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Phylogeny and morphological evolution of tribe Menispermeae (Menispermaceae) inferred from chloroplast and nuclear sequences

Wei Wang[a,b], Heng-Chang Wang[c], Zhi-Duan Chen[a,*]

[a]State Key Laboratory of Systematic and Evolutionary Botany, and Herbarium, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, PR China
[b]Graduate School of the Chinese Academy of Sciences, Beijing 100039, PR China
[c]Department of Systematics and Taxonomy, Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan, Hubei 430074, PR China

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Abstract

The Menispermaceae family contains ca. 72 genera with 450 species that are almost entirely tropical. Its phylogeny at the tribal level has never been examined using molecular data. Here we used DNA sequences of the chloroplast matK gene and trnL-F regions, and the nuclear ITS region to study the delimitation and position of the tribe Menispermeae within the family and its subtribal monophyletic groups. Family-wide phylogenetic analyses of the chloroplast data produced two strongly supported clades. The first clade contains two subclades: Coscinieae including Arcangelisia and Anamirta, and Tinosporeae sensu lato including Fibraureae, supported by morphological characters, such as traits of the cotyledon, stylar scar and embryo. The second clade consists of the tribes Menispermeae sensu DC. and Tiliacoreae Miers. All our analyses surprisingly recognized that tribe Menispermeae is not monophyletic unless tribe Tiliacoreae is included, suggesting that characters of cotyledon and stylar scar are very important for the infrafamilial classification, and that endosperm presence vs. absence was over-emphasized in traditionally tribal division of the family. Our topologies indicate a secondary loss of endosperm. The monophyly of two subtribes of the tribe Menispermeae, Stephaniinae and Cissampelinae, is supported by the cpDNA and ITS data, as well as by morphological characters, including aperture types and shapes, and colpal membrane features of pollen grains, and sepal number of male flowers. The Cocculinae was recognized as a paraphyletic group containing the remaining genera of the tribe Menispermeae.

Keywords: Cotyledon; Endosperm; Menispermeae; Menispermaceae; Molecular phylogeny; Stylar scar

Introduction

The Menispermaceae family (Ranunculales), consisting of ca. 72 genera with 450 species, is primarily restricted to the tropical lowlands and montane forests. There are also a few temperate outliers, most notably Menispermum, which is disjunct between eastern North America and eastern Asia. The family is characterized by dioecy, petiole often swollen at base, drupes with stylar scars (Miers, 1851; Diels, 1910; Kessler, 1993). The family has customarily been divided into tribes based largely on characters of the fruits (endocarp

*Corresponding author. Tel.: +8610 62836434; fax: +8610 62590843.
E-mail address: zhiduan@ibcas.ac.cn (Z.-D. Chen).

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morphology) and seeds (endosperm presence or absence and rumination and embryo straight or curved). In his monograph of the Menispermaceae family, Diels (1910) recognized eight tribes (Fig. 1), which were accepted by most subsequent workers, such as Troupin (1956) for the family in Africa, Krukoff and Barneby (1971) for America, Forman (1968, 1975, 1978, 1985) for Asia to the Pacific, and Luo (1996) for China. However, Forman (1975) considered that the traditional number of tribes was too high. Accordingly, Kessler (1993) presented a system with five tribes (Fig. 1), followed by Mabberley (1997) and Wu et al. (2003). Nevertheless, the subdivisions have not been evaluated in a phylogenetic context.

The main differences between the two classification systems of Diels (1910) and Kessler (1993) pertain to the taxonomic treatment of Anamirta, Coscinium and Arcangelisia (Fig. 1). Diels (1910) created the tribe Anamirteae including the above three genera, but the name was replaced by Coscinieae Hook. f. et Thoms. based on the priority rule of the International Code of Botanical Nomenclature (Forman, 1982). However, Kessler (1993) placed Arcangelisia in the tribe Tinosporeae, and Anamirta and Coscinium in the tribe Fibraureae. Pachygoneae of Kessler (1993) corresponds to the three tribes of Diels (1910), Triclisieae (= Tiliacoreae Miers; Forman, 1982), Hyperbaeneae and Peniantheae, except Pachygone (see below). All members in the group share endosperm absence (Fig. 1).

Another divergence from the traditional classification was proposed by Barneby (1972) – that the tribe Tinosporeae sensu lato should include the tribe Fibraureae (sensu Diels, 1910). This was accepted by Forman (1985) and Harley (1985), but other authors continued to retain Fibraureae as a distinct tribe (e.g. Kessler, 1993; Luo, 1996; Mabberley, 1997).

As for the tribe Menispermeae, the delimitation of Kessler (1993) is almost congruent with that of Diels (1910) except for the exclusion of Pachygone. Diels (1910) placed Pachygone in the tribe Menispermeae, followed by Forman (1968) and Luo (1996). In his system, Kessler (1993) omitted the genus from the family. However, Pachygone should be posited in his tribe Pachygoneae because this tribe accommodates the genera with seeds lacking endosperm (Kessler, 1993), and considered as type genus of the tribe according to the International Code of Botanical Nomenclature since Kessler adopted Pachygoneae instead of Triclisieae Diels (Wu et al., 2003). Menispermeae, comprising 16 genera, is characterized as follows: stylar scar near the base; endocarps variously sculptured; endosperm present, non-ruminate; seed-cavity usually horse-shoe-shaped; curved around a well-developed condyle; and cotyledons not foliaceous (Kessler, 1993; Wu et al., 2003). According to carpel number and perianth symmetry of female flowers, Diels (1910) divided this tribe into three subtribes: Cocculinae (2–6 carpels, rarely 1), Cissampelinae (1 carpel, asymmetrical perianths of female flowers) and Stephaniinae (1 carpel, symmetrical perianths of female flowers). Forman (1968) considered the division of these subtribes inappropriate, but pollen morphology supports the recognition of the Stephaniinae and Cissampelinae (Harley and Ferguson, 1982). A preliminary investigation on the phylogeny of the tribe Menispermeae (Hong et al., 2001), using ITS sequence data, suggested that the unicarpellate taxa formed a clade and others with 2–6 carpels were paraphyletic.

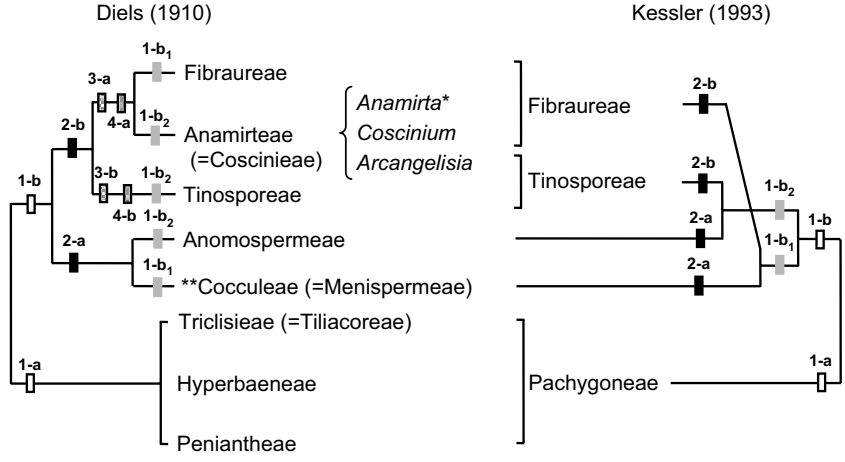

Fig. 1. Comparison of classification systems of Diels (1910) and Kessler (1993) of the Menispermaceae family. Boxes indicate morphological characters used by them: endosperm (1-a, absent; 1-b_1, non-ruminate; 1-b_2, ruminate), cotyledon (2-a, not foliaceous; 2-b, foliaceous), endocarp (3-a, not sculptured; 3-b, sculptured), perianth (4-a, not differentiated; 4-b, differentiated). *Indicates that endosperm in Anamirta is not ruminate like Fibraureae, but embryo is curved, distinguished from Fibraureae (Diels, 1910), and **shows that Pachygone without endosperm was placed in Menispermeae by Diels (1910) and omitted by Kessler (1993). The names in parentheses were corrected by Forman (1982).

The aims of this study were to investigate further the relationships of the genera traditionally placed in the tribe Menispermeae and to examine the monophyly of subtribes of Diels (1910) in the tribe using the chloroplast matK gene and trnL-F regions (trnL intron, and trnL [UAA] 3′ exon-trnF [GAA] intergenic spacer), and the internal transcribed spacer (ITS) of the nuclear ribosomal DNA. Furthermore, the chloroplast matK gene and trnL-F regions were used to examine the delimitation and systematic position of the tribe Menispermeae within the Menispermaceae family.

Materials and methods

Taxon sampling

We followed the system of Kessler (1993). Our study sampled 21 species of 10 out of 16 genera in the tribe Menispermeae, and nine representatives of three other tribes, Tinosporeae, Fibraureae and Pachygoneae (Table 1). We could not obtain material of Anomospermeae, the best characterized and most natural tribe within the family according to Kessler (1993), whose members are almost entirely neotropical. This sampling scheme represented the taxonomic diversity of the tribe Menispermeae sensu DC. and the diversity of morphological characters of the family, which were traditionally used for the classification of the Menispermaceae family, such as presence or absence of endosperm and embryo straight or curved, etc. (see below). Five species of Ranunculaceae and Berberidaceae, and two species of Lardizabalaceae were included in our sample set to serve as outgroups because they are most closely related to Menispermaceae (Hoot et al., 1999; Qiu et al., 1999; Hilu et al., 2003; Kim et al., 2004). Vouchers are deposited in the Herbarium, Institute of Botany, the Chinese Academy of Sciences, Beijing (PE).

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from silica-gel-dried leaves using the modified CTAB procedure of Doyle and Doyle (1987). The selected DNA regions were amplified with standard polymerase chain reaction (PCR). The matK gene, trnL-F and ITS regions were amplified using the primers matK-AF2 and matK-8R2 (Wang et al., accepted), trnF and trnR (Taberlet et al., 1991), and ITS-1 and ITS-4 (White et al., 1990), respectively. The PCR protocols used to amplify matK gene and trnL-F region followed Li et al. (2004), and ITS region followed Chen and Li (2004). The PCR products were purified using a GFX^{TM} PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA), were then directly sequenced. Additional primers, matK-mF2 and matK-mR2 for matK, were used for sequencing. Sequencing reactions were conducted using the DYEnamic^{TM} ET Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech). Sequences were analyzed using MegaBACE^{TM}1000 DNA Analysis Systems, following the manufacture's protocols. All sequences have been deposited at GenBank (see Table 1 for accession numbers). The resulting sequences were aligned using CLUSTAL X (Thompson et al., 1997), and further adjusted manually in BioEdit (Hall, 1999). Ambiguous positions were excluded from the phylogenetic analyses.

Phylogenetic analysis

In the first step, the chloroplast (matK and trnL-F) sequences were used to perform broader analyses on representatives of the Menispermaceae family to test the monophyly of the tribe Menispermeae and to explore their position within the family. Akebia quinata (Houtt.) Decne. and Sinofranchetia chinensis (Franch.) Hemsl. (Lardizabalaceae) were used as outgroups taxa in the analyses. A second series of analyses focused on the tribe Menispermeae, using Parabaena sagittata Miers and Tinospora sinensis (Lour.) Merr. as outgroups based on the results of the phylogenetic analyses of the cpDNA data. To test homogeneity between the chloroplast and nuclear data sets, 1000 replicates of the incongruence length difference test (ILD; Farris et al., 1994) was implemented in PAUP* 4.0b10 (Swofford, 2003) under the heuristic search constraints. Combinability of the two data sets prior to phylogenetic analysis was also assessed by visual comparison of the trees derived from the separate data partitions. If a taxon was placed in two different clades each with bootstrap support of 70% or higher in separate data partitions, these partitions would be considered incongruent (Mason-Gamer and Kellogg, 1996). Taxa causing such incongruence should thus be removed from the analysis (Vanderpoorten et al., 2003).

Because different analysis methods are sensitive to different biases in the data set, Baum et al. (1994) suggested that analyzing data with multiple algorithms is desirable and that clades consistently supported in different analyses might be considered more robust than those supported strongly by one search method but contradicted by another. Phylogenetic analyses for each matrix were carried out using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) methods in PAUP* version 4.0b10 (Swofford, 2003), PHYML version 2.4.3 (Guindon and Gascuel, 2003), and MrBayes version 3.0b4 (Ronquist and Huelsenbeck, 2003), respectively. For MP analyses, heuristic searches were conducted with 1000 replicates

Table 1. Species analysed in this study

Taxon	Voucher	Locality	GenBank Accession		
			matK	trnL-F	ITS
Menispermaceae					
Pachygoneae					
Albertisia laurifolia Yamamoto	Hong Y-P 99371	China, Hainan	EF143849	EF143880	EF143841
Pachygone valida Diels	Hong Y-P 99247	China, Yunnan	EF143850	EF143881	AY017393*
Pycnarrhena lucida (Teijsm. et Binn.) Miq.	Hong Y-P HN167	China, Hainan	EF143851	EF143882	EF143842
Tinosporeae					
Arcangelisia gusanlung Lo	Hong Y-P 99406	China, Hainan	EF143852	EF143883	
Aspidocarya uvifera Hook. f. et Thoms.	Hong Y-P 99190	China, Yunnan	EF143853	EF143884	
Parabaena sagittata Miers ##	Hong Y-P H346	China, Guizhou	EF143854	EF143885	
Tinospora sinensis (Lour.) Merr. ##	Wang H-C 109	Thailand, Samlan	EF143855	EF143886	
Fibraureae					
Anamirta cocculus (L.) Wight et Arn.	Wang H-C 103	Thailand, Chiang Mai	EF143856	EF143887	
Tinomiscium petiolare Hook. f. et Thoms.	Hong Y-P H142	China, Yunnan	EF143857	EF143888	
Menispermeae					
Cissampelos pareira L.	Wang H-C BN-001	China, Yunnan	EF143858	EF143889	EF143843
Cocculus laurifolius DC.	Hong Y-P 99401	South China Bot Gard (cult.)	EF143859	EF143890	AY017392*
Cocculus orbiculatus (Linn.) DC.	Hong Y-P H419	China, Guizhou	EF143860	EF143891	AY017391*
Cocculus trilobus (Thunb.) DC.	Hong Y-P H310	China, Guizhou	DQ478611	EF143892	EF143844
Cyclea barbata Miers	Hong Y-P 99405	China, Hainan	EF143861	EF143893	AY017405*
Cyclea hypoglauca (Schauer) Diels	Chen Z-D et al. 9812108	China, Guangdong	EF143862	EF143894	AY017406*
Cyclea polypetala Dunn	Hong Y-P 99379	China, Hainan	EF143863	EF143895	AY017407*
Cyclea tonkinensis Gagnep.	Hong Y-P 99242	China, Yunnan	EF143864	EF143896	EF143846
Cyclea wattii Diels	Hong Y-P 99235	China, Yunnan	EF143865	EF143897	EF143845
Diploclisia affinis (Oliv.) Diels	Hong Y-P H149	China, Guangxi	EF143866	EF143898	EF143847
Diploclisia glaucescens (Bl.) Diels	Hong Y-P 99403	South China Bot Gard (cult.)	EF143867	EF143899	AY017390*
Hypserpa nitida Miers	Hong Y-P 99378	China, Hainan	EF143868	EF143900	AY017388*
Menispermum dauricum DC.	Hong Y-P 99095	China, Beijing (cult.)	DQ478613	AF335293*	AY017395*
Pericampylus glauca (Lam.) Merr.	Chen Z-D et al. 9812095	China, Guangdong	EF143869	EF143901	AY017389*
Sinomenium acutum (Thunb.) Rehd. et Wils.	Hong Y-P H006	China, Henan	EF143870	EF143902	AY017394*
Stephania brachyandra Diels	Hong Y-P H043	Xishuangbanna Tropical Bot Gard (cult.)	EF143871	EF143903	AY017401*
Stephania cephalantha Hayata	Hong Y-P H231	China, Guangxi	EF143872	EF143904	AY017400*
Stephania chingtungensis Lo	Hong Y-P 99219	China, Yunnan	EF143873	EF143905	AY017397*
Stephania elegans Hook. f. et Thoms.	Hong Y-P 99191	China, Yunnan	EF143874	EF143906	AY017396*
Stephania longa Lour.	Hong Y-P H101	China, Guangxi	EF143875	EF143907	AY017399*
Stephania succifera Lo et Y. Tsoong	Hong Y-P 99421	China, Hainan	EF143876	EF143908	AY017403*
Stephania tetrandra S. Moore	Wang Wei 068	China, Guizhou	EF143877	EF143909	EF143848
Berberidaceae					
Caulophyllum robustum Maxim.			AB069832*	AF325911*	
Nandina domestica Thunb.			AB069830*	AF325912*	

Table 1. (continued)

Taxon	Voucher	Locality	GenBank Accession		
			matK	trnL-F	ITS
Ranunculaceae					
Caltha palustris L.			AB069845*	AJ496610*	
Enemion raddeanum Regel	Chen Z-D 2090	China, Jilin	AB069846*	EF143910	
Hydrastis canadensis L.	Chen Z-D 2002016	USA (cult.)	AB069849*	EF143911	
Lardizabalaceae					
Akebia quinata (Houtt.) Decne. #	Chen Z-D et al. 960637	China, Jilin	EF143878	AF335297*	
Sinofranchetia chinensis (Franch.) Hemsl. #	Wang Wei SX040	China, Shanxi	EF143879	AF335284*	

Tribe names according to Kessler (1993). *Sequence taken from GenBank. Outgroups for the broader and narrower phylogenetic analyses are indicated by # and ##, respectively.
Note: Omitted by Kessler (1993), Pachygone should be placed in the tribe Pachygoneae based on his classification (endosperm absent; see text).

of random addition, one tree held at each step during stepwise addition, tree-bisection-reconnection (TBR) branch swapping, MulTrees in effect, and steepest descent off. Gaps were treated as missing data, characters were equally weighted, and their states were unordered. Internal branch support was estimated with 1000 bootstrap replicates (Felsenstein, 1985) as described above. Likelihood analysis was performed in PHYML (Guindon and Gascuel, 2003) using a GTR substitution model with invariant sites and additional among site rate variation modeled as a discrete gamma distribution (Yang, 1994). ML parameter values were then optimized, with a BIONJ tree as a starting point (Gascuel, 1997) with the appropriate parameters. Nodal robustness on the ML tree was estimated by the non-parametric bootstrap (1000 replicates). Bayesian analyses were accomplished in MrBayes version 3.0b4 using the best-fit models [experimentally determined using Modeltest version 3.06 (Posada and Crandall, 1998)]. We ran four chains of the Markov Chain Monte Carlo, sampling one tree every 1000 generations for 1,000,000 starting with a random tree. The first 50 trees were the 'burn in' of the chain, and phylogenetic inferences were based on those trees sampled after generation 50,000. The posterior probability (PP) was used to estimate nodal robustness.

Morphological characters

The inference of character evolution was performed using parsimony method in MacClade 4.06 (Maddison and Maddison, 2003) with different characters coded as states of an unordered multistate character. The distribution of six morphological characters, upon which tribal classification of the Menispermaceae family has been traditionally based, was investigated. In addition, the distribution of eight morphological characters, upon which subtribal classification of the tribe Menispermeae have been based, was also examined. Information on morphological features was taken from the literature, particularly Diels (1910), Forman (1968, 1975, 1978, 1985), Kessler (1993) and Luo (1996) for general morphological characters, and Ferguson (1975) and Harley and Ferguson (1982) for pollen characters. Because sampling issues might affect the character reconstructions (Omland, 1999), characters of other taxa, not sampled in this study, are also consulted and compared.

Results

Analysis of Menispermaceae

The aligned matrix of matK sequences had 1266 nucleotides in length, 498 of which were variable sites and 320 parsimony-informative. 1204 positions were aligned for trnL-F data. After 60 ambiguous positions were excluded, 1144 characters were included in the phylogenetic analyses, 383 of which were variable and 230 parsimony-informative sites. Because there is no recombination in the chloroplast DNA, we combined the matK and trnL-F data in the analyses. The aligned matrix of the combined cpDNA data had 2410 characters with 881 variable and 550 parsimony-informative sites. Parsimony analysis generated three maximally parsimonious trees (MPTs) of 1511 steps, with a consistency index (CI) of 0.74, a retention index (RI) of 0.76, and a rescaled consistency index (RC) of

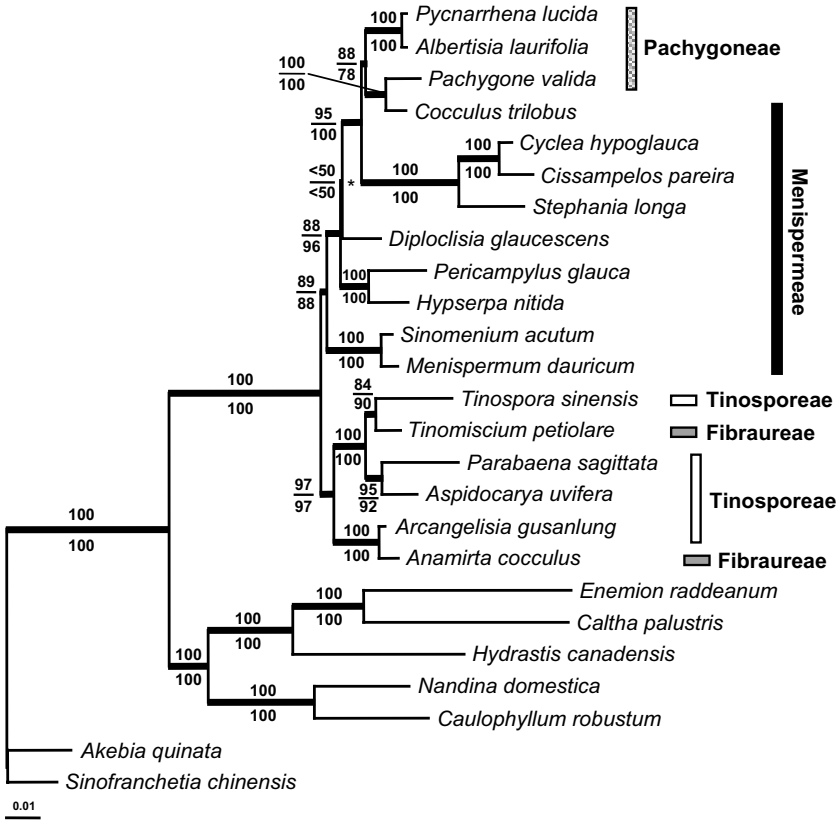

Fig. 2. ML tree inferred from the matK and trnL-F data. The results of ML bootstrap analysis are shown above the branches, whereas the values below the branches result from MP bootstrap analysis. Bootstrap values >50% are shown. The thick branches represent >95% Bayesian posterior probability. *Indicates the nodes not found in the strict consensus tree. Kessler's (1993) classification is shown on the right.

0.57. ML and BI analyses yielded identical trees that were also almost the same as those retrieved with MP analysis except that one node was collapsed in the consensus tree. Except the collapsed node, support of no node was less than 75% bootstrap (BS) values in MP and ML analyses, nor less than 95% PP in BI; bootstrap support values of three nodes differed by more than 5% between MP and ML (Fig. 2). The Menispermaceae family was monophyletic with strong support and two major clades were identified in the broader analyses. Menispermeae and Pachygoneae (sensu Kessler, 1993) formed a single clade. Within it, Pachygone (tribe Pachygoneae) and Cocculus (tribe Menispermeae) formed a clade, sister to the remaining Pachygoneae. Because these results indicate that the latter tribe does not include the type genus Pachygone, hereafter we apply the name Tiliacoreae Miers (Forman, 1982) to the remaining members of this tribe. The second major clade included two subclades: Arcangelisia (tribe Tinosporeae) and Anamirta (tribe Fibraureae) formed a subclade, and Tinomiscium (tribe Fibraureae) and Tinospora, Aspidocarya and Parabaena (tribe Tinosporeae) formed the other subclade (Fig. 2).

Character evolution in the Menispermaceae family

Six morphological character reconstructions are shown in Fig. 3. According to the character of cotyledon, the family may be divided into two groups, which agrees with molecular phylogenetic analyses (Fig. 3a): one with non-foliaceous cotyledons (tribes Menispermeae and Tiliacoreae) and the other with foliaceous cotyledons (tribes Tinosporeae and Fibraureae). Menispermeae and Tiliacoreae have the stylar scars near the base with exception of Pycnarrhena, whose stylar scar is on the terminal and ventral side. Taxa with terminal stylar scar and taxa with lateral stylar scar form a clade, respectively (Fig. 3b). The reconstruction of the evolution of endosperm indicates that endosperm absent is nested in non-ruminate endosperm, and ruminate endosperm occurs in Arcangelisia, Parabaena and Tinospora, distributed in three different clades (Fig. 3c). The straight embryo appears in Albertisia and most genera of the tribes Tinosporeae and Fibraureae, positioned in different clades in the cpDNA tree. The curved embryo appears in the tribe Menispermeae, Anamirta and Arcangelisia (Fig. 3d). Endocarp not sculptured

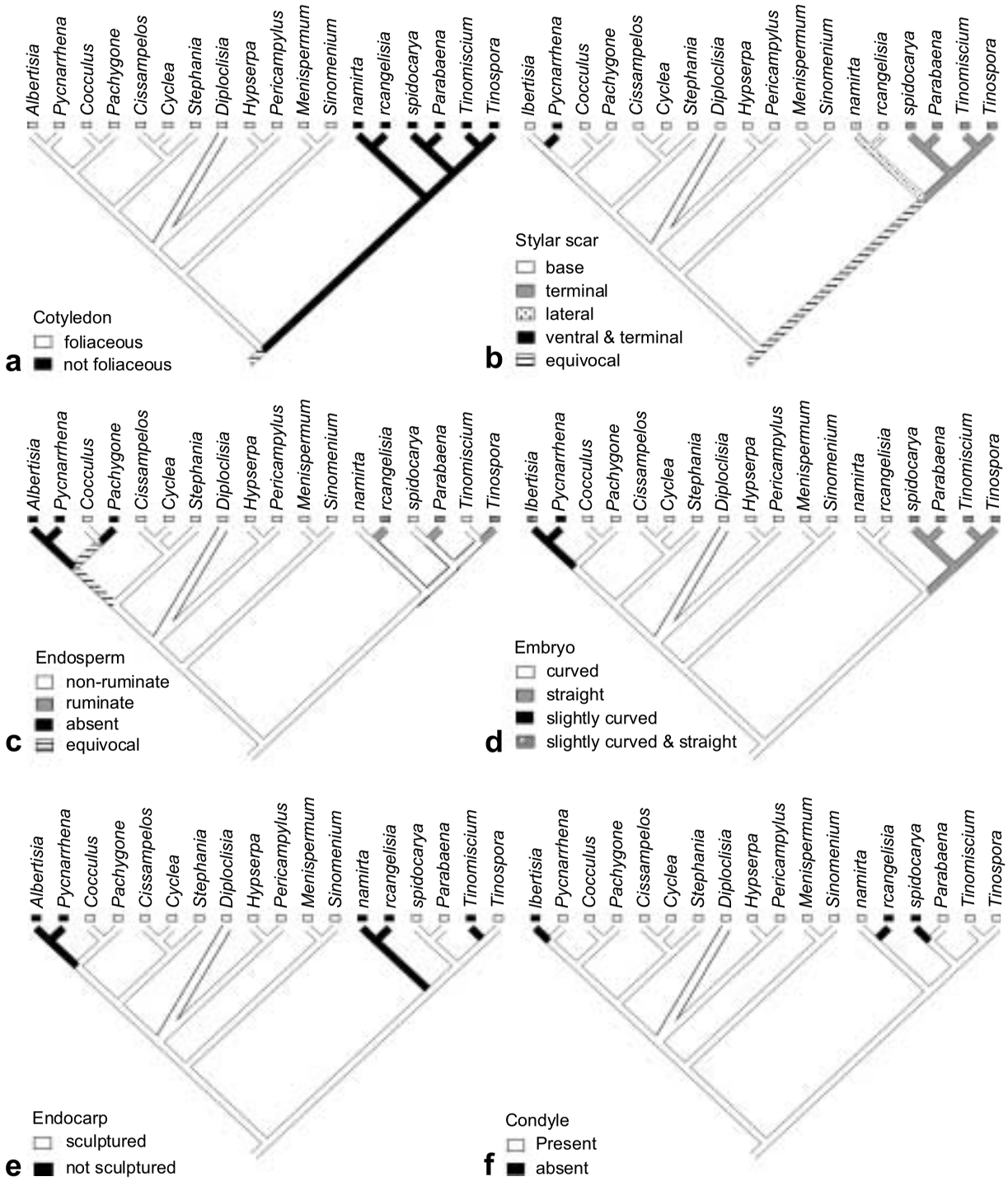

Fig. 3. Parsimony inference of character evolution using MacClade (Maddison and Maddison, 2003): (a) cotyledon; (b) stylar scar; (c) endosperm; (d) embryo; (e) endocarp; and (f) condyle.

(Fig. 3e) and condyle absent (Fig. 3f) occurs in three different clades.

Analysis of tribe Menispermeae

The aligned matrix of the matK sequences for the narrower analyses comprised 1236 aligned positions, of which 295 were variable and 184 parsimony-informative. The trnL-F data matrix had 1084 nucleotides in length. After 5 bp polyT tracks were excluded, 1079 characters were included in the phylogenetic analyses, 213 of which were variable sites and 120 parsimony-informative. The aligned matrix of the combined cpDNA data had 2315 characters with 508 variable

and 304 parsimony-informative sites. MP analysis produced six MPTs of 685 steps, with a CI of 0.84, RI of 0.91, and a RC of 0.77. ML and BI analyses resulted in identical trees that are highly congruent with those from MP analysis except that one node was collapsed in the consensus tree, Cyclea was monophyletic (BS = 51%) and Tiliacoreae was sister to a clade containing Cocculus, Pachygone, the Stephaniinae and Cissampelinae (BS = 55%). In addition, two nodes were supported with less than 75% BS in MP and ML analyses, of which one received less than 95% PP support in BI; bootstrap support values of two nodes differed by more than 5% between MP and ML (Fig. 4). The aligned matrix of ITS regions has a length of 714 characters, with 381 variable and 313 parsimony-informative sites. MP analysis produced six MPTs of 976 steps, with a CI of 0.65, RI of 0.80, and a RC of 0.52. ML and BI analyses resulted in identical trees that are largely congruent with those from MP analysis except that two nodes were collapsed in the consensus tree and Pachygone was sister to Cocculus orbiculatus (Linn.) DC. and Cocculus trilobus (Thunb.) DC. with poor support. Additionally, six nodes were supported less than 75% BS in MP analysis, of which five nodes were supported with less than 75% BS in ML analysis and five nodes received less than 95% PP support in BI; furthermore, bootstrap support values of five nodes differed by more than 5% between MP and ML (Fig. 5).

The ILD tests indicated that the cpDNA and ITS data sets were not incongruent (P = 0.17). However, conflict between the trees of the two data sets (Figs. 4 and 5) indicated a discrepancy in the position of Stephania chingtungensis Lo. This species was sister to Stephania cephalantha Hayata with strong support in the cpDNA data. Contrarily, it paired with Stephania succifera Lo et Y. Tsoong, which together with Stephania brachyandra Diels was strongly supported as a clade in the ITS data. We then excluded Stephania chingtungensis, and reanalyzed the chloroplast and ITS data independently. The ILD test gave a P value of 0.91. We thus performed the

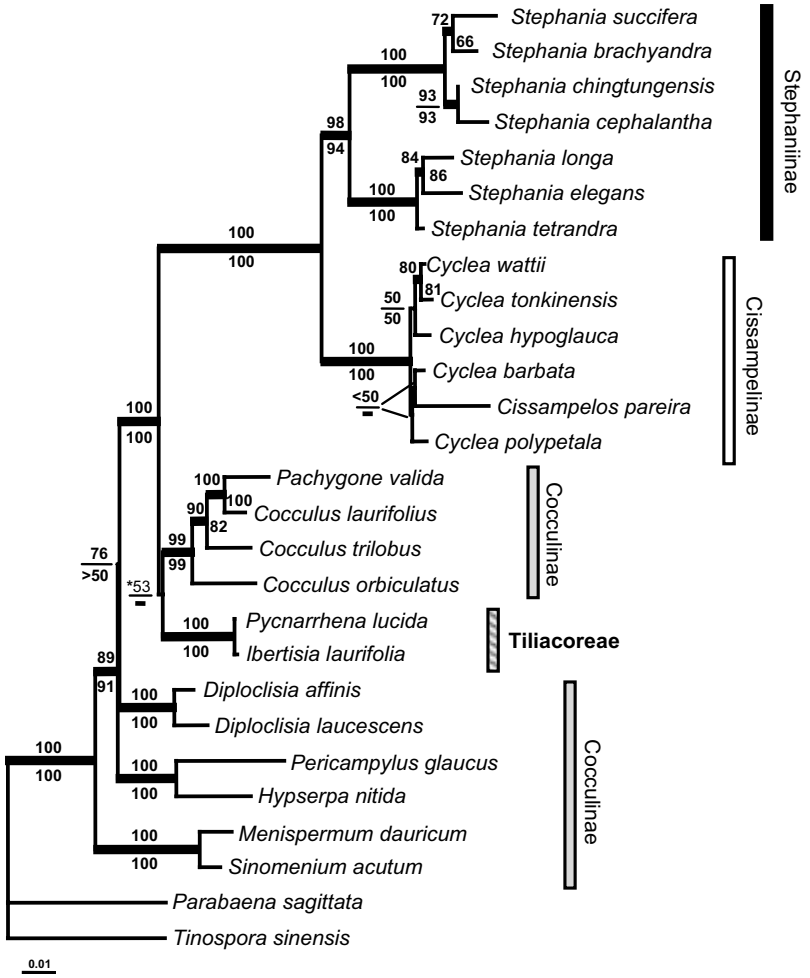

Fig. 4. ML tree inferred from the cpDNA data. The results of ML bootstrap analysis are shown above the branches, whereas the values below the branches result from MP bootstrap analysis. Bootstrap values >50% are shown. The thick branches represent >95% Bayesian posterior probability; *indicates the nodes not found in the strict consensus tree; –indicates the topological discordance of related clades between the ML and MP trees. Diels' (1910) classification is shown on the right.

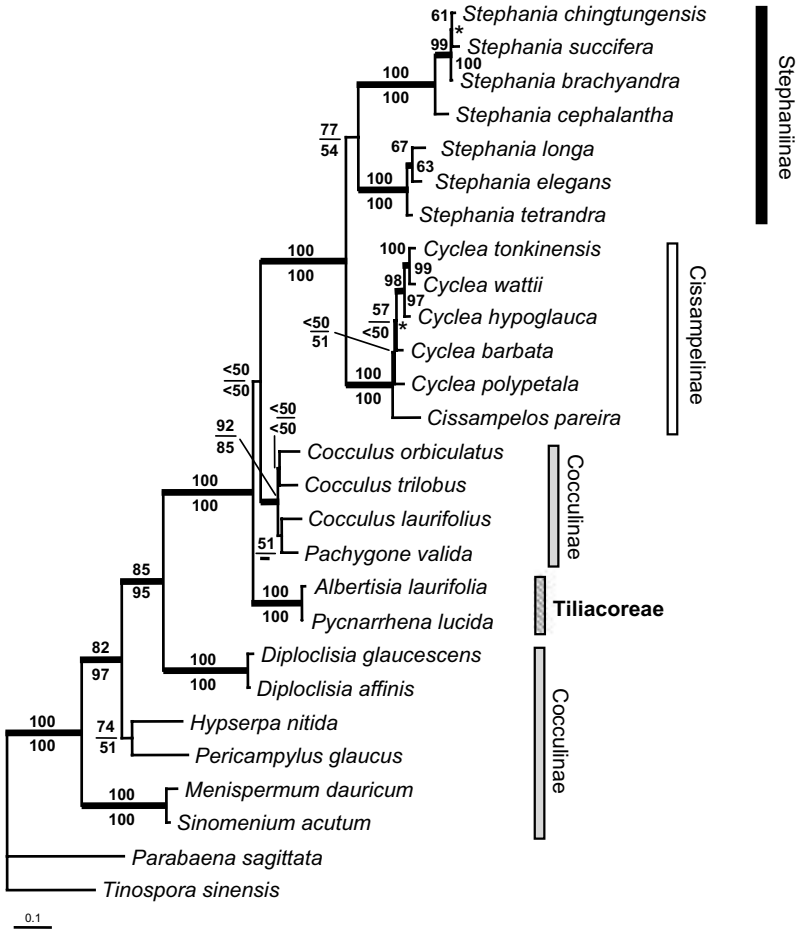

Fig. 5. ML tree inferred from the ITS data. The results of ML bootstrap analysis are shown above the branches, whereas the values below the branches result from MP bootstrap analysis. Bootstrap values >50% are shown. The thick branches represent >95% Bayesian posterior probability; *indicates the node not found in the strict consensus tree; –indicates the topological discordance of related clades between the ML and MP trees. Diels' (1910) classification is shown on the right.

combined analyses of the cpDNA and ITS sequences. The aligned matrix of the combined data consisted of 3029 positions, of which 888 were variable and 615 parsimony-informative sites. MP analysis produced three MPTs of 1659 steps, with a CI of 0.73, RI of 0.84, and a RC of 0.61. ML and BI analyses resulted in identical trees that are highly congruent with the tree of MP analysis except that two nodes were collapsed in the consensus tree. Additionally, support of one node was less than 75% in MP and ML analyses and 95% in BI, and bootstrap support values of five nodes differed by more than 5% between MP and ML (Fig. 6).

All data matrices, both separate and combined, produced trees with the almost same topology except the systematic position of the tribe Tiliacoreae and relationship between Cissampelos and Cyclea (Figs. 4–6). The Sinomenium–Menispermum clade was sister to the clade containing the remaining genera, followed by Pericampylus–Hypserpa. Diploclisia was sister to the clade containing the tribe Tiliacoreae plus Cocculus, Pachygone, Cyclea, Cissampelos and Stephania. Pachygone valida Diels was nested in Cocculus and sister to Cocculus laurifolius DC. The monophyletic Cissampelinae and Stephaniinae were both identified with strong support.

Character evolution in tribe Menispermeae

The occurrence of morphological characters in the tribes Menispermeae and Tiliacoreae, including pollen, was shown in Fig. 7. Eight such traits were plotted here against the strict consensus of three MPTs obtained from the combined chloroplast and ITS data. Taxa with one carpel and <6 stamens formed a clade containing the Stephaniinae and Cissampelinae. Triporate pollen apertures occur in the Stephaniinae, whereas the other two subtribes and the tribe Tiliacoreae are tricolporate. Sepal number of male flowers in the Cissampelinae is usually four to five, rarely six or three (Cyclea wattii Diels). Male flowers in the Stephaniinae and remaining taxa have six or more sepals, except Stephania tetrandra

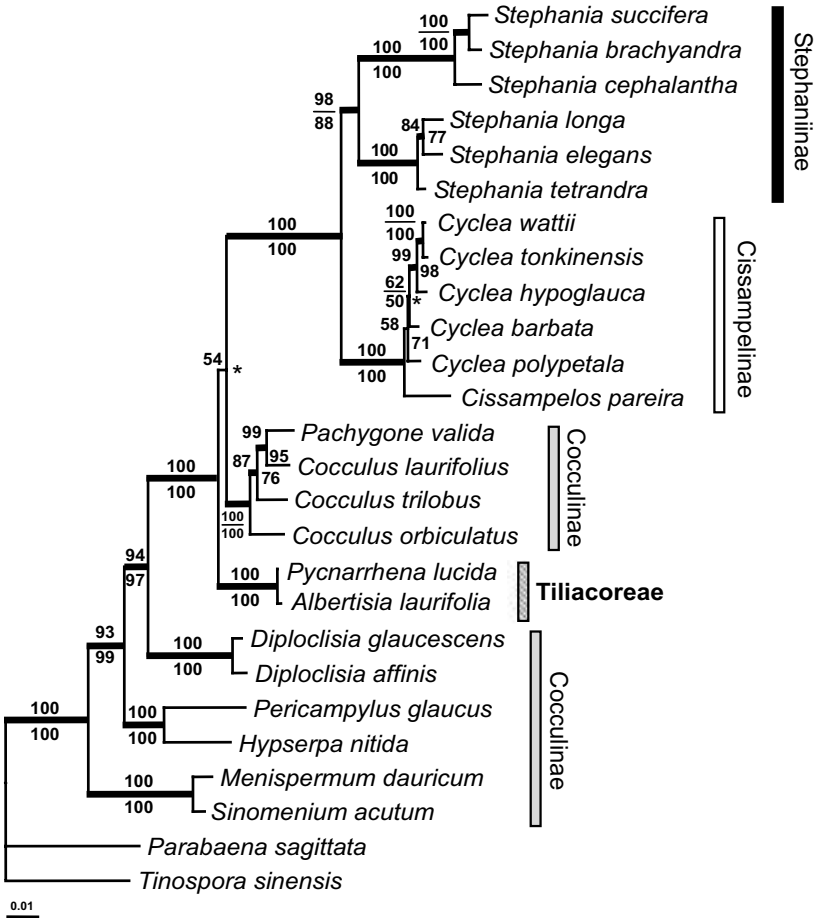

Fig. 6. ML tree inferred from the combined cpDNA and ITS data. The results of ML bootstrap analysis are shown above the branches, whereas the values below the branches result from MP bootstrap analysis. Bootstrap values >50% are shown. The thick branches represent >95% Bayesian posterior probability; *indicates the node not found in the strict consensus tree. Diels' (1910) classification is shown on the right.

S. Moore which has 4–5. Angulaperture and smooth colpal membrane of the Cissampelinae are contrary to fossaperture and granular, regulate or reticulate colpal membrane of the remaining genera. Synandrium and reticulate tectum occur in the Stephaniinae, Cissampelinae and tribe Tiliacoreae, and reticulate tectum occurs also in Pericampylus.

Discussion

Using the chloroplast sequences, we present a preliminary phylogenetic hypothesis for the Menispermaceae family (Fig. 2), which contrasts with the traditional classifications (Diels, 1910; Kessler, 1993). We know that for testing further the hypothesis, a comprehensive phylogenetic framework of the family is desired, but our hypothesis agrees with some morphological characters, such as cotyledon and stylar scar (Fig. 3a and b). Our sampling is poor in the tribes Tinosporeae and Pachygoneae. However, characters of cotyledon and stylar scar like Tinospora and Aspidocarya occur also in other genera of the tribe Tinosporeae (Forman, 1984; Kessler, 1993), not sampled in this study; the other Pachygoneae have foliaceous cotyledons like Albertisia (Kessler, 1993), and the stylar scar of some genera of the tribe is like Albertisia and tribe Menispermeae and of some genera like Pycnarrhena (Forman, 1975).

The establishment of tribe Coscinieae is strongly supported

The treatment of Anamirta, Coscinium and Arcangelisia differs notably between the classification systems of Diels (1910) and Kessler (1993) (Fig. 1). Diels (1910) established the tribe Anamirteae (= Coscinieae; Forman, 1982) for the three genera, whose key characters are the following: the perianth not differentiated into sepals and petals, the endocarp not sculptured, the cotyledons foliaceous and the endosperm ruminate. Although

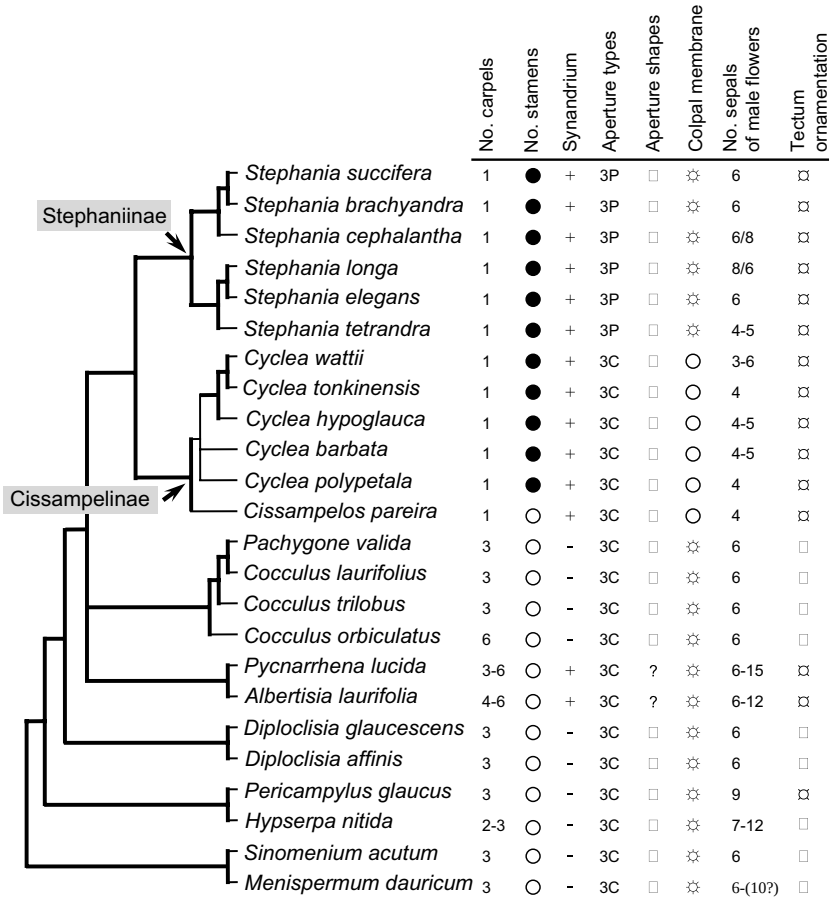

Fig. 7. Strict consensus of the shortest trees generated from the combined cpDNA and ITS data showing the distribution of selected character states. Branches in bold indicate BS > 75% in MP and ML trees and PP > 95% in BI. These characters are: number of carpels (1, 2–6), number of stamens (●, less than 6; ○, 6 or more), synandrium (+, present; −, absent), aperture type (3P, triporate; 3C, tricolporate), aperture shape (□, fossaperturate; □, angulaperturate; ?, missing data), sepal number of male flowers (4–5; 6 or more), colpal membrane (☼, granular, rugulate or reticulate; ○, smooth), tectum ornamentation (¤, reticulate; □, perforate).

Anamirta has non-ruminate endosperm like the tribe Fibraureae, its embryo is curved, distinguished from straight embryo of the tribe Fibraureae. The gross morphological characters (Forman, 1978), pollen morphology (Ferguson, 1978) and leaf-anatomical characters (Wilkinson, 1978) supported the placement of Arcangelisia close to Anamirta. However, Kessler (1993) abolished the tribe Anamirteae and placed Arcargelisia in the tribe Tinosporeae and Anamirta and Coscinium in the tribe Fibraureae, according to the characters of endosperm and cotyledon. Based on the classification of Kessler (1993), endosperm is ruminate in the tribe Tinosporeae, but not in the tribe Fibraureae. However, Coscinium has ruminate endosperm, distinguished from other members of Fibraureae of Kessler (1993), suggesting that the classification might be inappropriate. Our analyses strongly recognized that Arcangelisia and Anamirta formed a clade and the other four genera of the tribes Fibraureae and Tinosporeae studied formed another clade. Lateral stylar scar and curved embryo also support that the two genera are distinguished from other genera of the tribe Fibraureae and Tinosporeae. These results suggest that it is reasonable to accept the tribe Coscinieae.

Barneby's (1972) broader concept of tribe Tinosporeae is supported

Fibraureae was traditionally characterized by the perianth not differentiated into sepals and petals, the endocarp not sculptured and the endosperm not ruminate (e.g., Diels, 1910; Kessler, 1993; Luo, 1996; Mabberley, 1997). However, these characters are not synapomorphies for the tribe; Forman (1985) thus considered that tribe Fibraureae was one of the least well defined tribes within the family. The perianth of Arcangelisia in the tribe Coscinieae is also not differentiated into sepals and petals. In fact, the petals with their involute margins are clearly distinct from the sepals

in Tinomiscium (Forman, 1985; Luo, 1996). Endocarp not sculptured appears in the tribe Fibraureae, Arcangelisia and the tribe Tiliacoreae, distributed in three remote clades (Fig. 5e). Not only the tribe Fibraureae but also Aspidocarya (tribe Tinosporeae) and tribe Menispermeae have non-ruminate endosperm. Our reconstruction of the evolution of endosperm suggested that rumination has evolved independently at least three times (Fig. 5c). Boesewinkel and Bouman (1984) considered that rumination was due to irregular growth activity of the seed coat, or the endosperm itself, during later stages of seed development. Ruminate endosperm may occur in seeds of primitive and advanced taxa (Vijayaraghavan and Prabhakar, 1984). When describing the new genus Borismene, Barneby (1972) placed it in the tribe Tinosporeae, but he considered that it was most closely related to Fibraureae. His explanation was that tribe Tinosporeae sensu lato should include tribe Fibraureae. Like the tribe Tinosporeae, Fibraureae has a terminal stylar scar, the seed containing endosperm, and the embryo with thin and flat cotyledons. Thus, Forman (1985) considered that it seemed difficult to justify the recognition of the tribe Fibraureae as a distinct tribe and accepted Barneby's (1972) broader concept of the tribe Tinosporeae. Pollen characters also support this recommendation because distinctly elongate endoapertures, which is a comparatively uncommon form of endoaperture throughout the angiosperms, occurs in Tinomiscium and Fibraurea but also in Aspidocarya and Tinospora (Harley, 1985). Our present results strongly suggest that Tinomiscium, placed historically in the tribe Fibraureae, is nested in Tinosporeae and sister to Tinospora (Fig. 2), which also supports Barneby's (1972) view, although more taxon sampling is needed to verify this conclusion.

Tribes Menispermeae and Pachygoneae are not monophyletic

Pachygone was recently placed in the tribe Pachygoneae in a concept that also included the taxa previously placed in tribe Tiliacoreae (Kessler, 1993; Wu et al., 2003) based primarily on the absence of endosperm as a uniting character. However, our analyses strongly indicate that this genus is sister to Cocculus of the tribe Menispermeae (Figs. 2, 4–6), in agreement with earlier treatments by Diels (1910), Forman (1968) and Luo (1996). The remaining 'Pachygoneae' (bettered named Tiliacoreae) and the Pachygone–Cocculus clade formed a strongly supported clade, embedded within the tribe Menispermeae (Figs. 2, 4–6), which was also supported by cotyledon and stylar scar characters (Fig. 3a and b). Although endosperm was traditionally considered as the most useful and important character for the subdivision of Menispermaceae, the reconstruction of the evolution of endosperm suggested that in the family endosperm absence was secondarily lost (Fig. 3c). Other families of Ranunculales, all contain copious endosperm, which also suggests that endosperm presence is a synplesiomorphy for the Menispermaceae family and that endosperm absence is derived.

Subtribal divisions of tribe Menispermeae

According to carpel number and perianth symmetry of female flowers, Diels (1910) divided the tribe Menispermeae into three subtribes. The Cocculinae, with 2–6 carpels, is distinguished from the other two subtribes that have only one carpel per flower. The Stephaniinae (including only the genus Stephania) is characteristic by symmetrical female perianth, whereas in the Cissampelinae (including Cyclea and Cissampelos) the perianths of female flowers are asymmetrical. However, because the perianths of female flowers in Stephania venosa (Bl.) Sprengel and Stephania glandulifera Miers are asymmetrical, Forman (1968) considered that there was insufficient means of separating Stephania from the Cissampelinae. The inflorescence of Stephania laetificata (Miers) Benth. is not composed of umbelliform cymes or discoid capitula, which is a key character to Stephania; however, it is similar in form to the inflorescences of some species of Cissampelos. Thus, Forman (1968) considered that the two genera might be combined if generic limits in the family were reviewed as a whole. Our ITS and cpDNA data supported the monophyletic Stephaniinae and Cissampelinae and paraphyletic Cocculinae (containing tribe Tiliacoreae).

Carpel and stamen characters supported Diels' (1910) division where the Stephaniinae and Cissampelinae have one carpel and <6 stamens, whereas other genera have >2 carpels and six stamens (Fig. 7). Triporate pollen distinguishes the Stephaniinae from the other two subtribes, which are tricolporate (Fig. 7). Sepal number of male flowers in the Cissampelinae is usually four to five, rarely six or three in Cyclea wattii Diels, whereas in remaining taxa, sepal number of male flowers is six or more, except for Stephania tetrandra (4–5). Therefore, >6 sepals in male flowers may be a synplesiomorphy for tribe Menispermeae and <6 sepals may have been evolved independently in S. tetrandra and the Cissampelinae (Fig. 7). Angulaperture and smooth colpal membrane emphasize the distinctiveness of the Cissampelinae in contrast to fossaperture and granular, rugulate or reticulate colpal membrane in the other taxa (Fig. 7).

Tiliacoreae, Cissampelinae and Stephaniinae were strongly suggested each as a monophyletic clade, but the relationships among them were not consistent in the separate analyses (Figs. 2, 4–6). The characters of synandrium and reticulate tectum supported the relationship between Tiliacoreae and Stephania, Cyclea and

Cissampelos. However, more carpels (>2) and stamens (>6) supported that Tiliacoreae are related to Cocculus and Pachygone (Fig. 6).

Although Cissampelos and Cyclea formed a clade, monophyly of Cyclea was not well recognized (Figs. 4–6). Evolutionary rate of Cissampelos was obviously faster than that of Cyclea (Figs. 2, 4–6), which indicated that they evolved heterogeneously. When we excluded Cissampelos and reanalyzed the data sets, the relationships among the other taxa were not variable. Generic delimitation is an especially difficult problem in the Menispermaceae (Forman, 1968). In order to clarify the delimitation of the two genera, more sampling (including taxon density and genes) is required.

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