

60 million years of co-divergence in the fig–wasp symbiosis

Nina Rønsted^{1,*}, George D. Weiblen², James M. Cook³, Nicolas Salamin^{4,†},
Carlos A. Machado⁵ and Vincent Savolainen¹

¹Molecular Systematics Section, Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3DS, UK

²Department of Plant Biology, University of Minnesota, Saint Paul, MN 55108, USA

³Department of Biological Sciences and NERC Centre for Population Biology, Imperial College London,
Silwood Park Campus, Ascot SL5 7PY, UK

⁴Department of Botany, University of Dublin, Trinity College, Dublin 2, UK

⁵Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85721, USA

Figs (*Ficus*; ca 750 species) and fig wasps (Agaoninae) are obligate mutualists: all figs are pollinated by agaonines that feed exclusively on figs. This extraordinary symbiosis is the most extreme example of specialization in a plant–pollinator interaction and has fuelled much speculation about co-divergence. The hypothesis that pollinator specialization led to the parallel diversification of fig and pollinator lineages (co-divergence) has so far not been tested due to the lack of robust and comprehensive phylogenetic hypotheses for both partners. We produced and combined the most comprehensive molecular phylogenetic trees to date with fossil data to generate independent age estimates for fig and pollinator lineages, using both non-parametric rate smoothing and penalized likelihood dating methods. Molecular dating of ten pairs of interacting lineages provides an unparalleled example of plant–insect co-divergence over a geological time frame spanning at least 60 million years.

Keywords: co-divergence; co-speciation; molecular dating; *Ficus*; Agaoninae; phylogeny

1. INTRODUCTION

Figs have diversified extensively in terrestrial ecosystems throughout the tropics and subtropics. The distinctive fig inflorescence (syconium) is exclusively pollinated by female agaonine wasps that deposit their eggs in some of the flowers (figure 1; Cook & Rasplus 2003). Coevolutionary studies have centred on the specificity and stability of the exchange of pollination services for the rearing of pollinator offspring (Cook & Rasplus 2003; Molbo *et al.* 2003). Interspecific coevolution involves reciprocal, selected changes in the traits of interacting species, whereas co-divergence can arise purely from the maintenance of a specialized association between two lineages (Page *et al.* 1996; Page 2003; Percy *et al.* 2004). Patterns of co-divergence are expected in fig–pollinator relationships owing to extreme host fidelity, and comparisons of fig or fig wasp phylogenies with the classification of the other partner support this possibility (Weiblen 2000; Machado *et al.* 2001; Joussetin *et al.* 2003). Molecular phylogenetic trees of figs and their pollinators are suggestive of co-speciation (Herre *et al.* 1996; Weiblen & Bush 2002), but until now there has been no assessment of temporal congruence, namely whether dates of divergence are correlated between interacting lineages (Page 2003; Percy *et al.* 2004).

We inferred the phylogeny of 146 diverse *Ficus* species, representing all major lineages throughout the tropics, based on nuclear ribosomal DNA sequences

using both maximum parsimony and Bayesian reconstruction methods. We also obtained an independent estimate of fig wasp phylogeny (Machado *et al.* 2001). We estimated divergence times for figs and pollinators using independent fossil calibrations for each partner, with non-parametric rate smoothing (NPRS) and penalized-likelihood (PL) dating methods (Sanderson 1997, 2002). We then identified interacting fig and pollinator lineages and compared their respective ages to test for co-divergence. Compared with previous studies (Weiblen 2000; Joussetin *et al.* 2003), the present paper includes both dense sampling of *Ficus* species and appropriate outgroups. With a large underlying dataset, we present the first quantitative test of temporal congruence in the fig and pollinator diversification.

2. MATERIAL AND METHODS

(a) Taxon sampling

Total genomic DNA was extracted from 91 taxa of *Ficus* using CTAB (Doyle & Doyle 1987). In addition, 65 ribosomal internal transcribed spacers (ITS) DNA sequences and 39 external transcribed spacers (ETS) sequences were retrieved from GenBank/EBI (following papers by Weiblen (2000) and Joussetin *et al.* (2003)), resulting in a total sample of 146 taxa of *Ficus*. Included material, voucher information, origin and GenBank/EBI accession numbers (AY730059–AY730144 and AY730145–AY730233) are listed in the electronic supplementary material. Matrices have been deposited in TREEBASE (see <http://www.treebase.org>). Our sampling covers all fig sections *sensu* Berg (1989, 2003a–e, 2004), except for

* Author for correspondence (n.ronsted@kew.org).

† Present address: Department of Ecology and Evolution, University of Lausanne, 1015 Lausanne, Switzerland.



Figure 1. Fig-wasp symbiosis: the example of *Ficus macrophylla* Desf. ex. Pers. and its pollinator, *Pleistodontes froggatti* Mayr. Photograph by JMC, Brisbane, Australia, 2003.

three new and small sections in subgenus *Sycomorus* (Berg 2004); section *Hemicardia* with three species restricted to the Sino-Himalayan region, section *Boscheria* with two species occurring from the Philippines to New Guinea and section *Papuasyce* with three species from New Guinea to Fiji.

Previous studies (Weiblen 2000; Jousselin *et al.* 2003) have shown that ITS and ETS sequences of *Ficus* are so divergent from those of other genera (e.g. *Morus*, *Broussonetia*, *Brosimum* and *Artocarpus*) in family Moraceae, that no satisfactory alignment could be performed. As a result, rooting the tree of *Ficus* has been problematic and *Ficus* section *Pharmacosyceae* has often been used as the root based on preliminary analyses of 11 *rbcL* sequences (Herre *et al.* 1996) and indications from morphology (Berg 1989). Other molecular studies based on various plastid regions (Herre *et al.* 1996; Sytsma *et al.* 2002; Datwyler & Weiblen 2004) have, however, shown that the tribe Castilleae is more closely related to *Ficus* than any of the previously attempted outgroups. We collected DNA sequences of *Poulsenia*, *Castilla*, *Sparattosyce* and *Antiaropsis* in Castilleae, finding them to be alignable with *Ficus* and therefore suitable as outgroups.

For the phylogenetic analyses of the pollinating wasps, we used the dataset generated by Machado *et al.* (2001). This matrix includes mitochondrial cytochrome oxidase subunit I (*cox1* = COI) gene sequences of 816 nucleotides for 36 of the associated pollinator species, representing 15 out of 20 agaonine genera.

(b) PCR amplification and DNA sequencing

A total of 1230 aligned nucleotide positions across the ITS and ETS (Baldwin *et al.* 1995; Baldwin & Markos 1998) were amplified using primers 17SE and 26SE (Sun *et al.* 1994) and Hell1 and 18S ETS (Baldwin & Markos 1998), respectively. Standard automated sequencing protocols (Jousselin *et al.* 2003) were used except that DMSO was added to all reactions. Six taxa were only sequenced for ITS, because ETS could not be amplified (see electronic supplementary material).

(c) Phylogenetic reconstructions

The large number of *Ficus* sequences analysed prevented us from using maximum-likelihood (ML) searches, so we adopted the following strategy. We performed 500 heuristic search replicates of Subtree-Pruning and Re-grafting using maximum-parsimony (MP) criterion as implemented in PAUP* 4.0b10 (Swofford 2002). We then optimized ML branch lengths on one of the trees from the MP analysis using the save trees option in PAUP and the most suited HKY85 + γ model of molecular evolution, with all parameters estimated from the data (figure 2). The phylogenetic tree of wasps, containing far fewer taxa (36), was reconstructed directly under the ML optimality criterion using five Tree Bisection-Reconnection replicates in PAUP* 4.0b10 (Swofford 2002; figure 3).

Support was assessed by both Bayesian analyses and bootstrap re-sampling. Bayesian analysis was performed using MRBAYES 2.01 (Huelsbeck & Ronquist 2001). We used an HKY85 model of evolution (lset NST=2 RATES=equal). The analysis was performed with 1 000 000 generations on four Monte Carlo Markov chains with equal rates and trees sampled every 10 generations (mcmc NGEN=1 000 000, PRINTFREQ=100, SAMPLEFREQ=10, NCHAINS=4). We plotted generation number against the likelihood scores to locate the 'burn in'. The first 10 000 trees of low-posterior probability were deleted, and all remaining trees were imported into PAUP* 4.0b10 (Swofford 2002). A 50% majority rule consensus tree was produced to yield the posterior probabilities of clades. A total of 500 bootstrap replicates with TBR swapping, equal weighting and a limit of one random addition sequence per bootstrap replicate was performed.

(d) Dating phylogenies

We estimated divergence times for figs and pollinators using independent fossil calibrations for each partner and both NPRS (as implemented in TREEEDIT 1.0; Sanderson 1997; Rambaut & Charleston 2001) as well as PL (in r8s 2.0; Sanderson 2002, 2003) to account for deviations from the assumption of a molecular clock. Confidence intervals for ages were calculated by reapplying NPRS to 100 bootstrapped matrices. Sixty million years (Myr) old fossilized

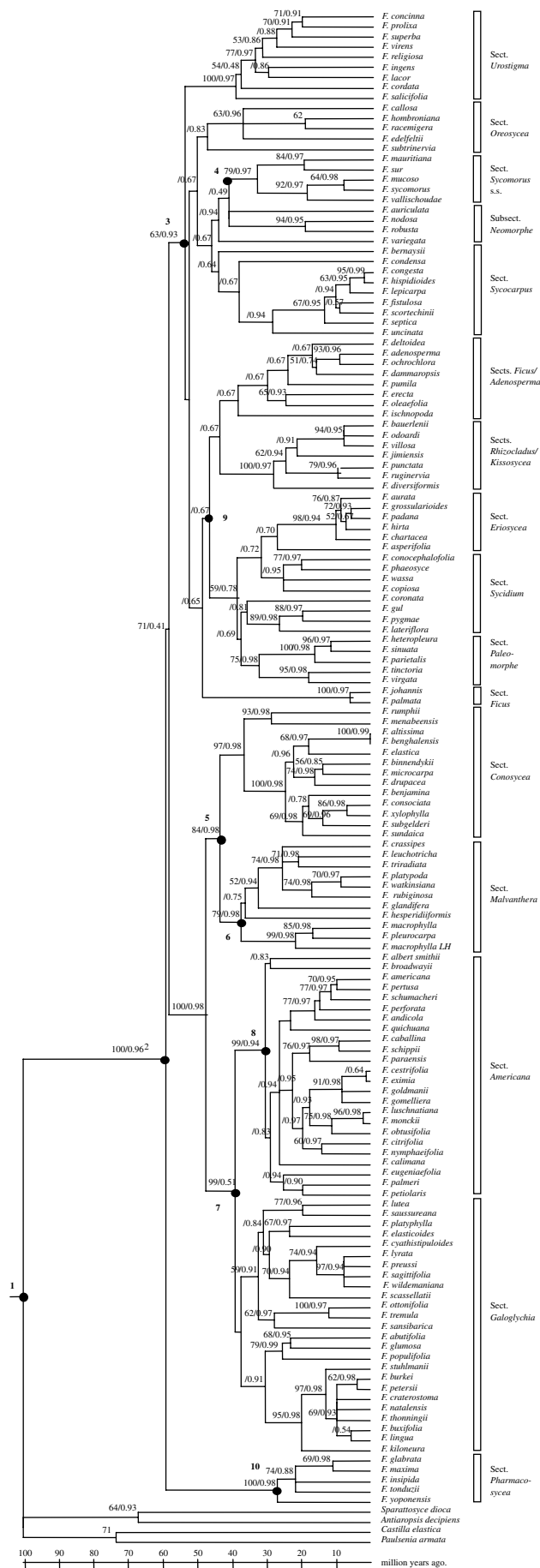


Figure 2. Ultrametric tree of *Ficus* (ITS+ETS). MP tree saved under estimated ML conditions and made ultrametric with NPRS; bootstrap percentages/posterior probabilities above branches.

achenes assigned to *Ficus* were used to constrain the minimum age of the fig radiation (Collinson 1989). We calibrated the wasp phylogeny using fossil *Pegoscapus* from Dominican amber, constraining the genus to be at least 21 Myr old (Machado *et al.* 2001).

The fig wasp symbiosis is regarded as extremely species specific. Although deviations from one-to-one species specificity are known (Molbo *et al.* 2003), associations between pollinator genera and *Ficus* sections are often congruent based on previous phylogenetic analyses (Herre *et al.* 1996; Weiblen 2000; Machado *et al.* 2001; Jousselin *et al.* 2003). We identified ten ecologically associated lineages of figs and pollinators and compared their ages (see figures 2–4 and electronic supplementary material). These 10 pairs represent possible co-cladogenetic events across the phylogeny at higher taxonomic levels for which the corresponding clades of figs and wasps were both resolved. A tanglegram generated with TREEMAP (Page 1995) showing the 10 nodes is provided in the electronic supplementary material. A plot of the age of wasp lineages against the corresponding fig lineages was constructed and regression analysis was performed following Percy *et al.* (2004; figure 4). To evaluate whether the correlation could be due to chance alone, sums of squares of perpendicular offsets from a perfect linear regression (slope = 1) were compared to 10 000 randomized sets of 10 pairs of ages drawn from the phylogenetic trees of figs and wasps.

3. RESULTS

(a) Phylogeny of fig species

The combined dataset, including 146 taxa of *Ficus* and four outgroups, represents by far the most comprehensive phylogenetic study of figs to date. Out of 1354 aligned nucleotides, 478 characters were parsimony informative. The MP analysis of the combined dataset generated 74 most parsimonious trees of 2010 steps with a consistency index of 0.52 and a retention index of 0.83. One of the trees is shown in figure 2 (MP and ML trees have been deposited in TREEBASE, see §2).

Our results are generally consistent with previous phylogenetic studies of figs (Weiblen 2000; Jousselin *et al.* 2003), with one notable exception. Figure 2 indicates that subgenus *Sycomorus* (Berg 2004) is not monophyletic due to the nesting of section *Adenosperma* with subgenus *Ficus*. We attribute this finding to error in phylogeny estimation owing to a lack of non-parametric bootstrap support and morphological evidence for the monophyly of subgenus *Sycomorus* (Berg 1989; Weiblen 2000). The inclusion of outgroups in the ITS/ETS analyses indicated that *Ficus* is monophyletic and strongly supported (100% Bootstrap support, BS, 0.96 Bayesian posterior probability, PP). The earliest diverging lineage is section *Pharmacosyce* (100% BS, PP=0.98), the sister group to the rest of the figs (71% BS, PP=0.41). Although weakly supported, this finding is consistent with morphology (Berg 1989) and a preliminary analysis of chloroplast DNA sequences (Herre *et al.* 1996).

The remainder of the figs split into two major groups, one including a well-supported subgenus *Urostigma* (100% BS, PP=0.98), but excluding section *Urostigma* itself. Within subgenus *Urostigma*, the well supported neotropical section *Americana* (99% BS, PP=0.94) may be sister to the African section *Galoglychia* (99% BS, PP=0.51), although section *Galoglychia* is not supported by

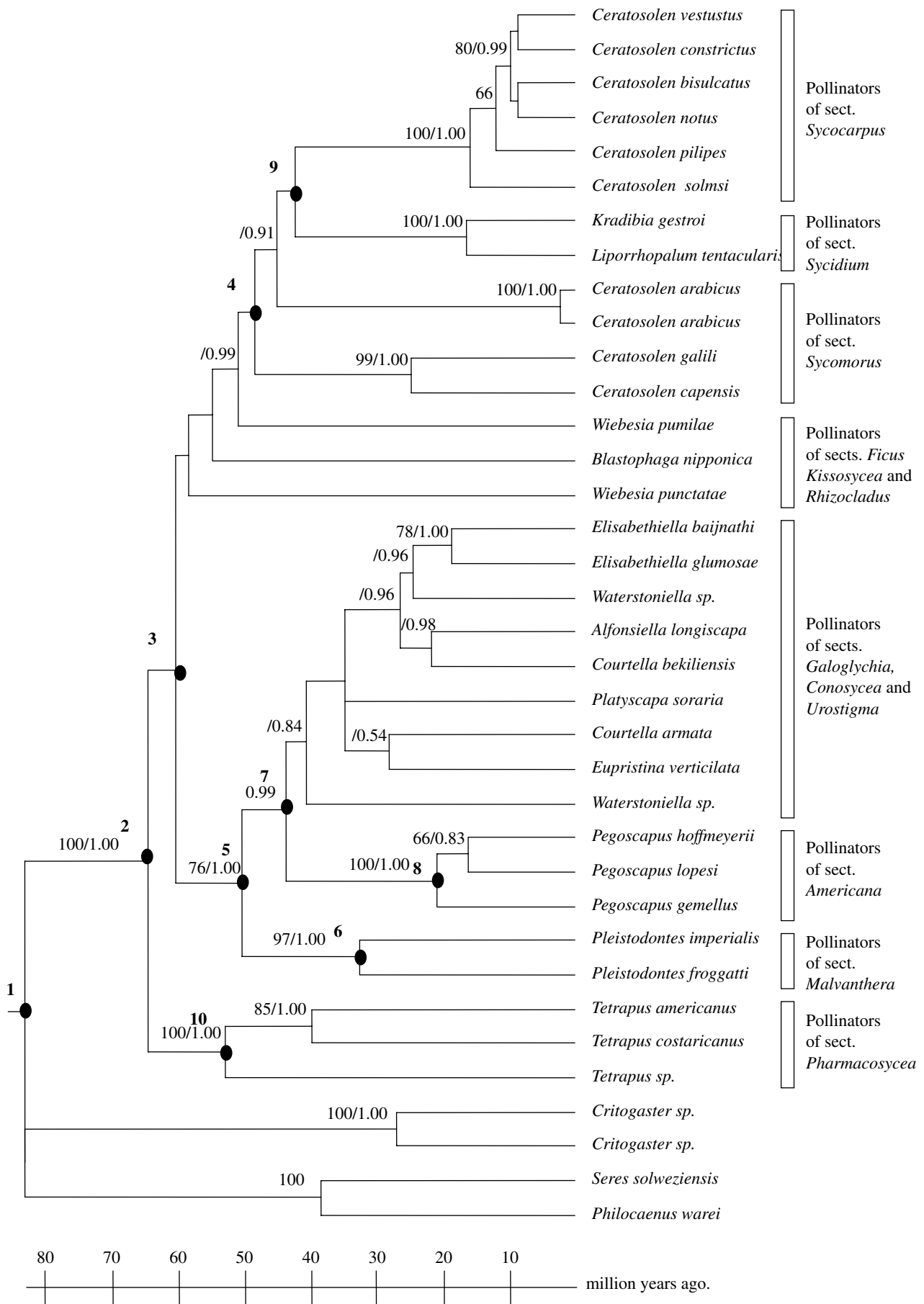


Figure 3. Ultrametric ML tree of fig pollinating Agonine wasps (*cox1*); bootstrap percentages/posterior probabilities above branches.

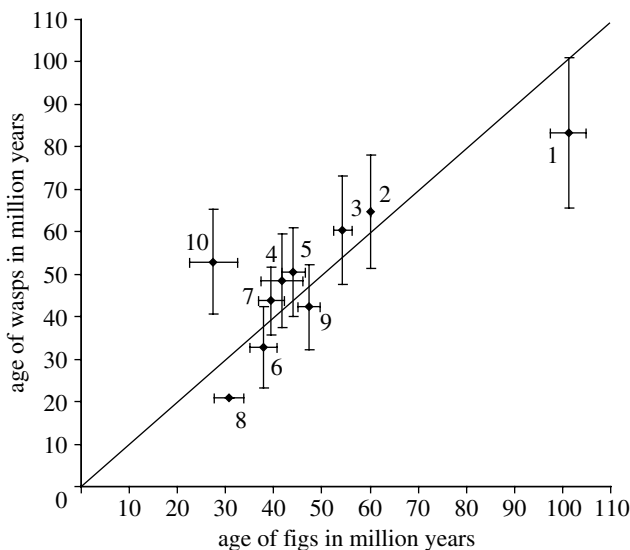


Figure 4. Temporal congruence of fig lineages and their associated pollinator wasp lineages based on independent, fossil calibrated molecular phylogenetic topologies. (1) *Ficus*/Agaoninae root nodes ($101.24 \pm 3.71/83.33 \pm 17.61$ Myr), (2) *Ficus*/Agaoninae crown groups (constrained to 60 Myr (Collinson 1989)/ 64.69 ± 13.30 Myr), (3) Dioecious *Ficus*/associated pollinator stem lineages ($54.34 \pm 1.84/60.49 \pm 12.76$ Myr), (4) *Sycomorus* s.s./associated *Ceratosolen* stem lineages ($41.70 \pm 4.41/48.52 \pm 10.94$), (5) *Malvanthera*/*Pleistodontes* stem lineages ($44.08 \pm 2.39/50.54 \pm 10.45$ Myr), (6) *Malvanthera*/*Pleistodontes* crown groups ($37.84 \pm 2.73/32.78 \pm 9.47$ Myr), (7) *Americana*/*Pegoscapus* stem lineages ($39.47 \pm 2.68/43.71 \pm 7.87$ Myr), (8) *Americana*/*Pegoscapus* crown groups (30.67 ± 2.98 Myr/constrained to 21 Myr; Machado *et al.* 2001), (9) *Sygidium*/*Kradibia* plus *Lipporhopalum* stem lineages ($47.28 \pm 2.28/42.25 \pm 9.95$ Myr), (10) *Pharmacosycea*/*Tetrapus* crown lineages ($27.47 \pm 5.03/52.89 \pm 12.28$ Myr).

BS > 50% and is paraphyletic with respect to section *Americana* in 35 of the 74 most parsimonious trees. Subgenus *Urostigma* also shows the Australasian section *Malvanthera* (79% BS, PP = 0.98) and the mainly Asian section *Conosycea* (97% BS, PP = 0.98) as sister clades (84% BS, PP = 0.98). *Ficus elastica*, which has traditionally been placed in a section of its own (*Stilpnophyllum*; Berg 1989; Jousset *et al.* 2003), or more recently with members of section *Malvanthera* Corner (*Stilpnophyllum* s.l.; Berg & Corner 2005) is here well embedded within section *Conosycea*. *Ficus rumphii* of the Asian section *Leucogyne* (two species; Berg 1989) also appears to be embedded in section *Conosycea*, where it groups with the Madagascan *Ficus menabeensis*.

The other major lineage (63% BS, PP = 0.93) is weakly supported by bootstrapping and resolution is poor. Section *Urostigma* (100% BS, PP = 0.97) is an early diverging lineage within this clade, which also includes sections *Oreosycea* (BS < 50%, PP = 0.83) and *Sycomorus* (79% BS, PP = 0.97) plus all of the dioecious figs. Subgenus *Synocia* (Berg 2003a,d) including the two root climbing sections *Kissosycea* and *Rhizocladus* is strongly supported (BS 100%, PP = 0.97).

(b) Dating the divergence of figs and their pollinating wasps

Figure 4 illustrates the respective ages plotted with standard errors for 10 interacting fig and pollinator lineages

(linear regression through the origin with $r = 0.968$ not significantly different from $r = 1$, $t = 0.165$, $p = 0.386$). Ages estimated from trees dated with NPRS and PL methods were highly correlated ($R^2 = 0.897$ and 0.960 for figs and wasps, respectively), although PL provided slightly younger ages depending on smoothing parameters. NPRS ages are shown in figure 4. Comparing 10 000 random sets of 10 pairs of ages taken from both phylogenetic trees showed that the pattern observed in figure 4 is highly significant ($p = 0.002$). The standard errors for the dates in the fig phylogeny were narrower than the intervals obtained for the wasp phylogeny, reflecting the denser taxon sampling and better resolution in the fig phylogenetic tree.

4. CONCLUSION

We have produced the most comprehensive phylogeny of figs to date and this supports the idea that section *Pharmacosycea* is the oldest section in the genus. With a dense taxon sampling, our phylogenetic analyses support the monophyly of most fig sections, especially within the monoecious subgenera *Urostigma* and *Pharmacosycea*. With over 750 species, *Ficus* is a large genus, and more detailed studies of phylogenetic patterns and evolutionary processes in the fig-wasp interaction should focus on smaller, more manageable subsets of species such as sections of the genus. Knowing the monophyly of a group of figs is a prerequisite for evaluating possible co-speciation in the mutualism. Not all clades are well supported in our analysis and future molecular systematic work should focus on the relationship between monoecious and dioecious figs (particularly the relationships of sections *Urostigma* and *Oreosycea*) and the sectional classification of dioecious figs.

Two previous molecular studies have estimated the date of origin of figs and their pollinating wasp. Machado *et al.* (2001) obtained an age interval of 75–100 Myr for the crown group of the wasps, a date that is older than available fossil evidence of *Ficus* by at least 15 Myr. More recently, Datwyler & Weiblen (2004) used three calibration points to date their phylogenetic tree of Moraceae based on *ndhF* sequences of over 80 taxa representing 33 genera. They obtained an estimate of 83 Myr for the root node of *Ficus*. We obtained confidence intervals of 98–105 Myr for the age of the root node of *Ficus* and 66–101 for the age of the root node of the wasps. The crown group of *Ficus* was constrained to 60 Myr by a fossil achene, and for the crown group of the wasps we obtained a confidence interval of 51–78 Myr (figure 4). Our results confirm previously published dates suggesting a time frame of 60–100 Myr ago for the origin of the fig-wasp association. However, the use of fossils for dating yields minimum age estimates, because the fossil record may not coincide with the earliest appearance. Confirmation of these dates could be given by analysing whether they are compatible with biogeographic scenarios for *Ficus* (see Machado *et al.* 2001; Zerega *et al.* in press). If the age estimates we have obtained are correct, this could imply long distance oceanic dispersal being an important process explaining the present distribution of *Ficus*. For instance, the south American section *Pharmacosycea* would have separated from the rest of the figs only 60 million years ago (node 2 on figure 2), which post-dates the separation of

South America from Africa (about 90–100 Myr ago) during the break up of Gondwana. Likewise, the American section *Americana* and the African section *Galoglychia* would have separated around 40–50 million years ago (node 7 on figure 2).

Phylogenetic double-dating has so far only been used to evaluate co-speciation between parasitic psyllids (Hemiptera) and their hosts in the genistoid legumes (Genisteae; Percy *et al.* 2004). The authors found that all but one of the putative co-speciation events were in fact asynchronous, indicating that the psyllids colonized hosts that had already diversified rather than co-speciating contemporaneously with their hosts. By comparison, the fig-pollinating wasp system exhibits strong evidence for co-diversification in at least 10 interacting lineages.

Coevolution between mutualistic partners and between hosts and parasites is a long-held hypothesis, but the prevalence of coevolution between interacting taxa is unknown, largely because only a small number of associations have been studied in sufficient detail to document long-term coevolution.

The best-documented case is that between pocket gophers (Geomysidae) and their chewing lice (Phthiraptera; see Hafner *et al.* 2003 and references therein). Independent phylogenies of host and parasite lineages, based on sequences of the mitochondrial *cox1* gene, show significant congruence both at high-taxonomic levels and within genera. Although lice may be transmitted horizontally between individuals, such dispersal relies on host-to-host contact, which is almost exclusively intraspecific among gophers. Other biological aspects, such as hair diameter, may also restrict the suitability of other potential host species for dispersing lice. Consequently, Hafner and co-workers suggest that the pattern of co-cladogenesis results primarily from lack of opportunity to colonize new host species.

Another classic system is the obligate pollination mutualism between the yucca (Agavaceae) and the yucca moth (Lepidoptera; Pellmyr 2003), but no analysis of parallel cladogenesis has yet been conducted due to the lack of phylogenetic estimates for the host plants.

In addition, an obligate pollination mutualism between *Glochidion* trees (Phyllanthaceae) and *Epicephala* moths (Gracillariidae) was recently described (Kato *et al.* 2003). Several different methods for investigating the level of co-cladogenesis between phylogenies indicated that there is a greater degree of correlation between the *Glochidion* and *Epicephala* phylogenetic trees than expected in a random association (Kawakita *et al.* 2004). Coevolution with pollinators has also been suggested in *Phyllanthus*, another genus in Phyllanthaceae (Kawakita & Kato 2004).

Likewise for palms, a diversity of insect pollination mutualisms have been described (Henderson 1986), but not yet studied in a phylogenetic framework.

All of these systems show deviations from perfect phylogenetic congruence, which could be due to host-shifting, independent speciation and/or extinction events, and error associated with phylogeny estimation. A number of studies have provided evidence that various hemipteran insect taxa, such as mealybugs (Baumann & Baumann 2005), white flies (Thao & Baumann 2004) and their primary bacterial endosymbionts, share phylogenetic histories. These systems tend to show perfect congruence, but this is consistent with a single infection of the hosts

with an ancestor of the endosymbionts followed by vertical transmission. Other studies have simply failed to demonstrate coevolution between associated partners. For instance, Desdevises *et al.* (2002) found that host–parasite associations between *Sparidae* (Teleostei) fishes and their parasites of the genus *Lamellogadus* (Monogenea) were due more to ecological factors than to coevolutionary processes.

Molecular dating showed that the yucca–yucca moth association arose at least 40 million years ago (Pellmyr 2003) and long-term co-divergence was recently reported for Simian foamy RNA viruses and old World primates (Switzer *et al.* 2005). The phylogenetic trees were remarkably congruent in both branching order and divergence times over 30 million years, strongly supporting co-speciation in this host–parasite system.

The strength of the relationship between the independently inferred ages of closely associated fig and pollinator lineages in the present study provides the most compelling evidence to date for long-term co-divergence in this now classical mutualism during at least the past 60 million years. Having established a scenario of parallel diversification of fig and wasp lineages, future studies should focus on the extent of co-speciation in the fig–wasp symbiosis based on manageable monophyletic groups of figs in comparison with the associated pollinators as exemplified by Weiblen & Bush (2002) for *Ficus* subgenus *Sycomorus* and the pollinating wasps of the genus *Ceratosolen*. Another promising line of investigation would be to examine whether the dates obtained in the present study are compatible with biogeographic scenarios and dates obtained for other groups and what implications these dates have for explaining the present distribution of figs and fig pollinators.

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