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Reconstructing the phylogeny of figs (*Ficus*, Moraceae) to reveal the history of the fig pollination mutualism

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Abstract
Figs (*Ficus*, Moraceae) constitute one of the largest genera of flowering plants with ca. 750 species worldwide. While the extraordinary mutualism between figs and their pollinating wasps has received attention for decades, we are only just beginning to reconstruct the phylogeny of both partners, a necessary framework for addressing a variety of questions concerning the evolution of mutualism. Here, we present phylogenetic analyses of 100 species of *Ficus*, representing all subgenera, sixteen out of nineteen sections, and two outgroups, using three nuclear markers. We explore the utility of the single copy nuclear encoded glyceraldehyde-3-phosphate dehydrogenase gene (*G3pdh*) for phylogeny reconstruction in *Ficus*, and evaluate infrageneric relationships based on *G3pdh* DNA sequences in combination with the nuclear ribosomal internal and external transcribed spacers (ITS and ETS). The *G3pdh* gene provides limited resolution within *Ficus*, but increases the proportion of well-supported clades when combined with ITS and ETS. Of the six subgenera traditionally recognized based on morphology and distribution patterns, only subgenus *Sycidium* is supported as monophyletic. We identify fifteen clades within *Ficus*, but the branching order of the early lineages of *Ficus* and some of the internal branches are not well supported and should be considered uncertain at present.

Keywords: *Ficus*, Moraceae, phylogeny, classification, ITS, ETS, *G3pdh*

1. Introduction

The figs (*Ficus* species, Moraceae) are among the largest genera of angiosperms with approximately 750 species of trees, epiphytes and shrubs in tropical and subtropical regions worldwide. Frodin (2004) ranked them as the twenty-first largest genus of seed plants. *Ficus* is one of the most diverse plant genera in regard to growth habit, with both deciduous and evergreen free-standing trees, small shrubs, creepers, climbers, stranglers, rheophytes and lithophytes (Harrison, 2005). The Asian-Australasian region has the richest and most diverse fig flora with over 500 species. By comparison, the richness of *Ficus* in Africa and the Neotropics is lower, with approximately 110 and 130 species, respectively. Roughly half of the *Ficus* species are monoecious, and the rest are functionally dioecious (Berg, 2003; Berg and Corner, 2005).

All members of the genus share the distinctive inflorescence (syconium), which is the site of an intricate mutualism with pollinating fig wasps of the subfamily Agaonidae (Cook and Rasplus, 2003). Figs are pollinated only by female wasps that lay their eggs exclusively in fig flowers where wasp larvae feed on some of the developing seeds. The fig-wasp interaction has persisted for over 60 million years (for example, Rønsted et al., 2005), and was thought to be mutually species-specific, but an increasing number of exceptions have been documented (Haine et al., 2006; Lopez-Vaamonde et al., 2002; Machado et al., 2005; Silvieus et al., in press). Persistence of obligate mutualisms over long periods of time is noteworthy given the risk of extinction associated with extreme specialization, and so the fig-wasp mutualism has long received attention as a model system for studying the comparative biology of

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mutualisms and co-evolution (Bronstein and McKey, 1989).

Our knowledge of the nature and extent of co-evolution in the fig-wasp mutualism has been limited, because an accurate evaluation of the history of the interaction requires that phylogenies of both partners be known (Page, 1996). Traditional classifications of *Ficus* (Table 1; Corner, 1965; Berg and Corner, 2005) and Agaonidae (for example, Wiebes, 1986) were primarily based on morphology and distribution patterns, but evolutionary relationships are more appropriately revealed by DNA sequence analyses (Herre at al., 1996; Jousselin et al., 2003; Weiblen, 2000).

The first molecular phylogenetic analysis of *Ficus* (Herre et al., 1996) included only fifteen species. This study was based on plastid *rbcL* and tRNA sequences, which provided poor resolution within in the genus. A study by Weiblen (2000) based on ITS sequences and morphology included 46, mainly dioecious, species of *Ficus*. Jousselin et al. (2003) used a combination of ITS and ETS data for 41 species of *Ficus* and included representatives of most sections of *Ficus*. These studies showed that Corner's (1965) classification was not phylogenetic and revealed homoplasy in characters such as growth habit and breeding system that had been used to recognize subgenera (Herre et al., 1996; Jousselin et al., 2003; Weiblen, 2000).

These studies also relied on a few exemplar taxa to represent large groups, which begs the question of whether these large groups are in fact monophyletic (Graybeal, 1998; Rønsted et al., 2006). Hence, our earlier research extended sampling of ITS and ETS to include 146 taxa of *Ficus* and outgroups (Rønsted et al., 2005). These results were in general agreement with previous molecular findings but several major relationships were not resolved or supported by these two markers alone. It is often observed that large plant genera such as *Ficus* have insufficient ITS and ETS variation to detect phylogenetic relationships among closely related species that may have diversified recently or major clades that may have diverged rapidly in the ancient past. Sequencing low-copy, protein-coding genes can provide additional information (Rønsted et al., 2006).

The intent of this paper is to explore the utility of glyceraldehyde-3-phosphate dehydrogenase (*G3pdh*) sequences for phylogeny reconstruction so to improve resolution and support of *Ficus* phylogeny, and to evaluate relationships within *Ficus* using three nuclear markers: the *G3pdh* gene combined with the nuclear ribosomal internal and external transcribed spacers (ITS and ETS).

2. Materials and Methods

Materials

We based our taxon sampling on a previously published phylogenetic study of *Ficus* combining ITS and ETS (Rønsted et al., 2005), although we included here three additional species from the heterogeneous section *Oreosycea*, namely *F. albipila, F. pseudojaca,* and *F. variifolia*. Total genomic DNA was extracted using the Qiagen DNeasy plant extraction kit (Qiagen Inc., Valencia, California, USA) from 20–30 mg of dried leaf-fragments or herbarium material. Two ITS, seven ETS and 79 new *G3pdh* sequences were produced for this study (see Table 3). In addition, 99 ITS, 81 ETS, and 18 *G3pdh* sequences were retrieved from GenBank. Information on these sequences is reported in Weiblen (2000), Jousselin et al. (2003), Machado et al. (2005), Rønsted et al. (2005), and Silvieus et al. (in press).

Our sampling included all *Ficus* sections recognized by Berg and Corner (2005) except for three small and recent sections in subgenus *Sycomorus* (Table 1). *Antiaropsis decipiens* and *Castilla elastica* were included as outgroups based on a previous study by Rønsted et al. (2005).

PCR amplification and DNA sequencing

The ITS and ETS regions (Baldwin et al., 1995; Baldwin and Markos, 1998), were amplified using primers 17SE and 26SE (Sun et al., 1994) or ITS4 and ITS5 (White et al., 1990), and Hel1 and 18S ETS (Baldwin and Markos, 1998), respectively. The *G3pdh* gene (Strand et al., 1997) was amplified using primers 7F and 9R (Strand et al., 1997). Standard automated sequencing protocols (Jousselin et al., 2003) were used to generate ITS and ETS sequences, except that DMSO was added to all reactions. *G3pdh* PCR reactions were prepared using ~20 ng genomic DNA, 1X *TaKaRa Ex Taq* buffer (2 nM $MgCl_2$), 1.25 unit *TaKaRa Ex Taq* DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan), 1 μM each primer, 0.2 mM each dNTP, 0.4% BSA (bovine serum albumine). DMSO was added to some of the reactions. Amplified products were purified with the Qiagen PCR purification kit (Qiagen Inc.) following the manufacturer's protocols. Amplification of *G3pdh* generally consisted of 3.5 min at 94°C followed by 36 cycles of: 1 min denaturation (95°C), 1 min annealing (49°C) and 2 min extension (72°C). After the last cycle, the temperature was kept at 72°C for a final 7 min extension and then lowered to 4°C.

Cycle sequencing reactions were carried out using the BigDye™ Terminator Mix (Applied Biosystems, Inc., Foster City, California, USA). The sequencing protocol consisted of 26 cycles of 10 sec denaturation (96°C), 5 sec annealing (50°C) and 4 min elongation (60°C). Sequencing reactions were run on ABI 377 or ABI 3130 Genetic Analyzers according to the manufacturer's protocols (Applied Biosystems). Both strands were sequenced for each region for the majority of taxa. For sequencing of the *G3pdh* gene of some taxa, internal primers 286F (TGT ATT CTG GTT GGG TTT C) and 437R (TTC TGA AGC

Table 1. Traditional classification of *Ficus* based on morphology and preliminary taxonomic implications of the present study.

Traditional subgenera and sections (Berg and Corner, 2005)	# of species	Clades in the combined analysis (Fig. 2)	BS (%)[1]	Comment
Subg. *Ficus*				
Sect. *Eriosycea*	28	Sect. *Eriosycea*	98	
Sect. *Ficus*	29	Subsect. *Ficus*	100	Subsect. *Ficus*
		Subsect. *Frutescentiae*	100	Including *F. pumila*. Sister to subg. *Sycidium* (74% BS)
Subg. *Pharmacosycea*				Polyphyletic
Sect. *Oreosycea*	55	Sect. *Oreosycea* s.s.	100	Excludes subser. *Albipilae*. May be sister to sect. *Sycomorus* s.l.
		Subser. *Albipilae*	60	Sect. *Oreosycea* subsect. *Pedunculatae* subser. *Albipilae*
Sect. *Pharmacosycea*	25	Sect. *Pharmacosycea*	80	First diverging lineage
Subg. *Sycidium*		Subg. *Sycidium*	97	Monophyletic. Sister to sect. *Eriosycea* (74%)
Sect. *Paleomorphe*	30	Sect. *Paleomorphe*	87	The relationship between sects. *Sycidium* and *Paleomorphe* is unclear
Sect. *Sycidium*	80	Not resolved		
Subg. *Sycomorus*				
Sect. *Adenosperma*	20	Sect. *Adenosperma* s.l.	90	Includes *F. dammaropsis* (sect. *Dammaropsis*). Sister to sect. *Sycocarpus* (84%)
Sect. *Bosscheria*	2	Not sampled		
Sect. *Dammaropsis*	5			*F. dammaropsis* is included in sect. *Adenosperma* s.l.
Sect. *Hemicardia*	3	Not sampled		
Sect. *Papuasyce*	3	Not sampled		
Sect. *Sycocarpus*	86	Sect. *Sycocarpus*	100	Sister to sect. *Adenosperma* s.l. (84% BS)
Sect. *Sycomorus*	18	Sect. *Sycomorus* s.l.	68	Includes former sect. *Neomorphe*. May be sister to sect. *Oreosycea* s.s.
Subg. *Synoecia*		Subg. *Synoecia* s.s.	99	Excludes *F. pumila*. The relationship between sects. *Rhizocladus* and *Kissosycea* is unclear. Subg. *Synoecia* may be sister to *Ficus* subsect. *Frutescentiae* (61% BS)
Sect. *Kissosycea*	28	Not resolved		
Sect. *Rhizocladus*	47	Not resolved		
Subg. *Urostigma*				Polyphyletic
Sect. *Americana*	100	Sect. *Americana*	100	May be derived within sect. *Galoglychia*
Sect. *Galoglychia*	72	Not resolved		The relationship between sects. *Americana* and *Galoglychia* is unclear
Sect. *Urostigma*	90	Sect. *Urostigma* s.s.	100	Excludes sect. *Conosycea* and Corners sect. *Leucogyne*. Not part of the clade with the remainder of the former subg. *Urostigma*
		Sect. *Conosycea* s.l.	94	Includes *F. elastica* and Corners sect. *Leucogyne*. Sister to sect. *Malvanthera* (96% BS)
Sect. *Stilpnophyllum*	20	Sect. *Malvanthera*	90	Excludes *F. elastica*. Sister to sect. *Conosycea* (96% BS)

[1]BS: Bootstrap percentages.

CTG ACA GTG AGG) were used, in addition to the primers used for amplification. For sequencing of the ITS region of some taxa, internal primers (GCT ACG TTC TTC ATC GAT GC) and (GCA TCG ATG AAG AAC GTA GC), were modified from ITS2 and ITS3 respectively (White et al., 1990), and used in addition to the primers used for amplification.

Phylogenetic reconstructions

Two new ITS, seven ETS, and 79 *G3pdh* sequences were edited and assembled using Sequencher 4.1.2™ software (Gene Codes Corp, Ann Arbor, Michigan, USA), and all sequences were aligned manually in PAUP v. 4.0b5 (Swofford, 2001). Phylogenetic analyses were conducted using PAUP with unordered and equally weighted characters. Firstly, the *G3pdh* region was analysed separately, whereas the ITS and ETS data were combined based on previous findings of data congruence (Jousselin et al., 2003; Rønsted et al., 2005), and secondly, we analysed all three DNA regions simultaneously. For 17 taxa, it was not possible to amplify all three regions, and these were

Table 2. Phylogenetic information for *Ficus* ITS, ETS and *G3pdh* sequences.

Analysis	# taxa	# characters	# variable characters	# pars. inf. characters	# Trees	Length	CI	RI	% clades resolved	% clades >50% BS	% clades >74% BS
ITS	101	820	369 (45%)	210 (26%)	–	–	–	–	–	–	–
ETS	88	515	290 (56%)	186 (36%)	–	–	–	–	–	–	–
G3pdh	97	762	313 (41%)	181 (24%)	135	566	0.72	0.85	47%	43%	24%
ITS+ETS	102	1335	659 (49%)	396 (30%)	275	1580	0.58	0.82	89%	75%	48%
ITS+ETS+*G3pdh*	102	2097	972 (46%)	577 (28%)	722	2214	0.60	0.81	87%	77%	58%

Pars. inf. = Number of potentially parsimony informative characters. # trees = Number of most parsimonius trees. CI = Consistency index. RI = Retention index. Percent resolved clades in the strict consensus tree and percent clades with more than 50 or 74 percent BS, are proportions of the possible number of clades (number of taxa – 1).

coded as missing data in the combined analysis. The ITS region was sequenced for all taxa, except *F. dicranostyla*. The ETS region was not sequenced for *F. albipila, F. bernaysii, F. chartacea, F. dammaropsis, F. hirta, F. hombroniana, F. lepicarpa, F. nodosa, F. ochrochlora, F. odoardii, F. padana, F. robusta*, and *F. variifolia*. The *G3pdh* region was not sequenced for *F. edelfeltii, F. ochrochlora, F. racemigera, F. rumphii*, and *F. variifolia*. None of the taxa with missing data were placed unexpectedly, indicating that the missing data was not a problem, as also suggested by Weins (2003). All analyses were performed under maximum parsimony (MP). Most parsimonious trees were obtained using 1,000 replicates of random taxon addition sequence performed using the heuristic search option in PAUP (Swofford, 2001) and tree bisection-reconnection branch swapping (TBR) with no limit on the number of trees saved. Levels of homoplasy in all datasets were assessed using the consistency index (CI) and the retention index (RI) as implemented in PAUP. Support was assessed using bootstrap re-sampling under MP (Felsenstein, 1985). Bootstrap analyses were carried out using 1,000 simple addition sequence replicates with TBR swapping. We considered bootstrap percentages (BS) between 50 and 74% as weak support, 75–89% as moderate support, and >90% BS as strong support.

3. Results

Phylogenetic information on ITS, ETS, and *G3pdh* sequences from *Ficus* are presented in Table 2. In all cases the RI was at least 0.81, and a single island of most parsimonious trees was found in each analysis (Table 2; Maddison, 1991).

Combined ITS and ETS analysis

The combined ITS and ETS analysis (not shown) gave the same overall topology and recovered most of the clades found in previous studies of the same regions (Jousselin et al., 2003; Rønsted et al., 2005), but support was poor for deep relationships among major clades.

G3pdh *analysis*

The *G3pdh* analysis (Fig. 1) provided poor resolution and support overall, which we attribute to lack of informative characters in the *G3pdh* dataset. Most of the clades shown correspond to clades found in the combined ITS and ETS analysis, but were not supported.

The combined three region analysis

One of the most parsimonious trees obtained by analysis of the combined dataset is shown in Fig. 2. Combined analysis recovered most of the clades identified in previous molecular phylogenetic studies (Herre et al., 1996; Weiblen et al., 2000; Jousselin et al., 2003; Rønsted et al., 2005), with a few noteworthy exceptions. All studies agree that section *Pharmacosycea* (100% BS) is sister to the remainder of *Ficus* (92% BS). We resolved two major lineages in the rest of the genus, labeled A and B (Fig. 2), although not well supported. Weakly supported lineage A (54% BS) included two clades, C, with sections *Adenosperma* (90% BS; including *F. dammaropsis*) and *Sycocarpus* (100% BS) as sister clades (84% BS), and D, with subgenus *Urostigma* excluding section *Urostigma* s.s. (100% BS). In clade D, there were two subclades, one with sections *Conosycea* s.l. (94% BS; including *F. rumphii* and *F. elastica*) and *Malvanthera* (90% BS) as sister groups (96% BS), and the other subclade (100% BS) included sections *Galoglychia* and *Americana* (100% BS). Section *Galoglychia* was paraphyletic to section *Americana*, but this was not supported by bootstrap and many branches collapsed in the strict consensus tree, resulting in a polytomy involving *Americana* and several lineages of section *Galoglychia*. Clade B and some of the deep divergences within it were not supported by the bootstrap and collapsed in the strict consensus tree, leaving a polytomy of five clades.

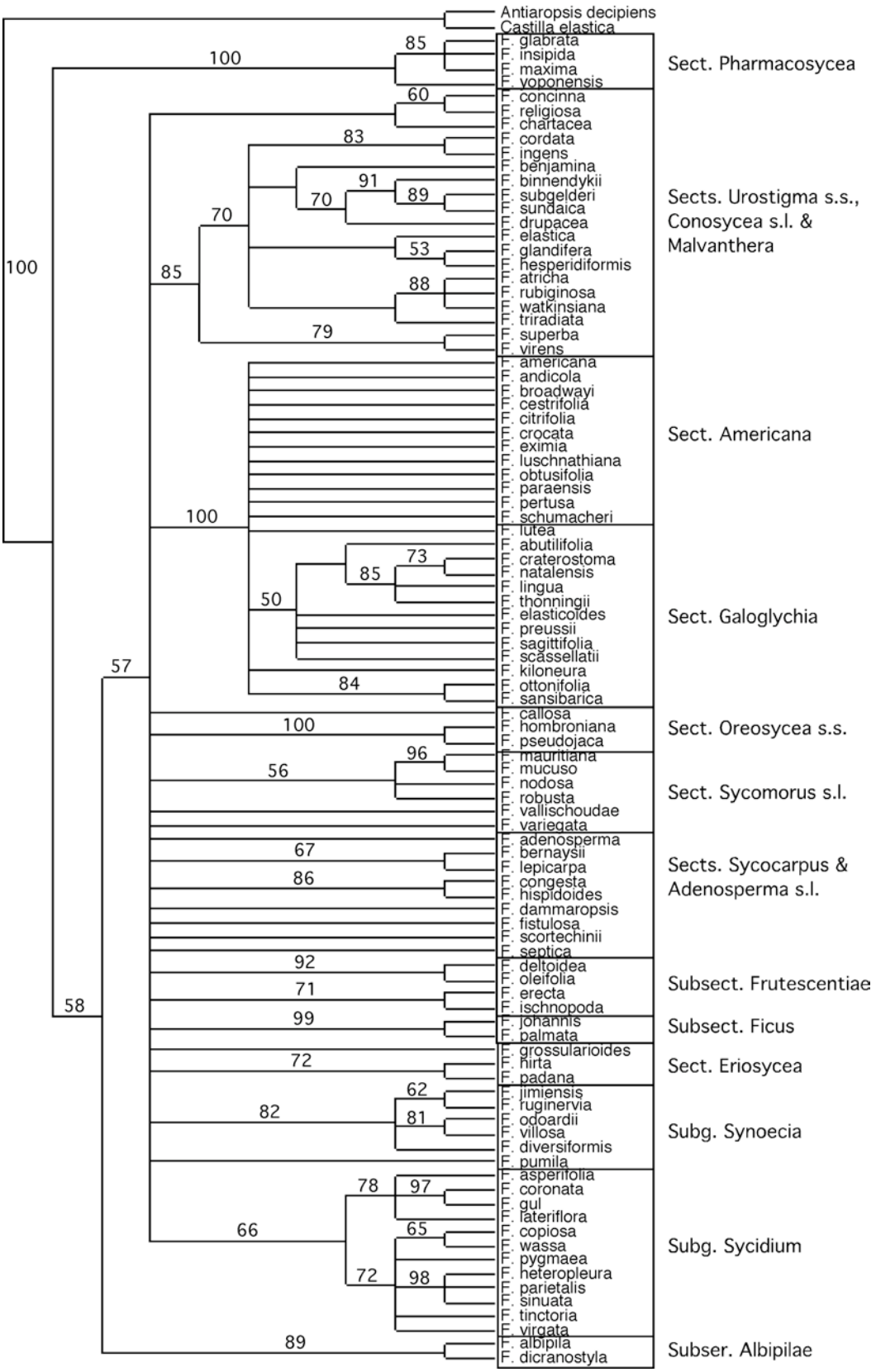

Figure 1. Strict consensus of 135 most parsimonious trees from analysis of the *G3pdh* sequences. Bootstrap percentages (above 50%) are indicated above the branches. *Antiaropsis decipiens* and *Castilla elastica* were used as outgroups.

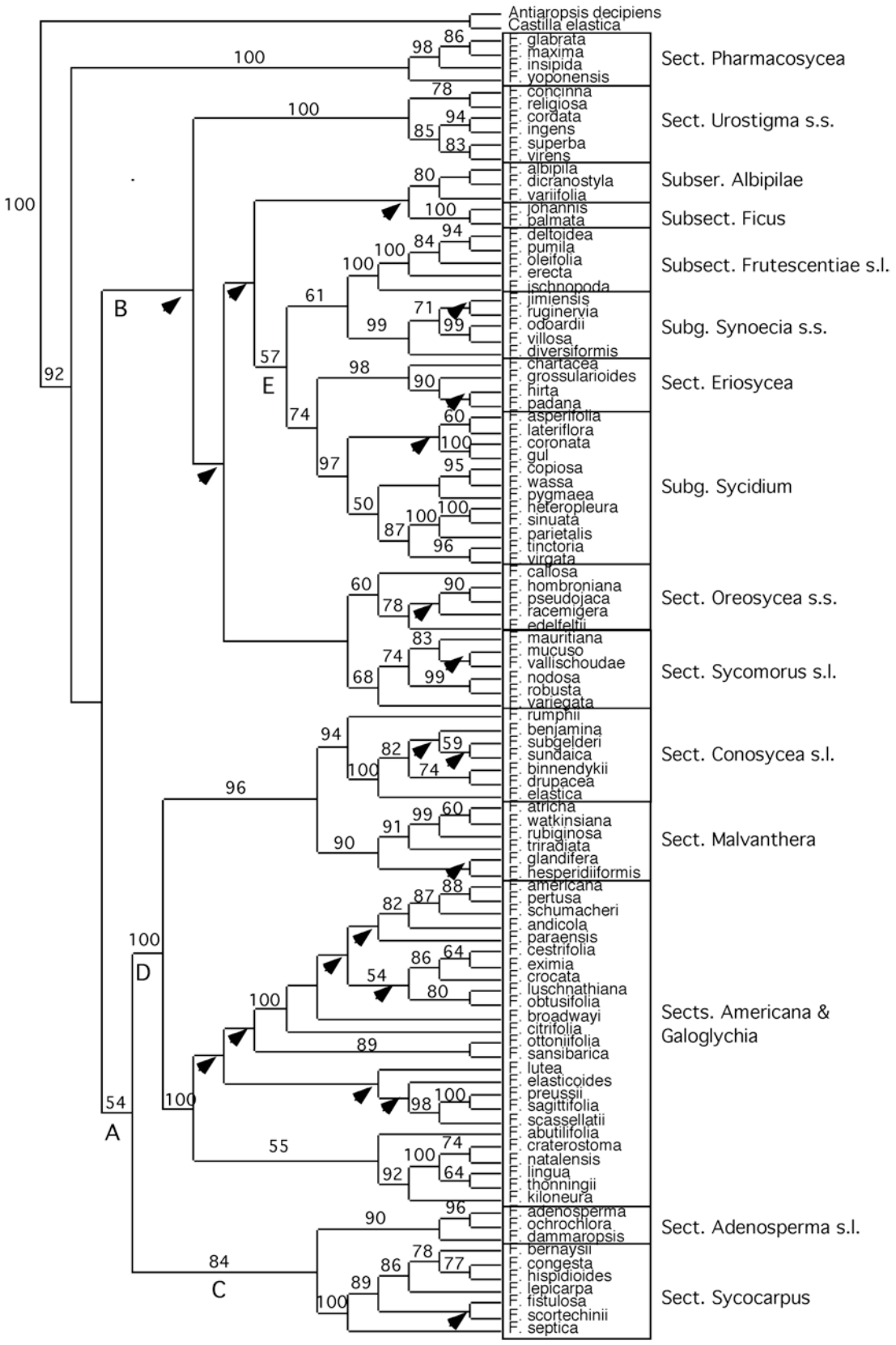

Figure 2. One of 722 most parsimonious trees obtained by the combined analysis of the ITS, ETS and *G3pdh* sequences. Bootstrap percentages (above 50%) are indicated above the branches and those branches that collapse in the strict consensus tree are indicated with arrowheads. *Antiaropsis decipiens* and *Castilla elastica* were used as outgroups. Fifteen clades of *Ficus* currently recognised by the authors based on the present study and traditional classifications (Corner, 1965; Berg, 1989; Berg and Corner, 2005) are indicated.

These included (1) section *Urostigma* s.s. (100% BS). (2) suber. *Albipilae* of section *Oreosycea* (80% BS), (3) subsection *Ficus* of section *Ficus* (100% BS), (4) clade E (57% BS), and (5) a clade (<50% BS) including section *Oreosycea* s.s (60% BS) as sister to section *Sycomorus* s.l. (68% BS including section *Neomorphe*). Clade E was divided into subgenus *Synoecia* s.s. (99% BS, excluding *F. pumila*) plus section *Ficus* subsection *Frutescentiae* (100% BS, including *F. pumila*) as sisters (61% BS), and section *Eriosycea* (98% BS) plus subgenus *Sycidium* (97% BS) as sisters (74% BS). Within subgenus *Sycidium*, section *Sycidum* was paraphyletic to section *Paleomorphe* (87% BS), but this was only weakly supported by the bootstrap (50% BS).

4. Discussion

Phylogenetic utility of G3pdh *for phylogeny reconstruction in* Ficus

The number of potentially parsimony informative characters (Table 2) increased from 396 in the combined ITS and ETS dataset to 577 in the three-region dataset. The percentage of clades resolved in the strict consensus tree out of the total possible (87%) and the proportion of clades with BS>50% for *G3pdh* (77%) were similar to the percentage resolved and supported by ITS+ETS alone (89% and 75%, respectively). However, the addition of *G3pdh* sequences increased the proportion of moderately supported clades (BS>74%) from 48% to 58% (Table 2).

All datasets in the present study showed limited resolution and support for the early-diverging lineages of *Ficus*, while more recent clades were strongly supported. There are several possible explanations for this pattern of limited basal resolution and support. Phylogenetic conflict could be generated by hybridization and introgression, incomplete lineage sorting, or erroneous comparisons of paralogous gene copies (Baldwin et al., 1995; Doyle, 1992). Since neither of the three gene regions alone unambiguously resolved the early divergence of major *Ficus* lineages, the poor resolution of the combined analysis is not likely due to different gene histories. We did not specifically look for paralogous gene copies by molecular cloning, but no heterogeneity indicative of multiple copies was observed in the electropherograms.

Another explanation could be saturation of DNA substitutions over the long history of the genus erasing phylogenetic information that might have supported the pattern of divergence among early *Ficus* lineages. Several studies have indicated that *Ficus* started to diversify at least 60 million years ago (for example, Rønsted et al., 2005). Lack of resolution could also be caused by rapid simultaneous radiation early in the evolutionary history of the genus as has been suggested in Araliaceae (Plunkett et al., 2004) and Arecaceae (Norup et al., 2006). Branches collapsing in the strict consensus tree of the combined analysis are relatively short (ten or fewer steps) which might indicate ancient and rapid diversification. Finally, the pattern could be caused by ancient hybridization events, which occurred when the lineages were distributed differently than they are today, as reported by Wendel et al. (1995) in *Gossypium*.

It is our hope that information from additional DNA regions will provide an explanation for the poor resolution of early lineages that we have observed so far and to provide a more robust hypothesis for the infrageneric classification and evolutionary history of *Ficus*.

The *G3pdh* region has the advantage of being easy to amplify and only one copy appears to exist in *Ficus* but even a combined analysis of ITS, ETS and *G3pdh* did not resolve the pattern of early diversification. Although adding taxa can sometimes increase accuracy in phylogenetic analyses, especially if they break up long branches (Graybeal, 1998), we argue that sequencing of additional DNA regions is needed to resolve the phylogeny of *Ficus*. Large genera often show low variability in ITS, and sequencing of low copy, protein coding regions, is a good alternative, but these are often not easily amplified (Rønsted et al., 2006). Possible candidates are plastid-expressed glutamine synthetase (*ncpGS*; Emshwiller and Doyle, 1999) and granule-bound starch synthase (GBSSI or *waxy*; Mason-Gamer et al., 1998), which have shown promise in preliminary analyses (Rønsted et al., 2006; Silvieus et al., in press). However, such regions are difficult to amplify, require high quality DNA templates, and may require molecular cloning.

In summary, ITS, ETS and *G3pdh* regions amplify well from high quality DNA and allow for comprehensive taxon sampling, but they are not likely to resolve the ancient or rapid diversification of closely related *Ficus* species. Low copy, protein coding, regions, may provide more resolution, but as such regions are difficult to amplify, this may limit the extent of taxon sampling. Missing data can influence both resolution and phylogenetic accuracy, but recent studies have shown the potential for placing even highly incomplete taxa when combining datasets that do not include identical taxa (Weins, 2003).

The best strategy for obtaining a comprehensive and well-resolved molecular phylogenetic hypothesis of *Ficus* may therefore be to combine the two different approaches. Firstly, one could sequence *G3pdh* in addition to ITS and ETS across a comprehensive set of *Ficus* species (250–300 species, i.e. about 1/3 of the genus). This dataset could then be used to identify monophyletic groups as well as problematic taxa. Secondly, additional regions (ncpGS and *waxy*) could be sequenced for a subset of taxa (e.g. 50 species) representing the clades identified by a three-gene approach.

Table 3. Voucher information and GenBank accession numbers for new sequences. Herbarium acronyms are given in parenthesis after accessions following Index Herbariorum (www.sciweb.nybg.org/science2/IndexHerbariorum.asp). Listed sequences are *G3pdh* unless otherwise specified. Liv. col: Living collection. PNG: Papua New Guinea

Taxon	Voucher specimen (Herbarium)	Locality	GenBank accession
Antiaropsis decipiens K. Schum.	Weiblen 1706 (MIN)	Papua New Guinea	EF092326
Castilla elastica Sess	Chase 19850 (K)	Living Collection, Kew	K19863013 EF092327
F. abutilifolia Miq.	Forest 326 (NBG)	Liv. col. (NBG)	EF092348
F. albipila (Miq.) King	GW1070 (MIN)	Java (BO)	EF092366
F. adenosperma Miq.	Weiblen 1764 (MIN)	Madang, PNG	ETS: EF092321 EF092374
F. americana Aubl.	Rønsted 154 (K)	Liv. col. (BG)	EF092339
F. andicola Standl.	Rønsted 145 (K)	Liv. col. (BG)	EF092340
F. asperifolia Miq.	S. Compton	Liv. col. (LDS)	EF092394
F. atricha D.J. Dixon	Rønsted 142 (K)	Liv. col. (BG)	EF092360
F. benjamina L.	Rønsted 179 (AU/ BKF/K)	Hat Yai, Thailand	EF092333
F. binnendykii Miq.	Chase 19871 (K)	Lic. col. (K)	EF092334
F. broadwayi Urb.	Rønsted 121 (K)	Liv. col. (BG)	EF092341
F. callosa Willd.	Rønsted 109 (K)	Liv. col. (BG)	EF092367
F. cestrifolia Schott.	Rønsted 139 (K)	Liv. col. (BG)	EF092342
F. chartacea King	Rønsted 177 (AU/BKF/K)	Sri Phang Nga, Thailand	EF092384
F. concinna Miq.	Rønsted 178 (AU/BKF/K)	Songkhla, Thailand	EF092328
F. copiosa Steud.	Weiblen 057 (A)	Madang, PNG	ETS: EF092324 EF092395
F. cordata Thunb. ssp. *salicifolia* (Vahl) C.C. Berg	Forest 329 (NBG)	Liv. col. (NBG)	EF092329
F. coronata Spin.	Rønsted 71 (C)	Liv. col. (C)	EF092396
F. craterostoma Mildbr. & Burret.	Forest 340 (NBG)	Liv. col. (NBG)	EF092349
F. crocata Miq (syn. *F.goldmanii* Standl.)	Rønsted 92 (C)	Liv. col. (C)	EF092343
F. deltoidea Jack	Rønsted 73 (C)	Liv. col. (C)	EF092378
F. dicranostyla Mildbr.	Rønsted 152 (K)	Liv. col. (BG)	EF092368
F. diversiformis Miq.	Samuel 3100 (K)	Kitugala, Sri Lanka	EF092392
F. drupacea Thunb.	Rønsted 114 (K)	Liv. col. (BG)	EF092335
F. elastica Roxb. ex Hornem.	Rønsted 90 (C)	Liv. col. (C)	EF092338
F. elasticoides De Wild.	Rønsted 128 (K)	Liv. col. (BG)	EF092354
F. erecta Thunb.	Rønsted 134 (K)	Liv. col. (BG)	EF092379
F. eximia Schott.	Rønsted 146 (K)	Liv. col. (BG)	EF092344
F. fistulosa Reinw. ex Bl.	Rønsted 111 (K)	Liv. col. (BG)	EF092375
F. glandifera Summerh.	Wheatley 297 (K)	Vanuatu	EF092361
F. grossularioides Burm.	Jousselin et al.	Labi Road, Totong, Brunei 1999	EF092385
F. gul Laut et K. Schum.	Takeuchi et al. 15019 (K)	Morobe, PNG	EF092397
F. hesperidiiformis King	Weiblen 825 (MIN)	Madang, PNG	EF092362
F. heteropleura Bl.	Rønsted 165 (AU/BKF/K)	Phang Nga, Thailand	EF092400
F. hirta Vahl	Rønsted 168 (AU/BKF/K)	Phang Nga, Thailand	EF092386
F. hombroniana Corner	Weiblen 1859 (MIN)	East Sepik, PNG	EF092369
F. ingens Miq.	Rønsted 106 (K)	Liv. col. (BG)	EF092330
F. ischnopoda Miq.	Rønsted 175 (AU/BKF/K)	Sri Phang Nga, Thailand	EF092380
F. jimiensis C. C. Berg	Weiblen 792 (A/LAE)	Eastern Highlands, PNG	EF092388
F. johannis Boiss. (syn. *F. carica* L.)	Rønsted 96 (C)	Liv. col. (C)	EF092381
F. kiloneura Hornby (syn. *F. fischeri* Mildbr. & Burret)	Forest 327 (NBG)	Liv. col. (NBG)	EF092350
F. lateriflora Vahl.	Fournel JF130 (REU 10236)	Mare Longue, Réunion	EF092398
F. lepicarpa Bl.	Rønsted 162 (AU/K)	Ranong, Thailand	EF092376
F. lingua De Wild. & T. Durand ssp. *lingua*	Rønsted 208 (K)	Mefou, Central Province, Cameroon	EF092351
F. luschnathiana Miq.	Rønsted 151 (K)	Liv. col. (BG)	EF092345
F. lutea Vahl	Rønsted 87 (C)	Liv. col. (C)	EF092347
F. mauritiana Lam.	Fournel & Michenaud JF89 (REU 10241)	Basse Vallé, Réunion Island	EF092371
F. mucuso Ficalho	Rønsted 129 (K)	Liv. col. (BG)	EF092372
F. natalensis Hochst.	Forest 333 (NBG)	Liv. col. (NBG)	EF092352
F. odoardii King	Weiblen 708 (A/LAE)	Eastern Higlands, PNG	EF092389

Table 3. Continued.

Taxon	Voucher specimen (Herbarium)	Locality	GenBank accession
F. oleifolia King	Weiblen 2287	(digital image, MIN)	ETS: EF092322 EF092382
F. ottoniifolia Miq. ssp. *macrosyce*	Rønsted 117 (K)	Liv. col. (BG)	EF092358
F. padana Burm.	Weiblen 1066 (MIN)	Java (BO)	EF092387
F. palmata Forssk.	FB/S2786 (BR)	Liv. col. (BR)	EF092383
F. parietalis Bl.	De Kok 1026 (K)	Borneo	EF092401
F. preussii Warb.	Rønsted 138 (K)	Liv. col. (BG)	EF092355
F. pseudojaca Corner	Weiblen 2341 (MIN)	Madang, PNG	ITS: EF092317 ETS: EF092320 EF092370
F. pumila L.	Weiblen 2686 (MIN)	Liv. col. (MIN)	EF092390
F. pygmaea Hiern.	Forest 332 (NBG)	Liv. col. (NBG)	EF092399
F. religiosa L.	Rønsted 86 (C)	Liv. col. (C)	EF092331
F. rubiginosa Desf. ex Ventenat.	Rønsted 89 (C)	Liv. col. (C)	EF092363
F. ruginervia Corner	Weiblen 854 (A/LAE)	Kalimantan, Borneo	ETS: EF092323 EF092393
F. sagittifolia Mildbr. & Burret	Chase 19852 (K)	Liv. col. (K)	EF092356
F. sansibarica Warb.	Rønsted 117 (K)	Liv. col. (BG)	EF092359
F. scassellatii Pamp.	Rønsted 110 (K)	Liv. col. (BG)	EF092357
F. scortechinii King	Rønsted 167 (AU/BKF/K)	Kao Phae Taew, Phang Nga, Thailand	EF092377
F. schumacheri (Liebm.) Griseb.	Rønsted 123 (K)	Liv. col. (BG)	EF092346
F. sinuata Thunb.	Rønsted 70 (C)	Liv. col. (C)	EF092402
F. subgelderi Corner	Jousselin et al.	Labi Road, Tutong, Brunei 1998	EF092336
F. sundaica Bl.	Weiblen 906 (MIN)	Liv. col. (MIN)	EF092337
F. superba Miq.	Rønsted 63 (C)	Liv. col. (C)	EF092332
F. tinctoria Forst. f.	Rønsted 99 (K)	Liv. col. (BG)	EF092403
F. thonningii Bl.	Forest 341 (NBG)	Liv. col. (NBG)	EF092353
F. triradiata Corner	Hyland 8336 (K)	Tablelands, Qlds, Australia	EF092364
F. vallis-choudae Delile.	Rønsted 126 (K)	Liv. col. (BG)	EF092373
F. variifolia Warb.	Styles 82 (K)	Bunyoro, Uganda	ITS: EF092318
F. variifolia Warb.	Rønsted 131 (K)	Liv. col. (BG)	ETS: EF092319
F. villosa Bl.	Chase 19851 (K)	Liv. col. (K)	EF092391
F. virgata Reinw. ex Bl.	Rønsted 65 (C)	Liv. col. (C)	EF092404
F. wassa Roxb.	Weiblen 051(A)	Madang, PNG	ETS: EF092325
F. watkinsiana F.M. Bailey	Rønsted 83 (C)	Liv. col. (C)	EF092365

Preliminary taxonomic implications

Our results only support the monophyly of subgenus *Sycidium* out of the six subgenera traditionally recognized primarily based on morphology (Berg and Corner, 2005, Table 1). Ultimately, it is our goal to provide a fully revised classification of *Ficus* reflecting the phylogenetic history of the genus but the results of the present study based on three nuclear regions must be considered with caution due to limited sampling, resolution, and support. According to three gene regions, however, we have identified fifteen clades of *Ficus* (Fig. 2, Table 1), most of which were strongly supported.

Subgenus Pharmacosycea: The traditional subgenus *Pharmacosycea* (Berg and Corner, 2005) is not monophyletic. Section *Pharmacosycea* is the sister group to the rest of *Ficus*. Section *Oreosycea* appears polyphyletic because members of subsection *Pedunculatae* subseries *Albipilae* (*F. albipila, F. dicranostyla* and *F. variifolia;* Berg and Corner; 2005) form a clade that is not closely related to the other included members of section *Oreosycea* s.s. (subsection *Glandulosae* and subsection *Pedunculatae* subseries *Vasculosa*). Berg and Corner (2005) noted overall similarities of vegetative characters in subsection *Glandulosae* and section *Adenosperma* of subgenus *Sycomorus*. However, according to three genes, neither of the two clades of sect. *Oreosycea* (subser. *Albipilae* and *Oreosycea* s.s.) can be placed near the dioecious subgenus *Sycomorus* with any confidence. It will be important to include more samples from section *Oreosycea*, and especially from the biogeographically diverse subsection *Pedunculatae*, before section *Oreosycea* is formally divided.

Subgenus Urostigma: As previous studies have also

shown (Jousselin et al., 2003; Rønsted et al., 2005), subgenus *Urostigma* is polyphyletic due to the placement of sect. *Urostigma* s.s. with some dioecious figs. Section *Malvanthera* sensu Corner (1965) and section *Conosycea* s.l. are strongly supported sister clades. Berg and Corner (2005) united sections *Malvanthera* and *Stilpnophyllum* (*F. elastica*) in their most recent treatment of the genus, but this is not appropriate as *F. elastica* clearly belongs to section *Conosycea* which also includes *F. rumphii*, one of two species in Corner's (1965) section *Leucogyne*, which Berg and Corner (2005) erroneously placed in section *Urostigma* subsection *Urostigma*. Sections *Americana* and *Galoglychia* are also closely related, but while section *Americana* is strongly supported, *Galoglychia* may be paraphyletic with respect to *Americana*. Three genes did not lend bootstrap to this arrangement and so more information is needed to test the monophyly of section *Galoglychia*.

Subgenus Sycomorus: The current circumscription of subgenus *Sycomorus* (Berg and Corner, 2005) is partly based on all members of the subgenus being pollinated by the wasp genus *Ceratosolen*. The subgenus was strongly supported as monophyletic in previous studies by Weiblen (2000; BS 95%) and Jousselin et al. (2003; BS 87%) but Rønsted et al. (2005) found subgenus *Sycomorus* to be polyphyletic due to the nesting of section *Adenosperma* within subgenus *Ficus*. The authors attributed this to possible error in phylogeny estimation given the lack of bootstrap support. In the present study members of subgenus *Sycomorus* form two clades (sect. *Sycomorus* s.l. and sects. *Adenosperma* plus *Sycocarpus*) that are separated by five nodes, but all branches supporting polyphyly of this subgenus have less than 54% BS. Sections *Adenosperma* (including *F. dammaropsis*) and *Sycocarpus* are sister clades, and form a weakly supported clade with members of subgenus *Urostigma* (excluding section *Urostigma*) in this study. Section *Neomorphe* was recently included as a subsection in section *Sycomorus* by Berg and Corner (2005) and section *Oreosycea* s.s. is sister to this clade, but with less than 50% BS.

Subgenus Sycidium: (Berg and Corner, 2005) is the only clearly monophyletic subgenus in our study. Section *Sycidum* appears paraphyletic with respect to a moderately supported section *Paleomorphe*, but this is only supported by 50 % BS, and additional sampling can test whether *Sycidium* is monophyletic.

Subgenus Synoecia: Subgenus *Synoecia* is monophyletic provided that *F. pumila* is excluded. This species appears to be an unusual member of section *Ficus* subsect. *Frutescentiae*. The subgenus is united by the root climbing habit, including heterophylly, but a few species, such as *F. laevis* Bl., show intermediate characters suggesting affinity to subgenus *Ficus*. Sampling of the subgenus is too limited to determine the relationship of sections *Rhizocladus* and *Kissosycea* at this time.

Subgenus Ficus: is clearly not monophyletic and can be split into three distinct lineages which are not each others closest relatives. Section *Eriosycea* with about 30 species appears to be sister to subgenus *Sycidium*. Section *Ficus* subsect. *Ficus* includes only *F. carica* (of which *F. johannis* can be considered a synonym) and its two relatives *F. iidaiana* Rehder et Wilson and *F. palmata*. The closest relatives of this subsection are uncertain, but they could be subseries *Albipilae* of section *Oreosycea*, although this is not supported in the consensus tree or by bootstrap. Section *Ficus* subsection *Frutescentiae* s.l. includes about 25 species, the majority of which occur in the Sino-Himalayan region, and the climbing *F. pumila* is an unusual member of this clade.

5. Conclusion

We have shown that the *G3pdh* gene provides limited resolution within *Ficus*, but increases the proportion of well-supported clades when combined with ITS and ETS. Based on the combined analysis, we identified fifteen clades within *Ficus* (Fig. 2, Table 1), most of which are strongly supported by the BS. Of the six subgenera traditionally recognized based on morphology, only subgenus *Sycidium* is supported as monophyletic. The exact branching order of the early lineages of *Ficus* as well as some of the internal branches are not well supported, however, and should be considered uncertain at present. We conclude that comprehensive taxon sampling is very important to achieve a phylogenetic classification of such a large genus. We must be careful to evaluate the monophyly of major *Ficus* groups in order to meaningfully compare their diversification with the pollinating wasps and to study the co-evolution of this extraordinary interaction.

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