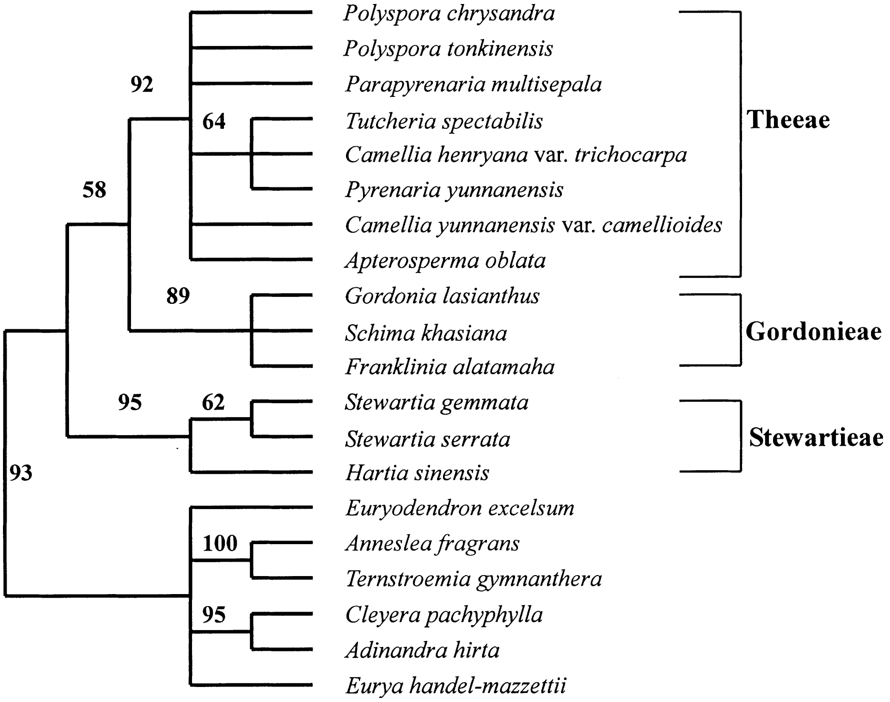

Fig. 3. The single most parsimonious tree based on the *mat*R sequences treating gaps as missing data (34 steps, CI = 0.9706, RI = 0.9863). Numbers above the lines represent the bootstrap values in 1000 replicates

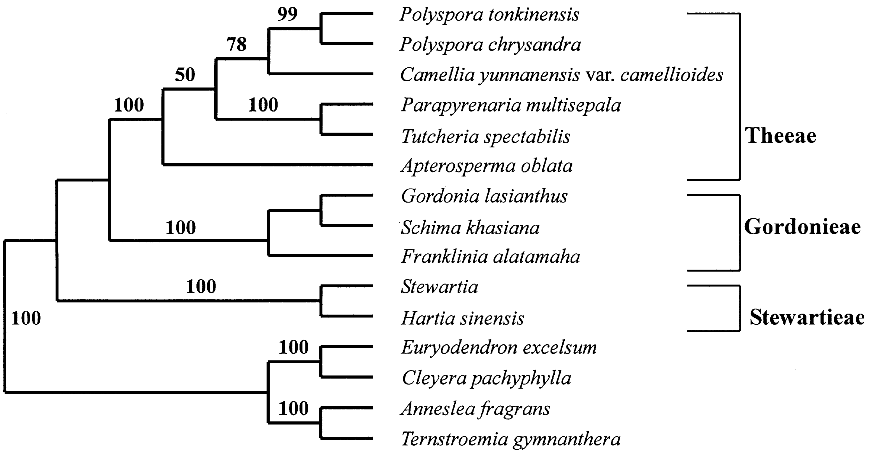

Fig. 4. The single most parsimonious tree based on a combined analysis of ITS, *trn*L-F and *mat*R sequences treating gaps as missing data (820 steps, CI = 0.7584, RI = 0.8120). Numbers above the lines represent the bootstrap values in 1000 replicates

paraphyletic as circumscribed as present. Together with *Franklinia* and *Schima, Gordonia* s.str, is located in Gordonieae, a position disband from *Polyspora*, which, combining with *Camellia, Pyrenaria* s.l. and *Apterosperma*, forms another major lineage, Theeae. These results provide new evidence for separating *Polyspora* from *Gordonia,* and at the same time, well mirror the differences between the two taxa in other aspects including the geographical disjunction,

As pointed out by Pitard (1902), in Theaceae, only *Gordonia* s.str., *Franklinia* and *Schima* show the subepidermal origin of periderm, while the rest of the genera including *Polyspora* share the "pericyclic" origin, In the analyses based on the three DNA data sets, the three genera (*Gordonia* s.str., *Franklinia* and *Schima*) with subepidermal origin of periderm exactly form a strongly supported monophyletic group (Figs. 1–3) and echo the anatomical homology. This result suggested that the difference in the origin of periderm is of phylogenetic significance in Theaceae, and is a valuable evidence for segregating *Gordonia.* The subepidermal origin of periderm is a good synapomorphy diagnosing Gordonieae in Theaceae.

Secondly, the differences in chromosome numbers between the American and Asian *Gordonia* species should be noted. Our earlier knowledge on chromosome numbers of *Gordonia* is restricted to four species, which share two different basic chromosome numbers, n = 15 and n = 18. The differences in the basic chromosome numbers just reflect the divergence between *Gordonia* s.str. and *Polyspora.* Three Asian species (*G. axillaris*, *G. excelsa* and *G. yunnanensis)* have 2n = 30 (n = 15) (Mehra and Sareen 1973; Mehra 1976; Oginuma et al. 1994a, b), and the only American species (*G. lasianthus*) has two different reports about its basic chromosome number, n = 15 (Santamour 1963) and the unique n = 18 (Bostick 1965). Although further study is needed to finally determine the chromosome number of *G. lasianthus,* it is possible that the earlier report of n = 15 (Santamour 1963) is erroneous, because n = 18 is the dominant basic chromosome number in *Schima* (Goldblatt 1981, 1988; Goldblatt and Johnson 1998). In four chromosome reports about *Schima wallichii*, only one is n = 15 (Malla et al. 1977), and the rest three reports as well as all the other species in this genus are n = 18. Recently we examined three Chinese species, *G. longicarpa, G. chrysandra* and *G. hainanensis,* all of which, unexceptionally, have n = 15 (Yang et al. to be published).

The mergence of *Polyspora* and *Gordonia* was mainly based on the overall similarity of the capsular fruits and the apically winged seeds. In all cases, the fruits are loculicidally dehiscent capsules, usually of five carpels. Mature fruits have a persistent columella and often retain some bracts and/or sepals. The seeds have an apical wing, a feature not found elsewhere in Theaceae. However, the morphological differences between both genera are obvious. G. *lasianthus*, the unique representative of *Gordonia* in North America, has long pedicels and clearly differentiated bracteoles and sepals (very similar to *Schima*), while *Polyspora* or the Asiatic species of *Gordonia* have extremely short pedicels, bracteoles gradually passing into sepals and forming a graduated series of about ten perules (Sealy 1958, Keng 1980). The present molecular analyses suggest that the morphological differences should not be underestimated or neglected, and the similarities in fruit and seed are possibly not homologous. Developmental studies on the seeds of the sister genus *Schima* of *Gordonia* s.str. and representatives of *Polyspora* by Tsou (1998) confirm a different pattern of wing development for these two genera, thus it is very necessary to add developmental data of *Gordonia* s.str.

Conclusion

Gordonia is not a monophyletic group and the North American and Chinese *Gordonia* species should be separated into two genera. *G. lasianthus*, the only North American species forms the monotypic genus *Gordonia* s.str., and the Chinese species might be classified into another genus which should be given the name *Polyspora*, the earliest synonym for the Asiatic *Gordonia* species, according to the law of priority of the International Code of Botanical Nomenclature.

Because of the limited sampling in the present study, more effort is required for finally clarifying the circumscription of *Gordonia* s.str. and *Polyspora*. It is possible that some non-Chinese Asiatic *Gordonia* as well as some *Laplacea* have to be included into *Gordonia* s.str. or *Polyspora*. Prince and Parks (2001) provided a significantly useful example. Their result revealed that *Gordonia lasianthus* and *G. brandegeei* form a separate strong branch from the remaining *Gordonia* s.l. taxa, which means that *Gordonia* s.str. may not be a monotypic genus. In fact, *G. brandegeei* is originally a member of *Laplacea* and is the new name of *Laplacea grandis* (Keng 1980). This result challenges the monophyly of *Laplacea*. A further and comprehensive investigation about *Laplacea* will be of great significance for confidently addressing the relationships between *Gordonia* s.str. and *Polyspora*.

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