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Further evidence for paraphyly of the Celtidaceae from the chloroplast gene *mat*K

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Abstract. Based on the chloroplast *mat*K gene sequence, a phylogenetic analysis of the Urticales in its traditional circumscription and its putative affinities produced three equally most parsimonious trees with tree length = 1527 steps, CI = 0.6863 and RI = 0.6352, indicating that the Ulmaceae s. l. are polyphyletic while the Celtidaceae are paraphyletic, and particularly, *Cannabis* and *Humulus* in the Cannabaceae are consistently nested within the Celtidaceae. Therefore, the present data strongly suggest that the Cannabaceae should be merged with the Celtidaceae to form a monophyletic group. According to the present study, the Celtidaceae including Cannabaceae are more closely related to the Moraceae and Urticaceae than to the Ulmaceae s. str.. *Gironniera* and *Aphananthe* are both basal clades of the Celtidaceae rather than members of Ulmaceae s. str.. The Rhamnaceae and Rosaceae are the closest relatives of the traditional Urticales, which is very congruent with the newest system of flowering plants put forward by APG.

Key words: Celtidaceae, Ulmaceae, Rosales, *mat*K, molecular phylogeny.

Introduction

The two angiosperm families, Celtidaceae and Ulmaceae, usually treated as two subgroups of the Ulmaceae s. l. (Cronquist 1981) in the Urticales, have been controversial in taxonomy for decades (Engler and Prantl 1893; Thorne 1968, 1983, 1989; Dahlgren 1983, 1989; Cronquist 1981, 1988; Ueda et al. 1997; Wiegrefe et al. 1998). The arguments mainly focused on: (a) Are the Ulmaceae s. l. monophyletic or polyphyletic? (b) Are the Celtidaceae and Ulmaceae independent families or subgroups (subfamily or subtribe) of the Ulmaceae s. l.? (c) What is the relationship between the Celtidaceae and the other families of Urticales? (d) Where should some problematic genera such as *Gironniera* Gaud., *Aphananthe* Planch. be assigned to? In the past years, studies on fossil (Zavada and Crepet 1981, Manchester 1989), distribution (Grudzinskaya 1967), flavonoid chemistry (Giannasi 1978), wood anatomy (Sweitzer 1971), leaf venation, fruit type and embryology (Hutchinson 1967), vernation patterns (Terabayashi 1991), pollen morphology (Zavada 1983, Takahashi 1989), seed coat morphology (Takaso and Tobe 1990), karyomorphology (Oginuma et al. 1990), phylogenetic analysis (Zavada and Kim 1996) and molecular analyses (Sytsma et al. 1996, Ueda et al. 1997, Wiegrefe et al. 1998) have come to some conclusions: the Ulmaceae s. l. are polyphyletic rather than

Table 1. The materials

Taxa	Sources	GenBank Accession No.	References
Ulmaceae			
Ulmus parvifolia Jacq.	Botanic Garden, Institute of Botany, Beijing	AF345321	Present study
Zelkova schneideriana Hand. -Mazz.	Botanic Garden, Institute of Botany, Beijing	AF345328	Present study
Hemiptelea davidii (Hance) Planch.	Botanic Garden, Institute of Botany, Beijing	AF345322	Present study
Celtis sinensis Pers.	Jinan, Shandong, China	AF345316	Present study
Pteroceltis tatarinowii Maxim.	Jinan, Shandong, China	AF345324	Present study
Gironniera subaequalis Planch.	South China Botanic Garden, Guangzhou	AF345319	Present study
Aphananthe aspera (Thunb.) Planch.	South China Botanic Garden, Guangzhou	AF345320	Present study
Trema tomentosa (Roxb.) Hara	South China Botanic Garden, Guangzhou	AF345325	Present study
Moraceae			
Morus alba Linn.	Botanic Garden, Institute of Botany, Beijing	AF345327	Present study
Broussonetia papyrifera (Linn.) L'Herit. ex Vent	Botanic Garden, Institute of Botany, Beijing	AF345326	Present study
Cannabaceae			
Cannabis sativa L.	Jinan, Shandong, China	AF345317	Present study
Humulus lupulus L.	Botanic Garden, Institute of Botany, Beijing	AF345318	Present study
Urticaceae			
Boehmeria platanifolia Franch. et Sav.	Yantai, Shandong, China	AF353579	Present study
Malvaceae			
Hibiscus syriacus L.	Botanic Garden, Institute of Botany, Beijing	AF345329	Present study
Ranunculaceae			
Cimicifuga acerina (Sieb. et Zucc.) Tanaka	Jinfo Mountain, Nanchuan, Sichuan, China	AF353578	Present study
Juglandaceae			
Juglans nigra Linn.		U92851	Manos and Steele, 1997
Betulaceae			
Betula papyrifera Marsh.		U92853	Manos and Steele, 1997
Fagaceae			
Fagus grandifolia Ehrh.		U92861	Manos and Steele, 1997
Rhoipteleaceae			
Rhoiptelea chiliantha Diels & Hand. -Mazz.		U92852	Manos and Steele, 1997
Hamamelidaceae			
Liquidambar orientalis Mill.		AF015651	Li et al., 1997
Rosaceae			
Rosa stellata Wooton		AB012000	Unpublished
Rhamnaceae			
Ziziphus obtusifolia Gray		AF049848	Unpublished

monophyletic although the Ulmaceae s. str. are a monophyletic group with little doubt; the Celtidaceae and Ulmaceae s. str. should be treated as independent families, moreover, the Ulmaceae s. str. are the sister group of the Celtidaceae plus all other families in the Urticales. Nevertheless, there are still some questions unresolved, and some new interesting questions appeared. The Cannabaceae, traditionally placed in the Moraceae, were nested within the celtidaceous clade while the Celtidaceae and Ulmaceae were undoubtedly included in the Urticales in the molecular phylogeny (Sytsma et al. 1996, Ueda et al. 1997, Wiegrefe et al. 1998). Furthermore, the current system of flowering plants put forward by APG (1998) implied that the Celtidaceae and Ulmaceae might be closely related to the Rosaceae and Rhamnaceae in the Rosales even though the putative relatives of the Urticales are still controversial. (Berg 1977, Cronquist 1981, Dahlgren 1983, Thorne 1983, Judd et al. 1994).

Although the previous molecular phylogenetic studies of the Ulmaceae and their relatives have resulted in significant contributions to the recognition of some generic relationships of the Ulmaceae s. l., the phylogeny generated from those studies was not well resolved due to the slow evolution rate of *rbc*L gene (Ueda et al. 1997) or poor sampling of Urticales and its close relatives in the cpDNA restriction site analysis of Wiegrefe et al. (1998). Therefore, further studies on the molecular phylogeny of the Celtidaceae and Ulmaceae or Urticales are still needed. Among the different markers frequently used in recent molecular systematics, the *mat*K gene (≈1500 bp size) which is located within the intron of the chloroplast gene *trn*K has been demonstrated to be highly potential for providing insight into evolutionary and systematic problems (Hilu and Liang 1997). Particularly, at lower taxonomic levels, such as genus, family, the *mat*K gene provides better resolution than the most widely used chloroplast gene *rbc*L. Moreover, it has been revealed that the variation of the *mat*K gene has similar rate in closely related lineages and a relatively even distribution at three codon positions (Wang and Shu 2000; Wang et al. 1998, 2000; Johnson and Soltis 1995). The *mat*K gene has been used effectively in addressing systematic questions in the families Pinaceae (Wang et al. 2000), Cupressaceae s. l. (Gadek et al. 2000, Kusumi et al. 2000), Taxaceae and Cephalotaxaceae (Wang and Shu 2000, Cheng et al. 2000), Brassicaceae (Koch et al. 2001), Saxifragaceae (Johnson and Soltis 1994, 1995), Polemoniaceae (Steel and Vilgalys 1994, Johnson and Soltis 1995), and Poaceae (Hilu et al. 1999).

Here, we report a *mat*K gene phylogeny of the Urticales or Rosales, and mainly discuss the following questions: What should the Celtidaceae and Ulmaceae be assigned to? Are the Celtidaceae monophyletic or paraphyletic? How to treat the genera *Gironniera* and *Aphananthe* based on molecular analyses?

Materials and methods

Materials. We sampled three of six genera of the Ulmaceae, five of nine genera of the Celtidaceae, representatives of the other three families (Moraceae, Urticaceae and Cannabaceae) in the Urticales. Since the closest relatives of the Urticales, including Hamamelidae (Cronquist 1981), Malvales and Euphorbiales (Dahlgren 1989, Thorne 1989), are still being debated vigorously (Berg 1977, Dahlgren 1983, Thorne 1983, Judd et al. 1994), and the members of Urticales have been merged with Rosales by APG (1998) although previous molecular analyses (Chase et al. 1993, Gunter et al. 1994, APG 1998, Swensen 1996) and morphological data (Hufford 1992) suggest that Rhamnaceae and Rosaceae are better outgroup candidates, we also sampled a representative from each of the two orders Malvales (*Hibiscus*) and Ranunculales (*Cimicifuga*), and used *Cimicifuga acerina* (Ranunculaceae) as the outgroup. Additional seven *mat*K gene sequences used in the present study were recovered from GenBank (Table 1).

DNA extraction, amplification and sequencing. Total DNA isolation followed the protocol of Rogers and Bendich (1988), using fresh or silica gel dried leaf tissue from a single individual.

Polymerase Chain Reaction (PCR) was used to amplify the *mat*K gene with the primers

Table 2. Pairwise distances between taxa
Below diagonal: Total character differences. Above diagonal: Mean character differences

	1	2	3	4	5	6	7	8	9	10
1 *Ulmus*	–	0.01327	0.04018	0.11823	0.11715	0.10610	0.09643	0.11799	0.10033	0.10786
2 *Zelkova*	16	–	0.04975	0.12935	0.12050	0.11000	0.10033	0.12218	0.10282	0.11120
3 *Hemiptelea*	61	60	–	0.13012	0.12469	0.11671	0.10568	0.12803	0.10531	0.11120
4 *Celtis*	179	156	197	–	0.08117	0.07738	0.08762	0.08117	0.10199	0.11120
5 *Pteroceltis*	140	144	149	97	–	0.06644	0.07531	0.06862	0.08368	0.09456
6 *Gironniera*	160	132	176	117	79	–	0.06217	0.06897	0.07833	0.08739
7 *Aphananthe*	146	121	160	133	90	94	–	0.08117	0.07131	0.07776
8 *Trema*	141	146	153	97	82	82	97	–	0.08619	0.09456
9 *Morus*	121	124	127	123	100	94	86	103	–	0.04264
10 *Broussonetia*	129	133	133	133	113	104	93	113	51	–
11 *Cannabis*	178	152	190	130	83	112	121	87	113	124
12 *Humulus*	156	132	172	102	66	86	93	68	88	102
13 *Boehmeria*	233	194	238	233	181	200	199	176	170	167
14 *Hibiscus*	211	216	215	223	205	195	196	210	196	193
15 *Cimicifuga*	295	249	301	322	251	291	284	253	235	236
16 *Juglans*	138	111	141	157	120	155	150	111	104	107
17 *Betula*	140	111	143	153	122	153	147	111	104	106
18 *Fagus*	146	108	160	169	123	164	164	116	112	109
19 *Rhoiptelea*	134	107	141	148	115	145	142	107	104	105
20 *Liquidambar*	230	194	238	240	188	208	201	187	163	168
21 *Rosa*	200	165	206	222	170	201	193	170	148	165
22 *Ziziphus*	117	117	123	138	116	109	104	120	102	114

*trn*K-3914F and *trn*K-2R (Johnson and Soltis 1995). The volume of the amplification reaction mixture was 50 μL, containing 10–50 ng of total DNA, 12.5 pmol of each primer, 0.2 mM of each dNTP, 2 mM $MgCl_2$ and 1.5 Units of *Taq* DNA polymerase. Amplification was conducted in GeneAmp PCR System 9600 (Perkin-Elmer). PCR cycles were as follows: one cycle of 4 min at 70 °C, the 2 cycles of 60 s at 94 °C, 20 s at 50 °C, and 150 s at 72 °C, followed by 36 cycles of 20 s at 94 °C, 20 s at 50 °C, and 150 s at 72 °C, with a final extension step for 6 min at 72 °C. PCR products were purified using Genclean (BioDev). Sequencing was done on an ABI PRISM 377 DNA Sequencer using the ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction Kit. The internal sequencing primers were *mat*K-F_1:(5′-ACTGTATCGYACTATGTATCA-3′), *mat*K-AR_2:(5′-CTGCATATACGCGCAAATCG-3′) and *mat*K-AR_3:(5′-CGTAAATGASAAGATTGGTTAC-3′).

Phylogenetic analysis. Sequence alignments were made with CLUSTAL X (Thompson et al. 1997) and refined manually. Maximum parsimony analysis was performed using PAUP v.4.0 program (Swofford 1998) on the basis of nucleotide substitutions in aligned sequences. Heuristic searches were implemented with simple sequence addition, tree-bisection-reconnection (TBR) branch swapping and the MULTREES option. Rooting was determined by the outgroup method (Watrous and Wheeler 1981). All character states, including indels, were specified as unordered and equally weighted. Gaps were treated as missing data. To evaluate relative robustness of the clades found in the most parsimonious trees, the bootstrap analysis (Felsenstein 1985) was conducted with 500 replicates using the Heuristic search option.

Results

The aligned *mat*K sequences were 1589 bp in length, containing 785 variable nucleotide sites, of which 405 were parsimony-informative, and 15 indels of different length (1–18 bp). When Ulmaceae and Celtidaceae species were exclusively included in the alignment, the aligned sequence length was 1537 bp

11	12	13	14	15	16	17	18	19	20	21	22
0.11757	0.10304	0.15981	0.17701	0.19485	0.13939	0.14141	0.14762	0.13549	0.15252	0.13263	0.10864
0.12604	0.10945	0.16928	0.18121	0.20715	0.16372	0.16372	0.15953	0.15805	0.16126	0.13750	0.10864
0.12550	0.11361	0.16324	0.18037	0.19881	0.14242	0.14444	0.16178	0.14257	0.15782	0.13660	0.11421
0.08564	0.06719	0.15981	0.18708	0.21268	0.15859	0.15455	0.17088	0.14965	0.15873	0.14683	0.12813
0.06946	0.05523	0.15947	0.17343	0.21075	0.17991	0.18291	0.18468	0.17267	0.15772	0.14298	0.10771
0.07407	0.05688	0.13774	0.16442	0.19297	0.15752	0.15549	0.16684	0.14751	0.13811	0.13294	0.10177
0.07971	0.06126	0.13649	0.16443	0.18758	0.15152	0.14848	0.16582	0.14358	0.13294	0.12765	0.09656
0.07244	0.05662	0.15507	0.17707	0.21243	0.16642	0.16642	0.17417	0.16066	0.15688	0.14298	0.11142
0.09370	0.07297	0.14834	0.16443	0.19551	0.15339	0.15339	0.16544	0.15362	0.13549	0.12333	0.09471
0.10368	0.08528	0.14701	0.16314	0.19749	0.16018	0.15868	0.16342	0.15742	0.14082	0.13796	0.10585
–	0.02690	0.14335	0.17475	0.20343	0.15455	0.15152	0.17088	0.14762	0.14815	0.13492	0.11142
41	–	0.13443	0.16137	0.19551	0.14444	0.14343	0.15774	0.13549	0.13690	0.12169	0.09935
209	196	–	0.21201	0.23888	0.18477	0.18173	0.19919	0.17785	0.19077	0.18664	0.15385
209	193	240	–	0.18865	0.17365	0.17365	0.18441	0.17241	0.13591	0.17116	0.16541
308	296	349	226	–	0.19637	0.19335	0.20060	0.19052	0.14957	0.19391	0.20037
153	143	182	116	195	–	0.06040	0.07830	0.02775	0.13982	0.16057	0.15214
150	142	179	116	192	61	–	0.07136	0.04757	0.13678	0.15447	0.15897
169	156	196	123	199	79	72	–	0.06151	0.15416	0.18006	0.15385
146	134	175	115	189	28	48	62	–	0.13286	0.15666	0.14872
224	207	277	162	226	138	135	152	131	–	0.14409	0.13873
204	184	271	203	293	158	152	177	154	217	–	0.12418
120	107	162	176	215	89	93	90	87	149	133	–

with four 6 bp indels. The pairwise distances between taxa are shown in Table 2.

Parsimony analysis (Fitch 1977) with Heuristic search, employing *Cimicifuga acerina* as the outgroup, generated three equally most parsimonious trees with a tree length of 1527 steps, a consistency index (CI) of 0.6863 and a retention index (RI) of 0.6352. One of them is shown in Fig. 1. Based on our *mat*K phylogeny (Fig. 1), the strongly supported (97%) monophyletic Rosales, i.e. the sister group of Fagales, comprises traditional Urticales taxa, Rosaceae and Rhamnaceae, which is consistent with the results of *rbc*L analyses (Chase et al. 1993, Gunter et al. 1994) and congruent with the ordinal classification for the families of flowering plants (APG 1998). *Ziziphus* (Rhamnaceae) and *Rosa* (Rosaceae) are the closest relatives of the traditional Urticales which are split into two strongly supported clades: one is Ulmaceae s. str., the other comprises Celtidaceae, Cannabaceae, Moraceae and Urticaceae. Therefore, the Ulmaceae s. l. are not a monophyletic group. In particular, Cannabaceae, represented by *Cannabis* and *Humulus*, is robustly nested within the Celtidaceae, revealing that the Celtidaceae is paraphyletic. Moreover, both *Gironniera* and *Aphananthe* are strongly supported to be the basal groups of the Celtidaceae including Cannabaceae.

Discussion

The position of Celtidaceae and Ulmaceae. Traditionally, the Ulmaceae s. l., together with Moraceae (including Cannabaceae) and Urticaceae, were placed in Urticales of subclass Hamamelidae (Cronquist 1988), superorder Malvanae (Dahlgren 1989, Thorne 1989) or superorder Urticales (Takhtajan 1997). But in the new flowering plant system put forward by APG (1998), the traditional Urticales families, together with Rosaceae, Rhamnaceae, Elaeagnaceae, Dirachmaceae,

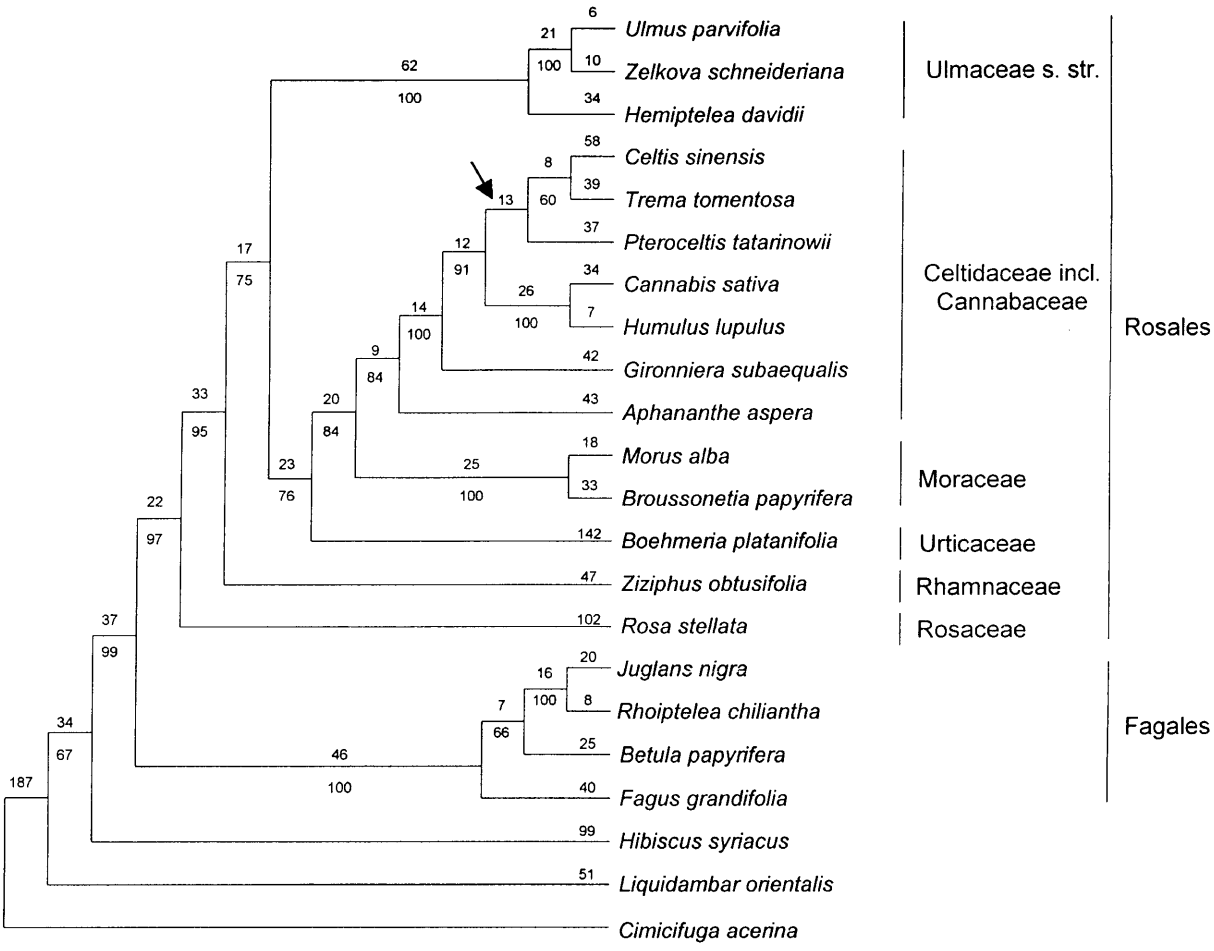

Fig. 1. One of the three equally most parsimonious trees obtained via cladistic analysis of *mat*K sequence data with *Cimicifuga acerina* as the outgroup (length = 1527; [CI] = 0.6863; [RI] = 0.6352). Numbers above the branches are branch lengths. Numbers below the branches represent bootstrap values. The arrow indicates that the clade will collapse on the strict consensus tree

Cecropiaceae and Barbeyaceae were assigned to the order Rosales within the Eurosid I clade. Based on the *rbc*L analysis of Juglandaceae and its relatives, Gunter et al. (1994) had ruled out that the presence of two rosid families (Rhamnaceae and Rosaceae) at the base of the Urticales suggests that homoplasy is the basis for many of the morphological similarities which Cronquist (1981) has considered to be characteristic of the subclass Hamamelidae. The result of present study is very congruent with that of *rbc*L gene sequence analysis (Gunter et al. 1994), and strongly supports the Rosales (bootstrap value of 97%) sensu APG (1998). In addition, the Fagales taxa sampled in this study form a strong clade, which is the sister group of the clade Rosales with 99% support, corresponding well with the new flowering plant system in which both Rosales and Fagales belong to the clade Eurosids I (APG 1998).

The delimitation of "monophyletic Celtidaceae". The previous morphological and molecular evidence has demonstrated that the genera of Celtidaceae might have a close relationship with the Cannabaceae, Moraceae and Urticaceae rather than with the Ulmaceae s. str., but this relationship was not strongly supported by the bootstrap test (Sytsma et al. 1996, Zavada et al. 1996, Ueda et al. 1997,

Wiegrefe et al. 1998). Especially, the genus *Cannabis* (Cannabaceae) was even suggested to be the sister group of the two genera *Pteroceltis* and *Celtis* in the Celtidaceae in the cpDNA restriction site analysis, but the result was in doubt because Cannabaceae was represented only by *Cannabis* and there were many difficulties in obtaining DNA of high quality and in mapping some restriction sites of *Cannabis* reliably (Wiegrefe et al. 1998). The present *mat*K gene sequence analysis further strongly demonstrates that the Ulmaceae s. l. are polyphyletic while the Celtidaceae are paraphyletic. Particularly, the Cannabaceae, represented by the two genera *Cannabis* and *Humulus*, are consistently nested within the Celtidaceae. As the basic chromosome number $x = 10$ is shared by portions of Celtidaceae and Cannabaceae alone in the traditional Urticales (Mehra and Gill 1974), the close relationship between the two families seems reasonable although Cannabaceae were traditionally placed within the Moraceae (Cronquist 1981, Humphries and Blackmore 1989). On the basis of the above discussions, we suggest that the Cannabaceae be merged with Celtidaceae to form a monophyletic Celtidaceae (Fig. 1).

The systematic position of *Gironniera* and *Aphananthe*. *Gironniera*, a very problematic genus, is isolated from other genera of the Celtidaceae based on its distinct exine sculpture of pollen (Takahashi 1989), vernation pattern (Terabayashi 1991) and derived reticulate seed coat surface sculpture shared with some Ulmaceae s. str. (Takaso and Tobe 1990). Moreover, its chromosome number ($n = 14$) and chromosome morphology are perhaps even of more phylogenetic interest. Ueda et al. (1997) found that *Gironniera* formed a clade together with *Pteroceltis*, *Chaetachme* and *Trema* in the *rbc*L gene tree, but the clade was not supported by the bootstrap test and morphological evidence. In the cpDNA restriction site analysis (Wiegrefe et al. 1998), *Gironniera* was not sampled. Our molecular data indicate that *Gironniera* is robustly supported to be basal to a clade comprising *Trema*, *Celtis*, *Pteroceltis*, *Humulus* and *Cannabis*, and distantly related to the Ulmaceae s. str. Therefore, it is better to include the genus *Gironniera* in the Celtidaceae rather than in the Ulmaceae even if it has many unique or intermediate characters.

Aphananthe differs from other genera of Celtidaceae in having asymmetrical ovule (Takaso 1987), flavonols (Giannasi 1978) and a reduced chromosome number of $n = 13$ (Oginuma et al. 1990). Moreover, Takaso and Tobe (1990) inferred that *Aphananthe*, with a distinct pattern of diversity in seed coat morphology and unique derived seed coat feature, may be in an isolated evolutionary line which diverged early from the ancestor of the Celtidaceae. However, *Aphananthe* has median or submedian simple centromere chromosomes like those of Celtidaceae (Oginuma et al. 1990). The placement of *Aphananthe* in Celtidaceae was weakly suggested by the previous molecular analyses (Ueda et al. 1997, Wiegrefe et al. 1998). In the present *mat*K phylogeny, the basal position of *Aphananthe* in the solid clade of Celtidaceae plus Cannabaceae is firstly revealed.

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