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Phylogenetic Framework for *Trema* (Celtidaceae)

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Abstract. We used ITS and *trn*L sequence data, analyzed separately and combined by MP, to explore species relationships and concepts in *Trema* (Celtidaceae), a pantropical genus of pioneer trees. Whether *Trema* is monophyletic or includes *Parasponia* is still unresolved. Three clades within *Trema* received moderate to high support, one from the New World and two from the Old World, but their relationships were not resolved. In the New World, specimens of *T. micrantha* formed two groups consistent with endocarp morphology. Group I, with smaller brown endocarps, is a highly supported clade sister to *T. lamarckiana*. Group II, with larger black endocarps, is poorly resolved with several subclades, including the highly supported *T. integerrima* clade. Both Old World clades contain Asian and African species, with three or more species in each region. *Trema orientalis* is not monophyletic: specimens from Africa formed a highly supported clade sister to *T. africana*, while those from Asia were sister to *T. aspera* from Australia.

Key words: *Trema*, *Parasponia*, Celtidaceae, tropical pioneer trees, ITS, *trn*L, molecular phylogeny.

Trema is a pantropical genus of fast-growing short-lived pioneer trees. Species grow in a range of tropical vegetation types from rainforests to dry savannas, from sea level to 3000 m elevation, and on a variety of very different soil types. Within these habitats, they are important components of successional vegetation following natural and man-made disturbances, rapidly colonizing landslides, volcanic ash deposits, roadsides and other disturbed areas (Elias 1970, Soepadmo 1977). Several species grow within mature forest, germinating in scattered tree fall gaps (Dalling et al. 1997, Silvera et al. 2003). As one of the few genera (and perhaps the only genus) that is both composed entirely of pioneer trees and distributed throughout the tropics, it offers a unique opportunity to study the ecology and evolution of the pioneer tree habit. However, the taxonomy and phylogeny of *Trema* are still poorly known.

The major challenge in the taxonomy of *Trema* Lour. has been the difficulty in finding good morphological characters to distinguish species. More than 75 published species names are listed in the International Plant Names Index (IPNI 2002), excluding subspecific taxa and synonyms under *Celtis* L. and *Sponia* Commers. ex Lamk. Blume (1856), who transferred many species from *Sponia* and *Celtis* to *Trema*, was apparently the most recent author to examine *Trema* on a world-wide basis. Because a world monograph is lacking, species

concepts vary considerably among regional floras. Thus estimates of the number of species range from 10–15 (Soepadmo 1977) to 55 (Elias 1970). Most authors note that the taxonomy of *Trema* is confused and poorly understood (e.g. Nevling 1960, Elias 1970, Soepadmo 1977). It is often difficult to assign many specimens to even the few species accepted in a region (e.g. Soepadmo 1977, Hewson 1989). Current trends have been toward recognizing fewer but more variable species. Although pragmatic, this approach precludes a deeper understanding the systematic relationships among species or their evolution and biogeography.

Given the uniformity of the small flowers among species, differences in fruit size and color and leaf characters such as size, shape and pubescence are frequently used to delimit species. Leaf characters, however, are subject to considerable ecotypic and ontogenetic variation. Mature leaves toward the tips of the growing branches differ in size, shape and pubescence from leaves near the branch base (Soepadmo 1977). There is also considerable variation between sun and shade leaves (Hewson 1989). St-Laurent et al. (2000a), however, claimed to distinguish species using discriminant analysis of many leaf characters. If species delimitation and relationships within *Trema* are to be resolved, new characters or new methods of analysis need to be deployed.

In this study, we use molecular sequence data from the chloroplast and nuclear genomes to address taxonomic problems within *Trema* and test phylogenetic relationships among species. Molecular markers such as *rbc*L, *trn*L, *ndh*F, and *mat*K have been used to address higher order relationships within Urticales. Results have supported separation of Celtidaceae from Ulmaceae s.l., inclusion of Cannabaceae within Celtidaceae, Ulmaceae as sister to all other families in Urticales, and position of Celtidaceae as sister clade to the remaining Moraceae and Urticaceae (Ueda et al. 1997, Wiegrefe et al.1998, Song et al. 2001, Sytsma et al. 2002). However, relationships of genera within Celtidaceae are still poorly resolved and relationships of species within genera unstudied (Sytsma et al. 2002). To examine these lower level relationships within *Trema*, we used the internal transcribed spacer (ITS) region of 18S-26S nuclear ribosomal DNA because it is a proven source of useful phylogenetic characters at this taxonomic level (Baldwin et al. 1995, Hershkovitz et al. 1999). As a complementary marker in the chloroplast genome, we used the non-coding *trn*L region (Taberlet et al. 1991). Both markers, present in high copy number, have been readily amplified and sequenced using universal primers from herbarium specimens of many species and proved suitable for *Trema* during preliminary trials.

The general objective of this study was to provide a framework for further revisionary and phylogenetic studies of *Trema*, including re-evaluation of morphological characters currently used to delimit species. Our specific questions relate to several taxonomic problems that could be addressed by a judicious but limited sampling of species.

1) Do New and Old World species form monophyletic groups? And within the Old World, do African and Asian species form monophyletic groups? If so, revisionary work can continue on a regional basis. More importantly, this has major implications for understanding the evolution and biogeography of the genus.

2) Is *Trema* monophyletic with respect to *Parasponia*? The Asian *Parasponia* Miquel, with five species, differs from *Trema* by having connate intrapetiolar stipules and imbricate perianth lobes on male flowers (Soepadomo 1977) and fixing nitrogen (Becking 1983). Recent molecular work using the chloroplast gene *rbc*L found *Trema* paraphyletic, with *Parasponia* nested within it (Sytsma et al. 2002), but only two species in each genus were studied.

3) In the New World, does the widespread but variable *T. micrantha* (L.) Blume represent one or more species? *T. micrantha* s.l. ranges throughout Central and South America and the Caribbean (Standley and Steyermark 1946,

Nevling 1960, Elias 1970, Burger 1977). Northern populations of *T. micrantha,* with densely pubescent leaves, are sometimes segregated as *T. florida* Britton or *T. micrantha* var. *floridana* (Britton) Standl. & Steyerm. (e.g. Standley and Steyermark 1946). In Panama, two ecologically and physiologically distinct groups within *T. micrantha* (Silvera et al. 2003) can be separated by leaf and endocarp characters (Parrish et al. 2001). We call these two groups the black and brown endocarp variants of *T. micrantha*.

4) What is the relationship of *T. micrantha* and its variants to other Neotropical species? *Trema lamarckiana* (Roem.& Schult.) Blume is widespread in the Caribbean and is distinguished from *T. micrantha* by its small leaves (Adams 1972, Howard 1988, Nicolson 1991). *Trema integerrima* (Beurl.) Standl., with entire leaf margins, is sometimes segregated from serrate margined *T. micrantha* (Standley 1933, Lasser 1971).

5) In Africa, does the widespread but variable *T. orientalis* (L.) Blume represent one species, as proposed by Polhill (1964), or several species, as proposed by St-Laurent et al. (2000a, b)? If there are multiple species, do they form a monophyletic group relative to Asian species? Lastly, is the African *T. orientalis* (L.) Blume truly conspecific with its Asiatic namesake?

Materials and methods

Taxon sampling. Origins and accession numbers of specimens analyzed are given in Appendix 1. In the New World, we sampled the geographic range of *Trema* using herbarium specimens and local variability using silica-dried leaves sampled from populations in four regions (southern Florida, Belize, Panama, and Ecuador). The three currently recognized species (*T. micrantha*, *T. lamarckiana*, *T. integerrima*) and the two endocarp variants of *T. micrantha* were each sampled at multiple sites. In the Old World, we sampled the geographic range of *Trema* using herbarium specimens only except for one silica-dried leaf sample from Thailand. The three African species proposed by St-Laurent et al. (2000) were analyzed from St-Laurent's vouchers (three samples each, at least one successful for both ITS and *trn*L). Four of five species reported for Malesiae and Australia (Soepadmo 1977, Hewson 1989), and additional species from elsewhere in the Old World, were included. Attempts to obtain sequences from *T. angustifolia* and typical *T. cannabina* failed. Three of five species of *Parasponia* (Soepadmo 1977), possibly congeneric with *Trema* (Sytsma et al. 2002), were also sampled. *Celtis* was chosen as an outgroup within Celtidaceae and *Ulmus* (Ulmaceae) as an outgroup within Urticales, as Ulmaceae is sister to the clade that includes Celtidaceae, Urticaceae and Moraceae (Sytsma et al. 2002). Sequences for *Parasponia* and outgroups were obtained from herbarium specimens or GenBank. All herbarium specimens were from the Natural History Museum (BM) except the St-Laurent vouchers from Ottawa (DAO). When phylogenetic analysis revealed one or more clades within a presumed species, each clade was given a unique label (e.g. I, II, IIa) congruent with the ITS, *trn*L and combined analyses.

DNA extraction. Whenever possible, we preferentially selected silica dried leaf samples over herbarium specimens as they gave consistently reliable results. Nonetheless, by selecting the apparently least degraded herbarium specimens and leaves on those specimens, we obtained excellent results from many herbarium specimens. Two methods of DNA extraction were used. For silica dried or fresh material, the hot CTAB method (Rogers and Bendich 1994) was employed. For herbarium specimens, the hot CTAB method was modified: following the SEVAC extraction phase, the aqueous layer was removed after centrifugation, then cleaned and concentrated using the GFX PCR DNA purification kit (Amersham Biosciences) following the manufacturer's instructions. DNA yields are markedly less using this modification and could not be visualized using ethidium bromide stained agarose electrophoresis. However, the DNA eluted from the GFX columns, when used directly in the PCR reactions, amplified and sequenced successfully from a wide range of specimens, some collected more than 80 years ago.

PCR amplification. Double stranded amplifications using the universal primers of the *trn*L gene region C-F (Taberlet et al. 1991) and of ITS-1 and ITS-2 including the 5.8S spacer (White et al. 1990) were performed using PCR (polymerase chain

reaction). The 1.5 ul aliquots of extracted DNA were amplified in 25 ul volumes containing 2.5 mM $MgCl_2$, 200 uM dNTP's, 1 uM Primers, 1x PCR Buffer and 0.5 U TAQ (Bioline). ITS reactions were augmented with Bovine Serum Albumin (Pääbo 1990) and Betaine 1.2 M (Chakrabarti and Schutt 2001): consistent amplification for ITS was achieved when these additional components where added. For *trn*L, thermal cycling conditions were 3 min at 94 °C, 30 cycles of 15 s at 94 °C, 30 s at 50 °C, 1 min at 72 °C, followed by a final extension cycle of 5 min at 72 °C. For ITS, cycling conditions were 1 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 54 °C, 3 min at 72 °C, with a final extension of 72 °C for 8 min.

Sequencing. PCR products were cleaned using GFX cleaning columns (Amersham Bioscience) according to the manufacturer's instructions. Depending on the concentration of amplified product as visualized on an ethidium bromide stained agarose gel, aliquots of PCR product were cycle sequenced using Big Dye v2.0 chemistry (PE Biosystems) and the original primers in 1/4 volume reactions. Products from the cycle sequence reactions were electrophoresed on an ABI 377 automated sequencer (PE Biosystems). Both amplified fragments were sequenced in both directions. The complementary sequences were aligned and edited using the Lazergene (DNAstar) software. IUPAC codes (Cornish-Bowden 1985) were used for ambiguous or polymorphic sites.

The small *rps4* gene fragment (*ca.* 600 bp), which codes for the chloroplast ribosomal subunit, was sequenced for a subset of 20 specimens from 13 species of *Trema* with *Zelkova serrata* Makino and *Celtis iguanaea* (Jacq.) Sarg. as outgroups (Yesson 2002). A phylogenetic analysis of this preliminary data set yielded only 18 informative sites. The resulting consensus of 8 most parsimonious cladograms (MPC; length = 43, CI = 0.41, RI = 0.34), was 18 steps longer than any MPC (Yesson 2002). Although there was good bootstrap support for the *Trema* clade, *rps4* provided little resolution within *Trema* and further work was discontinued.

Alignment. Alignment of sequences was carried out using Clustal X v1.8 (Thompson et al. 1997) and subsequently amended by hand using BioEdit (Hall 1999) on a PC. The sequence ends were trimmed to equal lengths at conserved regions. The *trn*L data set begins at the start of the *trn*L gene and continues to the end of the *trn*L-*trn*F intergenic spacer region. The ITS data set begins at the start of ITS-1, includes the 5.8S region, and continues until the end of ITS-2. These were exported to a Nexus file format for phylogenetic analysis. Little manual editing was required for *trn*L sequences. ITS sequences required greater manual intervention to produce an adequate alignment, particularly at the start of the ITS-2 region (positions 445–495). Full alignments are available from the corresponding author upon request.

Phylogenetic analysis. Many *trn*L sequences from different specimens were identical, as were several ITS sequences. In these cases, to reduce the size of the data matrix, only one copy of each unique sequence was used in the phylogenetic analysis. The sequence from one specimen was chosen for submission to Genbank: this specimen and all specimens with an identical sequence are given in Appendix 1. In the trees (Figs. 1–3), sample codes (from Appendix 1) identify all specimens with identical sequences. Characters were treated as Fitch unordered characters (Fitch 1971). Gaps were included and treated as missing data. Tree searches were conducted on a PC with the Windows version of NONA (Goloboff 1999) using the parsimony ratchet (Nixon 1999) with 200 iterations/replicates and 10 trees held per iteration. Relative support for clades was assessed using 10,000 bootstrap replicates in NONA. Values of the bootstrap percentage (BP) are shown on the trees (Figs. 1–3). Relative support for clades is summarized in the text as strong if BP was > 85%, moderate if BP was 71–85%, weak if BP was 50–70%, and absent if BP was < 50%. Characters were optimized onto the tree using the slow option of Winclada (Nixon 1999) to yield branch lengths. Before undertaking the combined analysis, the incongruence length difference (ILD) test of Farris et al. (1995) was carried out in NONA. This showed that the *trn*L and ITS data sets were not significantly incongruent (p = 0.33) and could be combined. Both genes were successfully sequenced from 36 specimens. In addition, consensus sequences for the combined analysis were generated for *T. micrantha* I from Panama (combining *trn*L from specimen PA4 and ITS from PA3) and the outgroup *Ulmus* (combining *trn*L from *U. glabra* and ITS from *U. americana*).

Insertion/deletion events: Indels often provide additional phylogenetically useful information, although there is yet no accepted standard method

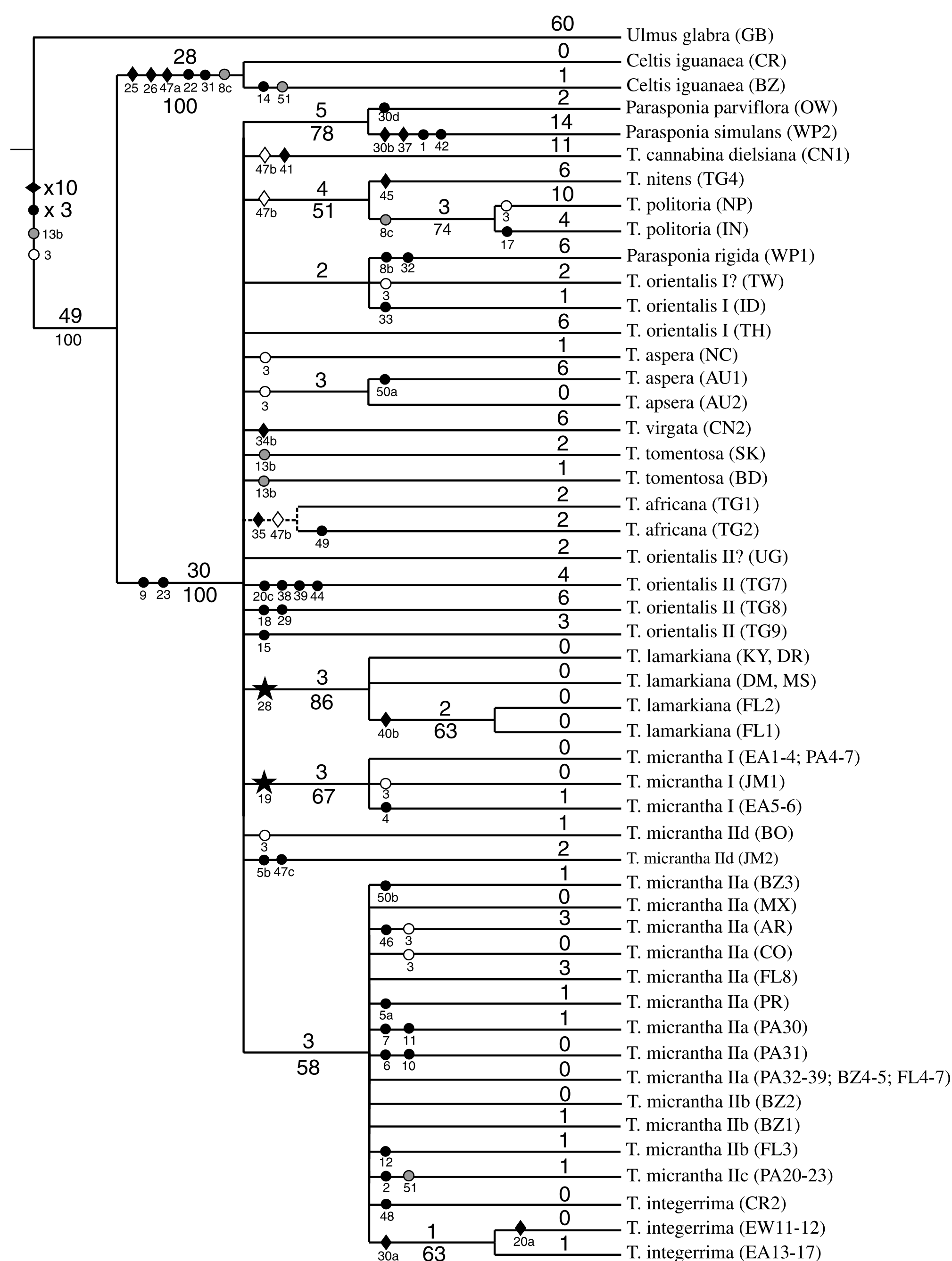

Fig. 1. Strict consensus of 46 maximum parsimony trees of the *trn*L region sequence data (length = 312, consistency index = 0.81, retention index = 0.71) showing branch lengths (above the line) and bootstrap values from 10,000 replicates (below the line if > 50%). Insertion/deletions (indels; Table 1) are displayed on the tree by indel length (circles, 1 bp; diamonds, 2–11 bp; stars, 70–90 bp) and degree of homoplasy (black symbols, unique indels occurring once on tree; grey symbols, non-unique indels occurring only once in *Trema* + *Parasponia*; white symbols, non-unique indels occurring multiple times in *Trema*). Small numbers below the indels correspond to indel number from Table 1. Dashed lines represent nodes that have collapsed in the strict consensus but receive some support from indels

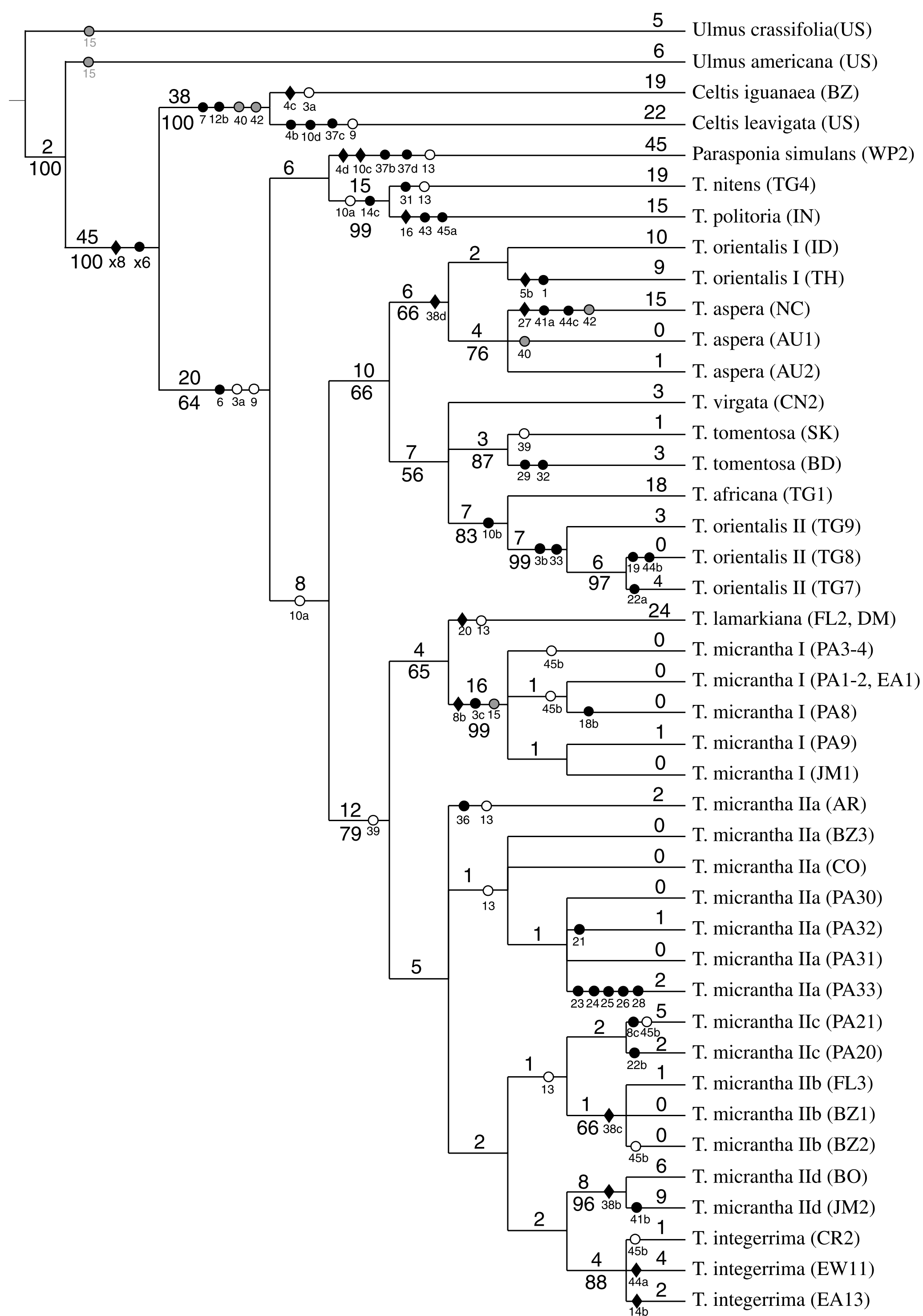

Fig. 2. One of 48 maximum parsimony trees of the ITS region sequence data (length = 506, consistency index = 0.69, retention index = 0.80) showing branch lengths (above the line) and bootstrap values from 10,000 replicates (below the line if > 50%). Insertion/deletions (indels; Table 2) are displayed on the tree by indel length (circles, 1 bp; diamonds, 2–8 bp) and degree of homoplasy (black symbols, unique indels occurring once on tree; grey symbols, non-unique indels occurring only once in *Trema;* white symbols, non-unique indels occurring multiple times in *Trema*). Small numbers below the indels corrspond to indel number from Table 2

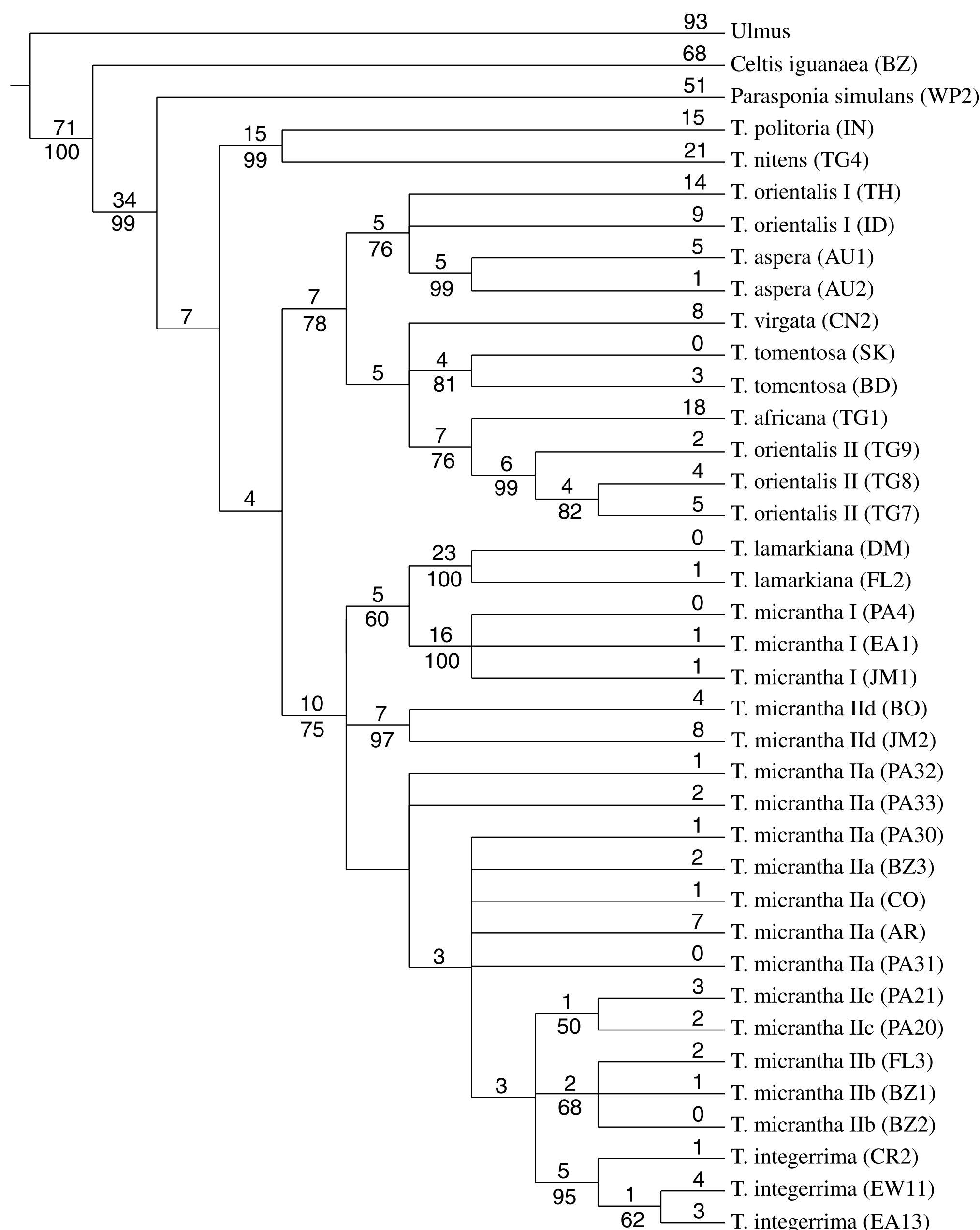

Fig. 3. Strict consensus of 32 maximum parsimony trees of the combined *trn*L and ITS region sequence data (length = 730, consistency index = 0.76, retention index = 0.75) showing branch lengths (above the line) and bootstrap values from 10,000 replicates (below the line if > 50%)

for coding gaps (Simmons and Ochoterena 2000, Freudenstein and Chase 2001). There were many indels in both the *trn*L and ITS sequence alignments. Indel regions were numbered sequentially from the start of each sequence. Two large indels (70–90 bp) in *trn*L were considered independent of smaller nested indels. Other overlapping or nested indels in one region were considered different unordered character states of that region. Coding overlapping and nested indels in this manner

identifies regions that would require complex indel coding for cladistic analysis (Simmons and Ochoterena 2000). Indels were usually scored relative to the outgroup *Ulmus*, but several homoplastic indels occurring in both *Ulmus* and a few *Trema* species were scored as simple insertions or deletions in both groups. Indels in the ITS sequence were more complex than those in *trn*L. To simplify coding of several ITS indels, we assumed that a primary indel event occurred between *Ulmus* and *Celtis*-*Parasponia*-*Trema*, and a secondary event in other species. Indels were not coded for positions 445–495 of ITS, as it was difficult to align sequences from the outgroups with *Parasponia* and *Trema* in this region, although it was not difficult to align *Trema* sequences in this region. Indels are displayed on the trees inferred from base characters, but were not analyzed separately or in combination with the base characters.

Results

***trn*L region.** We obtained sequences from 81 specimens in 16 presumed species, of which 49 were unique. The *trn*L data set for phylogenetic analysis consisted of these 49 unique sequences: these had 920 characters, of which 87 were parsimony informative. Sequences ranged from 731 to 840 bp long. (Nine of the 49 sequences were 10–30 bp short at the 5′ and/or 3′ end and 783–814 bp long.) Guanine-cytosine (GC) content ranged from 32 to 35%. The aligned length of *trn*L was 594 bp and the intergenic spacer region was 326 bp. Phylogenetic analysis of the *trn*L sequence data produced 46 most parsimonious cladograms (MPC) of length 293 with consistency index (CI) = 0.86 and retention index (RI) = 0.81. The strict consensus (Fig. 1) was 19 steps longer. The 46 MPCs contained many varying topologies due to the small number of parsimony informative characters within the data set. There were 66 insertion/deletion (indels) in 51 regions in the aligned sequence (Table 1): these are displayed on the strict consensus tree (Fig. 1). Of these, 61 indels (92%) occurred only once on the consensus tree. Of the five homoplasious indels, three occurred only once in the *Trema* + *Parasponia* clade, as well as in one of the outgroups.

There was strong support for a *Parasponia* + *Trema* clade (BP = 100%) but little resolution within it. In all MPCs, the three *Parasponia* species were grouped within *Trema*. In the majority of the MPCs, either *T. micrantha* I or *T. politoria* + *T. nitens* was basal to the rest of *Trema* + *Parasponia*. The three *Parasponia* species never formed a monophyletic group. *Parasponia parviflora* + *P. simulans* were moderately supported as sister species. *Parasponia rigida* grouped with two specimens of *T. orientalis* I in all MPCs, but this clade lacked bootstrap support. *Parasponia simulans* had a relatively large number of character changes (14 autapomorphies) and indels compared to most *Trema* species, but the other two *Parasponia* species did not.

Among Old World species, not all specimens of *T. orientalis* grouped together. Two specimens of *T. orientalis* I grouped with *Parasponia rigida* (see above). In 8 of the 46 MPCs, *T. orientalis* II formed a monophyletic group. Indels provided no support for groups within *T. orientalis*. Specimens of *T. africana* formed a group in over half of the MPCs; they also shared a unique 5 bp insertion. Australian *T. aspera* formed a consistent clade, but this lacks bootstrap support and excluded the New Caledonian specimen. Specimens of *T. politoria* formed a moderately supported clade and possessed a single bp deletion unique in *Trema* (but also found in *Celtis*). *Trema politoria* formed a weakly supported group with *T. nitens* that shared a 6 bp deletion. This deletion also occurred in *T. cannabina* var *dielsiana* and *T. africana,* but these never grouped with *T. politoria* + *T. nitens* or each other in any of the MPCs.

Among New World species, specimens of *T. micrantha* did not form a monophyletic group in any of the 46 MPCs. *Trema micrantha* I was a clade of three different sequences from 11 specimens weakly supported by bootstrap values and further supported by a unique large deletion (77 bp). This clade contained all specimens with brown endocarps and no specimens with black endocarps. This large indel is also present in partial sequences

Table 1. Insertion/deletion (indel) events in aligned *trn*L sequences. The base pair (bp) position of start of indel on aligned data, its size (bp) and type (+, insertion; −, deletion) and taxa exhibiting indel are indicated for each indel. Indels are scored relative to the outgroup *Ulmus* (except for several homoplasious indels). Indels regions are numbered sequentially. Overlapping or nested indels in the same region are identified by different letters except for two large indels (70–90 bp), which are coded as independent of smaller nested indels. The 66 indels (in 51 regions) are displayed on the strict consensus tree (Fig. 1). Origin of sample is indicated in parentheses when the indel does not occur in all samples of a taxon (see Appendix 1 for abbreviations)

Number	Position (bp)	Size and type	Taxa
1	2	1−	*Parasponia simulans*
2	3	1−	*T. micrantha* IIa (PA 20-23)
3	6	1−	*Ulmus, T. aspera, T. micrantha* I (JM1), *T. micrantha* IIa (AR, CO), *T. micrantha* IId (BO), *T. orientalis* I? (TW), *T. politoria* (NP)
4	8	1+	*T. micrantha* I (EA5–6)
5a	29	1+	*T. micrantha* IIa (PR)
5b	29	1+	*T. micrantha* IId (JM2)
6	32	1+	*T. micrantha* IIa (PA31)
7	42	1+	*T. micrantha* IIa (PA30)
8a	52	2−	*Ulmus*
8b	53	1−	*Parasponia rigida*
8c	54	1+	*Celtis, T. politoria*
9	56	1+	*Parasponia, Trema*
10	62	1−	*T. micrantha* IIa (PA31)
11	65	1−	*T. micrantha* IIa (PA30)
12	90	1−	*T. micrantha* IIb (FL3)
13a	91	5+	*Celtis, Parasponia, Trema*
13b	96	1+	*Ulmus, T. tomentosa*
14	106	1+	*Celtis* (BZ)
15	114	1−	*T. orientalis* II (TG9)
16	124	5+	*Celtis, Parasponia, Trema*
17	141	1+	*T. politoria* (IN)
18	145	1+	*T. orientalis* II (TG8)
19	170	77−	*T. micrantha* I
20a	181	8−	*T. integerrima* (EW)
20b	189	4−	*Celtis, Parasponia, Trema*
20c	189	1+	*T. orientalis* II (TG7)
21	209	1+	*Celtis, Parasponia, Trema*
22	211	1−	*Celtis*
23	227	1−	*Parasponia, Trema*
24	230	5−	*Celtis, Parasponia, Trema*
25	250	5+	*Celtis*
26	269	6+	*Celtis*
27	303	11−	*Celtis, Parasponia, Trema*
28	326	90−	*T. lamarckiana*
29	331	1+	*T. orientalis* II (TG8)
30a	335	3+	*T. integerrima* (EA, EW)
30b	336	2+	*Parasponia simulans*
30d	337	1+	*Parasponia parviflora*

Table 1 (continued)

Number	Position (bp)	Size and type	Taxa
30c	337	1+	*Ulmus*
31	360	1+	*Celtis*
32	453	1+	*Parasponia rigida*
33	464	1–	*T. orientalis* I (ID)
34a	586	5+	*Celtis, Parasponia, Trema*
34b	590	4+	*T. virgata* (CN2)
35	628	5+	*T. africana*
36	687	9–	*Celtis, Parasponia, Trema*
37	721	5+	*Parasponia simulans*
38	761	1–	*T. orientalis* II (TG7)
39	764	1–	*T. orientalis* II (TG7)
40a	770	8–	*Celtis, Parasponia, Trema*
40b	770	6+	*T. lamarckiana (FL1-2)*
41	826	10+	*T. cannabina dielsiana* (CN1)
42	840	1+	*Parasponia simulans*
43	852	2+	*Celtis, Parasponia, Trema*
44	858	1–	*T. orientalis* II (TG7)
45	867	6+	*T. nitens* (TG4)
46	879	1+	*T. micrantha* IIa (AR)
47a	892	8–	*Celtis*
47b	892	6–	*T. africana, T. cannabina dielsiana* (CN1), *T. nitens* (TG4), *T. politoria* (NP; IN?)
47c	897	1+	*T. micrantha* IId (JM2)
47d	902	1–	*Celtis, Parasponia, Trema*
48	906	1–	*T. integerrima* (CR2)
49	909	1–	*T. africana* (TG2)
50a	912	1–	*T. aspera* (AU1)
50b	913	1+	*T. micrantha* IIa (BZ3)
51	918	1–	*T. micrantha* IIc (PA 20–23), *Celtis* (BZ)

(obtained from a different primer set) of five additional specimens (Appendix 1) not used in the phylogenetic analysis: these also had brown endocarps. In contrast, the remaining 31 specimens with black endocarps, *T. micrantha* II, did not form a clade. Most specimens of *T. micrantha* II (IIa, IIb, IIc) formed a weakly supported group with *T. integerrima*, but the Bolivian and Jamaican specimens (IId) never appeared within it. *Trema integerrima* was also not monophyletic group because the Costa Rican specimen never grouped with the Ecuadorian specimens. Ecuadorian *T. integerrima* formed a weakly supported group that also possessed a unique 3 bp insertion. *Trema lamarkiana* was a strongly supported group further distinguished by a unique large deletion (90 bp) that did not overlap the large indel in *T. micrantha* I.

ITS region. We obtained sequences from 44 specimens in 15 presumed species, of which 42 were unique. The ITS data set for phylogenetic analysis consisted of these 42 sequences: these had 687 characters, of which 167 were informative. Sequences ranged from 592 to 619 bp long. (Three of the 42 sequences were 17–47 bp short at the end 3′ end and 562–584 bp long). GC content ranged from 60 to 66%. The aligned length of ITS-1 was 254 bp; ITS-2 was 257 bp; and 5.8S region was 176 bp. Phylogenetic analysis of the ITS sequence data produced 48 most

parsimonious cladograms (MPC) of length 506 with CI = 0.69 and RI = 0.80. Figure 2 shows one of these 48 trees. The strict consensus of these is 33 steps longer. The ITS data set was far more informative than the *trn*L data set and provided more resolution. Unfortunately, we tried but failed to obtain ITS sequences from some herbarium specimens used in the *trn*L analysis (see Appendix 1), including *Parasponia rigida* and *Trema cannabina* var. *dielsiana*. There were 71 insertion/deletion (indel) in 45 regions in the aligned sequence (Table 2), excluding a 50 bp region that was difficult to align (positions 445–495); these are displayed on one of the MPCs trees (Fig. 2). Of these, 62 indels (87%) occurred only once on the MPC illustrated. Of the 9 homoplasious indels, three occurred only once in *Trema*, as well as in one of the outgroups.

There was weak bootstrap support for a *Parasponia* + *Trema* clade, which had a unique single bp deletion. (Two homplasious indels at this node were shared with either *C. iguanaea* or *C. leavigata*.) In the 48 MPCs, *Parasponia simulans* was either sister to all *Trema* (16 MPCs) or sister to *T. nitens* + *T. politoria* (32 MPCs), which was in turn sister to the rest of *Trema*. Although a *Parasponia* + *T. nitens* + *T. politoria* clade was common among the MPCs, there was no bootstrap support for it. All *Trema* shared a single bp deletion absent from *Parasponia*, although this appears homoplasious in the topology of Fig. 2. *Parasponia simulans* had the highest number of autapomorphies (45) of any taxa in this study, and four unique indel character states.

There was moderate support for a New World clade of *Trema* (BP = 79%), which persisted in the consensus tree. A single bp insertion was shared by the 27 specimens of this clade, but it was also present in one specimen of *T. tomentosa*. In contrast, the Old World species did not form one clade but two. A *T. nitens* + *T. politoria* clade (OW1) was strongly supported (BP = 99%). These two species each had large numbers of autapomorphies (15–19) and unique indels. There was weak bootstrap support for a clade of the remaining Old World taxa (OW2).

Among Old World *Trema*, *T. orientalis* was not monophyletic. The Asian specimens, *T. orientalis* I, were sister to *T. aspera* from Australia and New Caledonia. The *T. orientalis* I + *T. aspera* clade (OW2a) had weak bootstrap support and a unique 2 bp insertion, and was retained in the strict consensus tree. The African specimens, *T. orientalis* II, formed a strongly supported group sister to *T. africana*. These African species, with the Indo-Asian *T. virgata* and *T. tomentosa*, formed a weakly supported clade (OW2b) that also persisted in the strict consensus tree.

Among New World *Trema*, the two endocarp variants of *T. micrantha* were not sister groups, a result congruent with the *trn*L analysis. The *T. micrantha* I clade was strongly supported (BP = 99%) and contained all specimens with brown endocarps and no specimens with black endocarps. This clade also had unique 5 bp and 1 bp insertions and a 1 bp insertion that occurred elsewhere only in *Ulmus*. A *T. micrantha* I + *T. lamarckiana* clade had only weak support and lacked distinctive indels, but was retained in the consensus tree. *Trema lamarckiana* has an unusually large number of autapomorphies (24), the highest amongst *Trema* taxa, and had a unique 2 bp deletion. The remaining specimens of *T. micrantha* with black endocarps, *T. micrantha* II, did not form a monophyletic group because specimens of *T. integerrima* were nested within it. The *T. micrantha* II + *T. integerrima* clade lacked bootstrap support and was not retained in the consensus tree, but contained three subclades that persisted in the consensus tree. The *T. integerrima* subclade had strong support (BP = 88%), but no unique indels. The *T. micrantha* IId subclade also had high support (BP = 96%) and both specimens shared a unique 2 bp insertion. The *T. micrantha* IIb clade had weak bootstrap support and all three specimens shared a unique 7 bp deletion. The *T. micrantha* IIa clade has no bootstrap support or unique indels, and was

Table 2. Insertion/deletion (indel) events in aligned ITS sequences. The base pair (bp) position of start of indel on aligned data, its size (bp) and type (+, insertion; −, deletion) and taxa exhibiting indel are indicated for each indel. Indel regions are numbered sequentially. Indels between positions 445 to 495 are excluded because of difficulties in alignment. Overlapping or nested indels in the same region are identified by different letters. Indels in the same region are considered independent events unless coded as primary (p) or secondary (s) events. The 71 indels (in 49 regions) are displayed on one of the 48 most parsimonious trees (Fig. 2). Origin of sample is indicated in parentheses when the indel does not occur in all samples of a taxon (see Appendix 1 for abbreviations). Indels are scored relative to the outgroup *Ulmus* (except for several homoplasious indels occurring only in *Ulmus* and a few *Trema* taxa)

Region	Position (bp)	Size and Type	Taxa
1	2	1+	*T. orientalis* I (TH)
2	39	1−	*Celtis, Parasponia, Trema*
3a (p)	41	1−	*Celtis iguanaea, Parasponia, Trema*
3b (s)	41	1+	*T. orientalis* II (TG 7-9)
3c (s)	41	1+	*T. micrantha* I
4a (p)	46	3+	*Celtis, Parasponia, Trema*
4b (s)	48	1−	*Celtis leavigata*
4c (s)	49	2+	*Celtis iguanaea*
4d (s)	49	2+	*Parasponia*
5a (p)	60	3+	*Celtis, Parasponia, Trema*
5b (s)	58	2+	*T. orientalis* I (TH)
6	70	1−	*Parasponia, Trema*
7	74	1−	*Celtis*
8a (p)	81	1+	*Celtis, Parasponia, Trema*
8b (s)	82	5+	*T. micrantha* I
8c (s)	82	1+	*T. micrantha* IIc (PA21)
9	93	1−	*Celtis laevigata, Parasponia, Trema*
10a (p)	99	1−	*Trema*
10b (s)	99	1+	*T. africana, T. orientalis*. II (TG7-9)
10c (s)	100	3+	*Parasponia*
10d (s)	103	1−	*Celtis leavigata*
11	125	2−	*Celtis, Parasponia, Trema*
12a (p)	130	4−	*Celtis, Parasponia, Trema*
12b (s)	134	1−	*Celtis*
13	170	1+	*Parasponia, T. nitens, T. lamarckiana, T. micrantha* IIa, IIc, IIb
14a (p)	191	3+	*Celtis, Parasponia, Trema*
14b (s)	189	2+	*T. integerrima* (EA13)
14c (s)	190	1+	*T. nitens, T. politoria*
15	202	1+	*Ulmus, T. micrantha* I
16	206	3+	*T. politoria*
17	221	1+	*Celtis, Parasponia, Trema*
18a (p)	228	1+	*Celtis, Parasponia, Trema*
18b (s)	229	1+	*T. micrantha* I (PA8)
19	237	1+	*T. orientalis* II (TG8)
20	250	2−	*T. lamarckiana*
21	264	1+	*T. micrantha* IIa (PA32)
22a	285	1−	*T. orientalis* II (TG7)
22b	286	1+	*T. micrantha* IIc (PA20)
23	307	1+	*T. micrantha* IIa (PA33)

Table 2 (continued)

Region	Position (bp)	Size and Type	Taxa
24	316	1 +	*T. micrantha* IIa (PA33)
25	331	1 +	*T. micrantha* IIa (PA33)
26	337	1 +	*T. micrantha* IIa (PA33)
27	387	8 +	*T. aspera* (NC)
28	435	1 +	*T. micrantha* IIa (PA33)
29	512	1 +	*T. tomentosa* (BD)
30	519	2 +	*Celtis, Parasponia, Trema*
31	522	1–	*T. nitens*
32	529	1 +	*T. tomentosa* (BD)
33	563	1 +	*T. orientalis*. II (TG7-9)
34	577	1 +	*Celtis, Parasponia, Trema*
35	591	1–	*Celtis, Parasponia, Trema*
36	619	1–	*T. micrantha* IIa (AR)
37a (p)	626	2 +	*Celtis, Parasponia, Trema*
37b (s)	624	1–	*Parasponia*
37c (s)	625	1 +	*Celtis leavigata*
37d (s)	628	1 +	*Parasponia*
38a (p)	633	3 +	*Celtis, Parasponia, Trema*
38b (s)	631	2 +	*T. micrantha* IId (BO, JM)
38c (s)	633	7–	*T. micrantha* IIb (BZ1-2, FL3)
38d (s)	636	2 +	*T. aspera, T. orientalis* I
39	644	1 +	*T. tomentosa* (SK), *Trema* (New World)
40	647	1–	*Celtis, T. aspera* (AU1)
41a	652	1 +	*T. aspera* (NC)
41b	652	1 +	*T. micrantha* IId (JM)
42	668	1–	*Celtis, T. aspera* (NC)
43	671	1 +	*T. politoria*
44a	674	2–	*T. integerrima* (EW11)
44b	674	1–	*T. orientalis* II (TG8)
44c	675	1 +	*T. aspera* (NC)
45a	680	1 +	*T. politoria*
45b	680	1 +	*T. micrantha* I (PA1-4,8; EA1), *T. micrantha* IIc (PA21), *T. micrantha* IId (BZ2), *T. integerrima* (CR2)

lost in the consensus tree, but it appeared in all but one of the 48 MPCs.

Combined dataset. We used combined sequences from 36 specimens in 12 presumed species from which both genes were successfully sequenced and two consensus sequences (Panamian *T. micrantha* I and outgroup *Ulmus*: see methods). The combined data set consisted of 38 unique sequences and had 1607 characters, of which 170 were informative. Phylogenetic analysis of this data set gave 32 MPCs of length 724, CI = 0.76, RI = 0.76. The strict consensus (Fig. 3) is 6 steps longer. Although the majority of informative characters originate from the ITS dataset, combining the ITS and *trn*L datasets altered the topology and support for some clades.

In the combined data set, *Parasponia* (only one species in this analysis) was sister to *Trema*, but this result received no bootstrap support. This topology resulted from the reduced number of taxa analyzed, rather than

the inclusion of the *trn*L dataset. When the ITS dataset for the same 38 sequences was analyzed separately, *Parasponia* was again sister to a monophyletic *Trema* and never formed a clade with *T. nitens* + *T. politoria* in any of the MPCs as it did in the full ITS dataset.

Within *Trema*, the highly supported *T. nitens* + *T. politoria* clade (OW1) remained sister to all other species, which comprised an Old World and New World clade. There was no bootstrap support for many nodes along the backbone of the consensus tree; therefore, relationships collapsed into a polytomy of four clades: *Parasponia*, *T. nitens* + *T. politoria* (OW1), other Old World species (OW2) and New World species. The topology remained the same as in the ITS analysis, but bootstrap support was lost for subclade OW2b, which included *T. orientalis* II, *T. africana, T. virgata*, and *T. tomentosa*. The New World *Trema* were better resolved in the strict consensus of the combined data set than in the ITS data set alone. *Trema micrantha* I was still a strongly supported monophyletic group and sister to *T. lamarkiana*. The *T. micrantha* IIb and IIc clades received weak support, and *T. integerrima* clade strong support, as subclades within the *T. micrantha* II group with black endocarps. However, the position of *T. micrantha* IId shifted toward the base of the New World clade rather than being sister to *T. integerrima* within the wider *T. micrantha* II group.

Discussion

The major objective of this study was to provide a framework for further revisionary, phylogenetic and phylogeographic studies of *Trema*. Because *Trema* has never been monographed in its entirety, there is much confusion in species concepts and nomenclature throughout its range. This study is the first world-wide phylogenetic analysis of *Trema*. It is also the first to use molecular sequence data as new characters for evaluating species delimitations and resolving species relationships in the genus. This extends the successful application of various molecular markers to higher level relationships within Celtidaceae and Urticales (Ueda et al. 1997, Wiegrefe et al. 1998, Song et al. 2001, Sytsma et al. 2002). Sequences from the *trn*L region of the chloroplast genome and ITS region of the nuclear genome proved successful in providing new informative characters for delimiting species, establishing relationships, and evaluating morphological characters.

We first evaluate evidence for the major clades within *Trema*. For each continental region, we then discuss whether the new molecular data support species concepts based on morphological data and discuss relationships among species. New World species are treated in more detail than African and Asian species because sampling within reputed species was more extensive. We conclude by discussing the relationship of *Trema* with *Parasponia* and biogeography of *Trema* in the context of Celtidaceae. Our results cast considerable doubt on current use of species names in most regions; therefore, the names used below should be considered as provisional. Much wider sampling within regions will be necessary before a clear picture emerges of species and their relationships.

Major clades within *Trema*. Specimens from the New World formed a moderately supported clade in both the ITS and the combined analyses, but not in the *trn*L analysis. Specimens from the Old World however, did not form a monophyletic group. The clade of *T. politoria* + *T. nitens* (clade OW1), from India-Nepal and Togo, respectively, was strongly supported in the ITS and combined analysis, and weakly supported even in the *trn*L analysis. The clade of the remaining Old World specimens (clade OW2) from Africa, Asia and Australia, was weakly to moderately supported in the ITS and combined analyses only. The *T. politoria* + *T. nitens* clade (OW1) was an unambiguous sister group to remaining *Trema* species in the combined analysis, but this did not have bootstrap support. Therefore, these three clades should be treated as a polytomy, with *Parasponia* as a fourth clade, until further species and/or markers can be

analyzed. In practical terms, this suggests that monographic work could continue separately in the New and Old World, but on a very large geographical scale in each region.

New World species. Specimens of *T. lamarckiana* (Roem. & Schult.) Blume formed a well-supported clade in all phylogenetic analyses. This clade was further characterized by a large 90 bp deletion in *trn*L, a smaller 2 bp deletion in ITS, and the largest number of autapomorphies found in any *Trema* ITS sequence. Morphologically, *T. lamarckiana* is distinguished primarily by its small leaves (< 6 cm long; Adams 1972, Stern and Sweitzer 1972, Howard 1988, Nicolson 1991). The molecular analysis supports the continued recognition of this widespread species found throughout the Caribbean and into southern Florida.

In contrast, specimens of *T. micrantha* (L.) Blume s.l., the most widespread and variable neotropical *Trema* (e.g. Burger 1977, Nevling 1960, Elias 1970), never formed a monophyletic group. Specimens of the two endocarp variants first identified in Panama (Dalling et al. 1997, Parrish et al. 2001, Silvera et al. 2003) were never grouped together. Endocarps of the brown variant are relatively small, lack prominent ridges and have a deeply cratered surface perforated with round pores; those of the black variant are relatively large and prominently ridged, and have an irregular surface perforated with irregular pores (Garwood unpublished). The *T. micrantha* I clade contained all specimens with brown endocarps, and none with black endocarps. It was strongly supported in the ITS and combined analysis and weakly supported even in the *trn*L analysis. *Trema micrantha* I was further characterized by a large 77 bp deletion in *trn*L (which did not overlap the large deletion in *T. lamarckiana*) and unique 8 bp and 1 bp insertions in ITS. The endocarp characters and molecular data together indicate that *T. micrantha* I is a distinct species.

The group of *T. micrantha* II specimens with black endocarps was not monophyletic. Several weakly to strongly supported subclades were scattered among unresolved specimens. Subclades IIb and IIc both had weak support in the combined analysis but lower support in ITS alone; their consistent sister group relationship, however, received no bootstrap support in either analysis. *Trema integerrima* was a strongly supported subclade nested within the broader *T. micrantha* II group in both the ITS and combined analyses, but not the *trn*L analysis. Thus, *Trema micrantha* II is paraphyletic relative to *T. integerrima*. *Trema integerimma* is distinguished from *T. micrantha* by entire margined leaves rather than serrate leaves (Standley 1933); it also has black ridged endocarps, but these are smaller than those of *T. micrantha* II (Garwood, pers. obs.). Subclade IId had very high support in the ITS and combined analyses. Its position shifted from sister group of *T. integerrima* in the ITS analysis to a position outside the *T. micrantha* IIa + IIb + IIc + *T. integerrima* clade in the combined analysis. In the *trn*L analyses, the two specimens comprising subclade IId were also outside the weakly supported *T. micrantha* IIa + IIb + IIc + *T. integerrima* clade, but they did not form a clade themselves. Thus, *T. micrantha* II is polyphyletic relative to subclade IId.

Differences in pubescence on the lower leaf surface (observed at 40 x) of the specimens analyzed provide further support for subclades within the *T. micrantha* II and *T. integerrima* group (Garwood, per. obs.). In *T. integerrima*, trichomes were very fine, sparse and appressed, and the upper leaf surface was not scrabrous (unlike most *Trema*). In subclade IIc, collected at 1000–1500 m in western Panama, dense minute white trichomes between the veinlets caused the surface to appear greyish-white. In subclade IIb, trichomes were short and erect. In subclade IId, trichomes were long, erect and somewhat thin. In the remaining unresolved specimens, IIa, trichomes were also long and erect but stouter and usually had enlarged bases. In contrast, the lower surface of *T. micrantha* I was nearly glabrous except for abundant large mounds resembling enlarged bases of trichomes (but without the hair) and

trichomes along the primary veins were appressed rather than erect.

The general congruence of morphological characters (trichomes and endocarps) with the molecular analysis suggests that the clades and subclades have taxonomic significance. However, it is difficult to decide what names or ranks to apply to clades identified in this analysis. Use of *T. lamarckiana* and *T. integerrima* seems straightforward. Using Adams (1972) to identify Jamaican specimens of *T. micrantha* I and IId, the former would be named *T. micrantha* and the latter *T. floridana*. However, *T. floridana* (or *T. micrantha* var. *floridana*) is usually applied to northern Central American and Florida populations (Standley and Steyermark 1946). Clade IId consisted of specimens from Jamaica and Bolivia and did not group with specimens from Belize or Florida. Clearly, further nomenclatural work is needed to determine which clade or subclade should be called *T. micrantha* and which of its many synonyms applied to the other groups.

The relationships of groups within neotropical *Trema* are not well resolved in either the ITS or combined analyses. There is weak support for a sister-group relationship between *T. micrantha* I and *T. lamarckiana*. Although there is much structure in the consensus tree of the combined analysis, most branches are unsupported and the subclades collapse to a polytomy.

African species. Polhill (1964) decided that only one widespread but variable species was present south of the Sahara and that this was identical to the Asiatic *T. orientalis* (L.) Blume. More recently, St-Laurent et al. (2000a, b), using discriminate analysis of leaf characters and SEM analysis of leaf trichomes, concluded that there were three species present in Togo and neighboring countries: *T. orientalis*, *T. africana* (Planch.) Blume and *T. nitens* (Hook. & Planch.) Blume. Our phylogenetic analysis of a sample of St-Laurent's specimens clearly shows that there are more than one species present in Togo. *Trema nitens* was part of the strongly supported OW1 clade, and therefore distantly related to *T. africana* and *T. orientalis*, which were part of clade OW2. *Trema africana* was supported as sister group to African *T. orientalis* II in the ITS and combined analyses within OW2. It was further distinguished from *T. orientalis* II by a 5 bp insertion in *trn*L and many autapomorphies in the ITS analysis. (For discussion of morphological characters and nomenclature of these species, see St-Laurent et al. 2000a.) There was no support for a close relationship between the African *T. orientalis* II and the Asian *T. orientalis* I within clade OW2.

Asian species. The number of recently recognized species is higher and problems of synonymy more complex in Asia than in Africa. Four species were recognized for *Flora Malesiana* (Soepadmo 1977). Species are, however, extremely variable: *T. orientalis* and *T. cannabina* Lour. each comprise "three rather but not completely distinct entities" (Soepadmo 1977). Of the Malesian species, all four extend into China (Yang and Lu 1996, Fu et al., unpubl. data), two range into India and Australia (Grierson and Long 1983, Hewson 1989, Khanna 1997), and two occur throughout the Pacific islands as far as Hawaii, although these do not always correspond closely to descriptions of Malesian species (Smith 1981, Florence 1997, Wagner et al. 1999). Excluding subspecies and probable synonyms, two additional species are reported from China (Fu et al., unpubl. data) and one from the Indian subcontient (Grierson and Long 1983, Khanna 1997). Our sample of 12 Asian specimens from six presumed species indicates that the Asian species do not form a monophyletic group.

Asian *T. orientalis* and *T. tomentosa* are usually considered closely related but distinguishable by the lower leaf surface being completely covered by minute hairs in the former but easily visible in the latter (Soepadmo 1977). We identified specimens of these two species using this diagnostic character. Specimens of *T. tomentosa* from Sikkim and Bangladesh formed a moderately supported group within subclade OW2b, which included *T. afri-*

cana, T. orientalis II, and *T. virgata*. Specimens of *T. orientalis* I from Thailand and Indonesia did not form a group, but formed a well-supported Asian clade with *T. aspera* (OW2a). Thus, Asian *T. tomentosa* and *T. orientalis* I do not appear closely related, although we were not able to compare sympatric specimens. *Trema aspera* (Brongn.) Blume is considered a synonym of *T. tomentosa* var. *viridis* (Planch.) Hewson in Australia (Hewson 1989). Because *T. aspera* consistently grouped with *T. orientalis* I, not *T. tomentosa,* in the ITS and combined analyses, we have retained *T. aspera*. The New Caledonian specimen of *T. aspera* grouped with the Australian specimens in the ITS analysis, but it differed from them by many autapomorphies and a unique 8 bp insertion.

We were unable to obtain sequences from specimens that closely matched the type of *T. cannabina* Lour., but were successful with *T. virgata* (Planch.) Blume, a presumed synonym (Soepadmo 1977, Fu et al. unpubl. data) and *T. cannabina* var. *dielsiana* (Hand.-Mazz.) C.J. Chen, both from China (Fu et al., unpubl. data). *Trema politoria* Planch. from northern India, Bhutan, Sikkim and Nepal was considered as a possible unrecognized synonym of *T. cannabina* (Grierson and Long 1983) and was identified as such on one specimen analyzed. We obtained *trn*L sequences from all three taxa, but not ITS from T. *cannabina* var. *dielsiana*. *Trema politoria*, as noted above, groups with *T. nitens* in all analyzes and never with *T. virgata* or *T. cannabina* var. *dielsiana.* In the *trn*L analysis, both *T. cannabina* var. *dielsiana* and *T. virgata* have relatively long branches but share few characters; the former has a unique 10 bp insertion and the latter a unique 4 bp insertion. This suggests that these three taxa are not closely related.

Relationship with *Parasponia*. The five Asian species of *Parasponia* (Miquel 1851) differ from *Trema* by having connate intrapetiolar stipules rather than free extrapetiolar stipules and imbricate perianth lobes on male flowers rather than induplicate valvate perianth lobes (Soepadomo 1977). *Parasponia* also fixes nitrogen (Becking 1983). Recent molecular work on Urticales using the chloroplast gene *rbc*L suggested that *Trema* is a paraphyletic group, with *Parasponia* nested within it (Sytsma et al. 2002). Their study included *T. micrantha, T. orientalis*, *P. parviflora* and *P. rigida*. Our study included many more species of *Trema* and three species of *Parasponia*. The *Trema* + *Parasponia* clade received strong support in the *trn*L and combined analyses, and weak support in the ITS analysis. All three *Parasponia* species were included in the *trn*L analysis: these did not form a monophyletic group in the consensus tree or in any of the MPCs. A *P. parviflora* + *P. simulans* clade received moderate bootstrap support. *Parasponia rigida* grouped with specimens of *T. orientalis I* from Taiwan and Sulawesi: this clade was retained in the consensus tree but lacked bootstrap support and did not contain *T. orientalis* I from Thailand. Only *P. simulans* was successfully sequenced for the ITS and combined analyses. In these, *P. simulans* was either sister to all *Trema* or sister to the *T. nitens* + *T. politora* clade, which was sister to all remaining *Trema*. In all three analyses, *P. simulans* had the highest number of autapomorphies of any taxa within the *Trema* + *Parasponia* clade; in the *trn*L analysis, the *P. parviflora* + *P. simulans* clade had more synapomorphies than other subclades. This suggests that at least *P. simulans* and *P. parviflora* sequences are quite different from *Trema*, although this does resolve their position as sister to or derived from within *Trema.* Given that the ITS region is highly informative, obtaining further sequences from the remaining four species of *Parasponia* should resolve their position relative to *Trema.*

Relationships within Celtidaceae and biogeography. Sytsma et al. (2002) recently reviewed relationships within Celtidaceae and Urticales. *Aphananthe*, *Gironniera* and *Chaetachme* are most likely early diverging genera within Celtidaceae, with *Trema* + *Parasponia* sister to a clade containing *Celtis*, *Pteroceltis*, *Humulus* and *Cannabis*. Celtidaceae have a good fossil record throughout the Northern

Hemisphere (Manchester 1989), so broad biogeographic patterns are known. Endocarps of the early diverging *Aphananthe* and *Gironniera* first appear in Maastrichtian to Oligocene deposits from Europe, North America and Asia (Manchester 1989). *Celtis* endocarps, permineralized during development and well represented in deposits lacking other plant fossils, appear in the Paleocene and Eocene of South and North America and first appear in the Eocene in Eurasia. The smaller endocarps of *Trema* and *Parasponia* are so far unknown as fossils; leaves attributed to *Trema* were reported from the Oligocene of Japan and diporate pollen attributed to *Trema* from the Eocene of North America (reviewed by Manchester 1989). Whether endocarps of *Trema* become permineralized like *Celtis* has not been studied, but *Trema* endocarps persist for long periods (> 10 yr) in the soil seed bank in tropical forests, much longer than seeds or fruits of most tropical taxa (Dalling et al. 1997). Therefore, looking for *Trema* endocarps (usually 1–3 mm in diameter) in finer sediments of fossil deposits of tropical floras from the early Tertiary might shed light on the early history and biogeography of this group.

Trema species now grow only in subtropical and tropical climates, although some species occur in cooler montane tropical forests up to about 3000 m. Free exchange of tropical taxa between the Old and New Worlds could only occur in the Paleocene and Eocene in the Northern Hemisphere because of cooling climates after the Eocene (Tiffney and Manchester 2001). This suggests the divergence of New and Old World clades of *Trema* may date back to the Paleocene or Eocene. Some clades might be much younger if either early *Trema* were more tolerant of cool temperate climates than living species or long distance dispersal has occurred more recently. *Trema* is found throughout the oceanic islands of the Indo-Pacific (Smith 1981, Florence 1997), including Hawaii (Wagner et al. 1999), and on oceanic islands in the Caribbean (Howard 1988, Nicolson 1991). This suggests that long distance dispersal has occurred repeatedly, perhaps promoted by a combination of small, long-lived and desiccation tolerant seeds and endozoochory by birds.

Future work. This study has provided the first phylogenetic framework for *Trema* and shown that molecular sequence data can provide valuable characters for systematic work in this genus. To resolve species delimitations, relationships, times of divergence and nomenclature of *Trema* throughout its range, a thorough monographic treatment combining molecular and morphological analyses is needed. Additional molecular markers will be required to resolve some relationships, such as those within the *T. micrantha* II complex. Given the importance of pubescence in distinguishing taxa, detailed analysis of trichome morphology using SEM will be essential, as already shown for some African and Asian taxa (Soepadmo 1977, St-Laurent et al. 2002b). Endocarp characters, not previously used for distinguishing among *Trema* species, merit further examination as they proved informative in delimiting neotropical *Trema* species. Finding fossil endocarps would provide a historical perspective, as they have done for *Celtis* (Manchester 1989). Lastly, a large-scale phylogeographic study is needed to understand the processes leading to this pattern of relationships. The morphological variability within currently recognized species should be considered an asset, not a liability, for understanding the role of biogeographic and evolutionary processes in this widespread pioneer tree genus.

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Appendix 1. Specimen data. Species found to be polyphyletic in this study (Figs. 1–3) are divided into groups (I, II, IIa-IId). The geographical origin, collection number, and year collected are given for each specimen. Collection dates between 1999 and 2002 indicate silica dried leaves and accompanying voucher specimens collected specifically for this study. Earlier collection dates (1864 – 1996) are herbarium specimens, mostly from the Natural History Museum (BM). A unique code linked to geographical origin distinguishes multiple samples of the same species. The original determination is included when it differs from our identification. All specimens are from the Natural History Museum (BM) except those from Togo, which are from Ottawa (DAO). Sequences for each marker from each specimen are complete (+ or GenBank accession number); partial (p); or absent (-, not attempted; #, unsuccessful). Identical sequences are marked by the same letter: sequences submitted to GenBank are distinguished from others (accession number and +, respectively). All sequences used in the study are new except for four outgroup sequences (GenBank numbers in bold)

Species			Specimen (year) [Original determination]	Sequence data	
Group	Sample Code	Origin		*trn*L	ITS
Celtis iguanaea (Jacq.) Sarg.					
	BZ	Belize	Whitefoord 9350 (1995)	AY488673	AY488719
	CR	Costa Rica	Haber & Bello 1745 (1985)	AY488672	–
Celtis leavigata Willd.					
	US	Texas, USA	GenBank	–	**AF174621**
Parasponia parviflora Miq.					
	OW	Old World	GenBank	**AF501596**	–
Parasponia rigida Merr. & L. M. Perry					
	WP1	West Papua, Indonesia	Brass 12459 (1939)	AY488675	#
Parasponia simulans Merr. & L. M. Perry					
	WP2	West Papua, Indonesia	Brass 13072 (1939)	AY488674	AY488720
Trema africana Blume					
	TG1	Togo	St-Laurent 96–43 (1996)	AY488690	AY488731
	TG2	Togo	St-Laurent 96–80 (1996)	AY488691	#
Trema aspera (Brongn.) Blume					
	AU1	Queensland, Australia	Adams 20038 (1959)	AY488681	AY488724
	AU2	Queensland, Australia	Michael 865 (-)	AY488682	AY488725
	NC	New Caledonia	Compton 511 (1914) [*Parasponia andersonii* Planch].	AY488680	AY488723
Trema cannabina var. *dielsiana* (Hand.-Mazz.) C.J. Chen					
	CN1	China	Bartholomew et al. 894 (1986)	AY488678	#
Trema integerrima (Beurl.) Standl.					
	CR2	Costa Rica	Dalling et al. s.n. (2001)	AY488718	AY488755
	EA13	Ecuador (Amazon)	Garwood 4545 (2001)	AY488717 D	AY488757

Appendix 1 (continued)

Species			Specimen (year)	Sequence data	
Group	Sample Code	Origin	[Original determination]	*trn*L	ITS
	EA14	Ecuador (Amazon)	Garwood 4546 (2001)	+ D	–
	EA15	Ecuador (Amazon)	Garwood 4514 (2001)	+ D	–
	EA16	Ecuador (Amazon)	Garwood 4540 (2001)	+ D	–
	EA17	Ecuador (Amazon)	Garwood 4542 (2001)	+ D	–
	EW11	Ecuador (west coast)	Garwood 4575A (2001)	AY488716 E	AY488756
	EW12	Ecuador (west coast)	Garwood 4575B (2001)	+ E	–
Trema lamarkiana (Roem. & Schult.) Blume					
	DM	Dominica	Ernst 1965 (1965)	AY488696 K	AY488735 C
	MS	Montserrat	Howard et al. 18957 (1979)	+ K	–
	DR	Dominican Republic	Howard 12093 (1950)	AY488695 J	#
	KY	Cayman Islands	Brunt 2049 (1967)	+ J	–
	FL1	Florida	Koop 12 (1999)	AY488698	#
	FL2	Florida	Koop 5 (1999)	AY488697	+ C
Trema micrantha (L.) Blume					
I	EA1	Ecuador (Amazon)	Garwood 4548 (2001)	AY488699 A	AY488737 B
I	EA2	Ecuador (Amazon)	Garwood 4525 (2001)	+ A	–
I	EA3	Ecuador (Amazon)	Garwood 4547 (2001)	+ A	–
I	EA4	Ecuador (Amazon)	Garwood 4553 (2001)	+ A	–
I	EA5	Ecuador (Amazon)	Garwood 4552 (2001)	AY488701 B	–
I	EA6	Ecuador (Amazon)	Garwood 4561 (2001)	+ B	–
I	JM1	Jamaica	Adams 7513 (1960)	AY488700	AY488740
I	PA1	Panama	Dalling 2 (1999)	p	+ B
I	PA2	Panama	Dalling 3 (1999)	p	+ B
I	PA3	Panama	Dalling 11 (1999)	p	AY488736 A
I	PA4	Panama	Dalling 78 (1999)	+ A	+ A
I	PA5	Panama	Dalling 65 (1999)	+ A	–
I	PA6	Panama	Dalling 82 (1999)	+ A	–
I	PA7	Panama	Dalling 84 (1999)	+ A	–
I	PA8	Panama	Dalling 6 (1999)	p	AY488738
I	PA9	Panama	Dalling 4 (1999)	p	AY488739
II a	AR	Argentina	Venturi 7580 (1928)	AY488706	AY488746
II a	BZ3	Belize	Garwood et al. 30 (1999)	AY488702	AY488742

Appendix 1 (continued)

II a	FL4	Florida	Koop 9 (1999)	+ H	#
II a	BZ4	Belize	Garwood et al. 14 (1999)	+ H	–
II a	BZ5	Belize	Garwood et al. 28 (1999)	+ H	–
II a	CO	Colombia	Little & Little 8231 (1944)	AY488707	AY488744
II a	FL5	Florida	Koop 7 (1999)	+ H	–
II a	FL6	Florida	Koop 8 (1999)	+ H	–
II a	FL7	Florida	Koop 10 (1999)	+ H	–
II a	FL8	Florida	Koop 1 (1999)	AY488709	–
II a	MX	Mexico	Nee et al. 25106 (1982)	AY488704	–
II a	PA30	Panama	Dalling 72 (1999)	AY488712	AY488741
II a	PA31	Panama	Dalling 83 (1999)	AY488714	AY488747
II a	PA32	Panama	Dalling 59 (1999)	AY488710 H	AY488745
II a	PA33	Panama	Dalling 60 (1999)	+ H	AY488748
II a	PA34	Panama	Dalling 52 (1999)	+ H	–
II a	PA35	Panama	Dalling 61 (1999)	+ H	–
II a	PA36	Panama	Dalling 66 (1999)	+ H	–
II a	PA37	Panama	Dalling 67 (1999)	+ H	–
II a	PA38	Panama	Dalling 69 (1999)	+ H	#
II a	PA39	Panama	Dalling 76 (1999)	+ H	–
II a	PR	Puerto Rico	Sustache s.n. (2001)	AY488713	–
II b	BZ1	Belize	Garwood et al. 23 (1999)	AY488703	AY488751
II b	BZ2	Belize	Garwood et al. 4 (1999)	+ H	AY488752
II b	FL3	Florida	Koop 2 (1999)	AY488715	AY488743
II c	PA20	Panama	Dalling 40 (1999)	AY488711 F	AY488750
II c	PA21	Panama	Dalling 62 (1999)	+ F	AY488749
II c	PA22	Panama	Dalling 39 (1999)	+ F	–
II c	PA23	Panama	Dalling 45 (1999)	+ F	–
II d	BO	Bolivia	Nee et al. 51812 (2001)	AY488705	AY488753
II d	JM2	Jamaica	Adams 11328 (1962)	AY488708	AY488754
Trema nitens (Hook. & Planch.) Blume					
	TG4	Togo	St-Laurent 97-53 (1996)	AY488679	AY488721
Trema orientalis (L.) Blume					
I	ID	Sulawesi, Indonesia	Martin 5034 (1985)	AY488688	AY488729
I	TH	Thailand	Maxwell & van de Bult 10 (2002)	AY488687	AY488730
I?	TW	Taiwan	Oldham s.n. (1864)	AY488686	#
II	TG7	Togo	St-Laurent 96-16 (1996)	AY488694	AY488732

Appendix 1 (continued)

Species			Specimen (year) [Original determination]	Sequence data	
Group	Sample Code	Origin		*trn*L	ITS
II	TG8	Togo	St-Laurent 96–2 (1996)	AY488693	AY488733
II	TG9	Togo	St-Laurent 97-11 (1996)	AY488692	AY488734
II?	UG	Uganda	Taylor 3113 (1935)	AY488689	#
Trema politoria Blume					
	IN	India	Kerr 2237 (1953)	AY488677	AY488722
	NP	Nepal	Williams 1149 (1950) [*Trema cannabina* Lour.]	AY488676	#
Trema tomentosa (Roxb.) H. Hara					
	SK	Sikkim	Stainton 5381 (1966)	AY488684	AY488727
	BD	Bangladesh	Rahman et al. 2554 (1998) [*Trema orientalis* (L.) Blume]	AY488685	AY488728
Trema virgata Blume					
	CN2	Yunnan, China	Forrest 12604 (1914)	AY488683	AY488726
Ulmus glabra Huds.					
	GB	London, UK	Yesson s.n. (2002)	AY488671	–
Ulmus americana L.					
	US	Texas, USA	GenBank	–	**AF176388**
Ulmus crassifolia Nutt.					
	US	Texas, USA	GenBank	–	**AF174641**

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