An extreme case of plant-insect co-diversification: figs and fig-pollinating wasps

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An Extreme Case of Plant-Insect Co-Diversification: Figs and Fig-Pollinating Wasps

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Running title: Co-diversification of Figs and Fig Wasps

Abstract.— It is thought that speciation in phytophagous insects is often due to colonization of novel host plants, because radiations of plant and insect lineages are typically asynchronous. Recent phylogenetic comparisons have supported this model of diversification for both insect herbivores and specialized pollinators. An exceptional case where contemporaneous plant-insect diversification might be expected is the obligate mutualism between fig trees (*Ficus* species, Moraceae) and their pollinating wasps (Agaonidae, Hymenoptera). The ubiquity and ecological significance of this mutualism in tropical and subtropical ecosystems has long intrigued biologists, but the systematic challenge posed by >750 interacting species pairs has hindered progress toward understanding its evolutionary history. In particular, taxon sampling and analytical tools have been insufficient for large-scale co-phylogenetic analyses. Here, we sampled nearly 200 interacting pairs of fig and wasp species from across the globe. Two supermatrices were assembled: on average, wasps had sequences from 77% of six genes (5.6kb), figs had sequences from 60% of five genes (5.5 kb), and overall 850 new DNA sequences were generated for this study. We also developed a new analytical tool, Jane 2, for event-based phylogenetic reconciliation analysis of very large data sets. Separate Bayesian phylogenetic analyses for figs and fig wasps under relaxed molecular clock assumptions indicate Cretaceous diversification of crown groups and contemporaneous divergence for nearly half of all fig and pollinator lineages. Event-based co-phylogenetic analyses further support the co-diversification hypothesis. Biogeographic analyses indicate that the present-day distribution of fig and pollinator lineages is consistent with an Eurasian origin and subsequent dispersal, rather than with Gondwanan vicariance. Overall, our findings indicate that the fig-pollinator mutualism represents an extreme case among plant-insect interactions of coordinated dispersal and long-term co-diversification.

[biogeography, coevolution, cospeciation, host switching, long branch attraction, phylogeny.]

Processes affecting the diversification of insects are crucial to understanding the origin of biodiversity, because most animals are either insect herbivores, or natural enemies (predators or parasitoids) of these phytophages (Novotny et al., 2002). As primary consumers, most insect herbivores are involved in antagonistic interactions with plants and, although herbivores often exhibit host-specific co-evolutionary adaptations to plant defences (Ehrlich and Raven, 1964), recent empirical studies have suggested that host plant lineages are generally older than their associated herbivores (Percy et al., 2004; Tilmon, 2008; McKenna et al., 2009). Such patterns of asynchronous plant-insect diversification are consistent with the general paradigm that insect speciation results from colonization of novel host plants and subsequent reproductive isolation (Percy et al., 2004; Tilmon, 2008; McKenna et al., 2009; Fordyce, 2010).

Phytophagous insects are often enemies of plants, but some engage in beneficial pollination mutualisms. A charismatic example involves the ca. 750 species of figs (*Ficus*, Moraceae) and their pollinating wasps (Hymenoptera, Chalcidoidea, Agaonidae) (Fig. 1). Agaonids are the only pollen vectors for fig trees and agaonid larvae feed exclusively on the flowers of their *Ficus* hosts. Each partner is thus entirely dependent on the other for reproduction. Figs are also a major resource for frugivores and most animal-dispersed tropical tree species interact with vertebrates that also consume figs (Howe and Smallwood, 1982). The fig-pollinator mutualism is therefore ecologically important in most tropical ecosystems (Shanahan et al., 2001). Many fig species reproduce irregularly, are relatively inaccessible in the forest canopy, or today are found only in rainforest remnants, such that coordinated sampling of *Ficus* and pollinator species for systematic study is difficult. These sampling challenges, coupled with the limitations of analytical tools for large data sets, have hindered progress towards understanding the global evolutionary history of the mutualism, despite the

fact that many details of this intricate symbiosis were described almost a century ago (Janzen, 1979; Wiebes, 1979; Weiblen, 2002; Cook and Rasplus, 2003; Herre et al., 2008).

Species-specificity in fig pollination appears to be extreme compared to most other insect pollination mutualisms. Most fig species are pollinated by only one or a few wasp species and most wasps are associated with just a single fig species (Cook and Rasplus, 2003; Molbo et al., 2003; Cook and Segar, 2010). Pollinators are specifically attracted to volatile compounds emitted by figs (Hossaert-McKey et al., 1994) and access to the specially modified inflorescences is by means of distinctive mandibular appendages and detachable antennae (van Noort and Compton, 1996). Pollination is either active (two thirds of the fig species) or passive (one third, mostly within subgenera *Pharmacosycea, Ficus, Synoecia* and *Urostigma*) (Kjellberg et al., 2001). Active agaonid wasps collect pollen from the anthers of their native figs and store it in thoracic pollen pockets (Galil and Eisikowitch, 1968; Ramirez, 1978). Once inside a receptive fig, they remove pollen from their pockets and deposit it on the flower stigma each time they lay an egg (Galil and Eisikowitch, 1968; Kjellberg et al., 2001). Passively pollinated figs produce large quantities of pollen through anther dehiscence and wasps are covered with pollen (Galil and Neeman, 1977) before flying away from their natal figs.

Closely matching fig and pollinator traits might be products of co-adaptation (Ramirez, 1974; Wiebes, 1979; Wiebes, 1982a; Kjellberg et al., 2001; Weiblen, 2004) but, regardless, trait-mediated interactions have the potential to simultaneously affect the evolution of reproductive isolation among pollinator and fig populations; this is because fig wasps breed exclusively in pollinated figs. This line of reasoning has underpinned the hypothesis that co-speciation might account for patterns of fig and pollinator diversity. However, this notion runs contrary to the paradigm that insect speciation generally involves

host-switching (Tilmon, 2008) and so it remains a controversial proposition that requires rigorous testing.

Under the co-speciation scenario, phylogenies of figs and pollinators are expected to show substantial congruence. There is some evidence for this pattern (Herre et al., 1996; Machado et al., 2005; Rønsted et al., 2005; Cook and Segar, 2010; Cruaud et al., 2011a), but recent studies have countered the underlying case for co-speciation with evidence of cryptic wasp species and relaxed partner specificity. At least 50 fig species are now known to have multiple pollinator species (Michaloud et al., 1985; Michaloud et al., 1996; Rasplus, 1996; Kerdelhué et al., 1997; Lopez-Vaamonde et al., 2002; Greeff et al., 2003; Molbo et al., 2003; Haine et al., 2006; Moe and Weiblen, 2010; Chen et al., in press) and as many as four different wasp species are known to pollinate a single fig species (Machado et al., 2005; Cook and Segar, 2010). Such cases occur in a broad taxonomic and geographic spectrum, although cases of pollinator species sharing multiple fig species have been reported mostly from monoecious figs in the Neotropics (Molbo et al., 2003) and the Afrotropics (Erasmus et al., 2007; Cornille et al., in press; McLeish and van Noort, in press). In any event, evidence of relaxed host specificity and some incongruent fig-pollinator phylogenies (Machado et al., 2005) suggest that host shifting is a viable alternative explanation for fig-pollinator diversification.

Co-speciation has been hypothesized for the vertically transmitted endosymbionts of insects (e.g. Moran, 2001; Jousselin et al., 2009) but this is not a plausible general model for the evolution of plant-insect associations, which are horizontally transmitted and not so integrated metabolically. Further, if the plant traits that mediate insect associations happen to be phylogenetically conserved, then host shifting among close relatives could also result in topologically congruent phylogenies (Percy et al., 2004). In addition, historical biogeography

has the potential to confound the explanation of such patterns if synchronous plant-insect dispersal to new environments, followed by geographic isolation, results in co-speciation.

Another useful approach is to investigate patterns of temporal congruence (Page and Charleston, 1998). Divergence time estimates for fig and pollinator clades are expected to be approximately equal in the event of co-radiation, whereas insect lineages are expected to be younger than hosts in the case of host shifting (Percy et al., 2004; Tilmon, 2008; McKenna et al., 2009).

Previous comparisons of fig and pollinator phylogeny have yielded rather different insights on the relative importance of host shifting and co-diversification depending on the taxonomic scope of sampling (Cook and Segar, 2010). Molecular phylogenetic trees appear roughly parallel when based on exemplars of *Ficus* sections and wasp genera (Herre et al., 1996; Jackson, 2004; Cruaud et al., 2011a), but such deep taxonomic sampling is unlikely to detect host shifts among close relatives (Machado et al., 2005). On the other hand, regional comparisons of particular fig and pollinator clades have tended to reject co-speciation in favour of host-switching (Machado et al., 2005; Marussich and Machado, 2007; Jackson et al., 2008; Jousselin et al., 2008), although not always (Weiblen and Bush, 2002; Silvieus et al., 2008). A global test for co-diversification therefore requires dense sampling of many fig and pollinator lineages across the entire geographic range, but a problem of this magnitude poses a further methodological challenge.

Tests of co-phylogenetic hypotheses often employ tree reconciliation methods that infer evolutionary processes such as co-speciation, host shifts, duplications and losses to account for topological incongruence between host and associate phylogenies (Page, 1994). This approach has the power to model the relative contributions of different evolutionary processes to a given phylogenetic pattern, but biologically realistic scenarios become computationally intractable for large numbers of taxa (Merkle and Middendorf, 2005; Ovadia

et al., 2011). Genetic algorithms that incorporate dynamic programming to efficiently locate and evaluate samples from an extremely large universe of event-based solutions hold promise in this regard (Conow et al., 2010).

Here, we extended the application of a genetic algorithm to event-based tree reconciliation analysis for co-phylogenetic problems involving >100 taxon pairs and applied randomization tests involving null models to test the co-divergence hypothesis on an unprecedented scale. Nearly 200 pairs of interacting fig and fig wasp species were sequenced at five fig loci (providing up to a total of 5.5 kb DNA sequence) and six wasp loci (up to a total of 5.6 kb). Two supermatrices were assembled. On average, wasps had sequences from 77% of six genes, figs had sequences from 60% of five genes, and overall we generated 850 new DNA sequences for the purpose of this study. Maximum likelihood analyses of fig and wasp data sets and Bayesian phylogenetic analyses under relaxed molecular clock assumptions enabled the comparison of distance, event-based, and temporal congruence. Inferences from historical biogeography based on our global sample of fig and pollinator clades provided additional insight on the relative roles of dispersal and vicariance with respect to alternative hypotheses of diversification.

MATERIALS AND METHODS

Taxonomic Sampling and DNA Sequencing

Ficus. —We sampled 200 fig species (more than 1/4 of the circa 750 described species) that represent all *Ficus* sections recognized by Berg and Corner (2005) (Appendix S1 in the Supplementary Material Online, doi: 10.5061/dryad.hr620). Four taxa belonging to the tribe Castilleae s.l., *Antiaropsis decipiens*, *Castilla elastica*, *Poulsenia armata* and *Sparattosyce*

dioica, were included as outgroups (Datwyler and Weiblen, 2004; Rønsted et al., 2005, Zerega et al., 2005; Clement and Weiblen, 2009). Total genomic DNA was extracted from 20-30 mg of dried leaf-fragments or herbarium material following Rønsted et al. (2008). *Ficus* phylogeny was reconstructed using five genes: ITS (891 bp), ETS (528 bp), glyceraldehyde 3-phosphate dehydrogenase (*G3pdh*, 769 bp), chloroplast expressed glutamine synthetase region (*ncpGS*, 1630 bp) and granule-bound starch synthase (*waxy* region, 1734 bp).

Amplification of ITS, ETS and *G3pdh* was performed following Rønsted et al. (2008). The *ncpGS* region (Emshwiller and Doyle, 1999) was amplified using Moraceae-specific primers 3F (5' GTT GTG ATT WAC CAT GCT) and 4R (3' AGA TTC AAA ATC GCC TTC) designed for this study. Amplification of *ncpGS* consisted of 4 min at 94°C followed by 36 cycles of: 1 min denaturation (94°C), 1 min annealing (50°C) and 2 min extension (72°C). After the last cycle, the temperature was kept at 72°C for a final 5 min extension and then lowered to 4°C. The *GBSSI* or *waxy* region (Mason-Gamer et al., 1999; Clement, 2008) was amplified using Moraceae-specific primers 3F (5' GAT CGY GTG TTT GTR GAT CAC C) and 10R (3' GCA ACT GAA TGA GAC CAC A). Amplification of *waxy* consisted of 3 min at 94°C followed by 2 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min, 2 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 2 min, 2 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 2 min, 2 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, and 24 cycles of 94°C for 1 min, 48°C for 1 min, 72°C for 2 min. After the last cycle, the temperature was kept at 72°C for a final 20 min extension and then lowered to 4°C. Amplified products were purified with the Qiagen PCR purification kit (Qiagen Inc.) following the manufacturer's protocols. ITS, ETS, *G3pdh* and *ncpGS* were sequenced directly from PCR products whereas *waxy* was cloned using the TOPO-TA PCR cloning kit (Invitrogen, Carlsbad, CA). Nine clones were screened for inserts, and plasmids were isolated from three of these using the Qiagen plasmid prep kit. Multiple copies of *waxy* are known in the Rosales (Evans et al., 2000) and therefore it was necessary to ensure that

phylogeny reconstruction was performed with orthologous copies. Two copies have been detected in Moraceae, GBSS1 and GBSS2, which were easily distinguished on the basis of size and intron alignment (Silvieus et al., 2008; W. Clement, unpublished data). Analyses were based solely on GBSS1 because GBSS2 was encountered less commonly in figs.

Cycle sequencing reactions were carried out following Rønsted et al. (2008). For sequencing of the *ncpGS* region, internal primers 1F (5' TCW TGW GCT GAA AAG CAT), 2F (TTT AAT CTC CAG ACT CSA), and 5F (5' TAG TTC ACT CTA AAG GGT) were designed for this study in addition to the primers used for amplification. Some 50% of the sequences were obtained from de novo sequencing for the purpose of this study and have been deposited in GenBank (Appendix S1). Other sequences, mostly deposited by co-authors, were obtained from existing databases.

Agaonidae. —93% of the 200 wasps and figs used in this study are true associates; i.e., even if they were not collected together simultaneously, the agaonid species is the pollinator of the fig species. In the very few cases where the corresponding agaonid was not available in our collection, we used instead the pollinator of a closely related species of fig (Appendix S2). This was always a wasp species that was a close congener of the actual pollinator. As the phylogenetic position of Agaonidae within the large and complex superfamily Chalcidoidea is as yet unknown (Gibson et al., 1999; Munro et al., 2011), four divergent members of the superfamily served as outgroups: *Sycophaga* (Sycophaginae), *Ficomila* (Eurytomidae), *Megastigmus* (Torymidae) and *Trichogramma* (Trichogrammatidae).

All material was collected alive in the field and fixed in 95% ethanol. With very few exceptions, Agaonidae sequences were obtained from the non-destructive extraction of a single wasp specimen (corpse kept as voucher). DNA was extracted from a single individual that was incubated at 56°C overnight (with gentle "shaking" steps by inverting the tubes) and

using the Qiagen DNeasy kit following the manufacturer's protocol. When destructive extraction was used, vouchers were selected among specimens sampled from the same tree and the same fig after careful identification by JYR, SvN and RU. Vouchers are deposited at CBGP, Montferrier-sur-Lez, France. To infer phylogenetic relationships between agaonid species, we combined two nuclear protein-coding genes [F2 copy of elongation factor-1a (*EF1a*, 516 bp), Wingless (*Wg*, 403 bp)]; two mitochondrial protein-coding genes [cytochrome c oxidase subunit I (*COI*, 1536 bp), cytochrome b (*Cyt b*, 749 bp)] and two ribosomal genes [28S rRNA (D2–D3 and D4–D5 expansion regions, 1520 bp), 18S rRNA (variable regions V3–5, 787 bp)]. Extraction, amplification and sequencing protocols follow Cruaud et al. (2010) for *CytB*, *COI* (barcode fragment), *Wg,* 28S and 18S rRNA, Weiblen (2001) for *COI* [C1-J-2183 (Jerry) - TL2-N-3014 (Pat) fragment], and Cruaud et al. (2011b) for *EF1a*. Both strands for each overlapping fragment were assembled using Geneious v5.4.2 (Drummond et al., 2007).

67% of the sequences were obtained from de novo sequencing for the purpose of this study and have been deposited in GenBank (Appendix S2). Other sequences, mostly deposited by co-authors, were obtained from public databases.

Phylogeny Reconstruction

Protein-coding genes and hypervariable regions were aligned using ClustalW 1.81 with the default settings (Thompson et al., 1994). Alignments of protein-coding genes were translated to amino acids using Mega 4 (Tamura et al., 2007) to detect frameshift mutations and premature stop codons, which may indicate the presence of pseudogenes. Alignment of sequences encoding rRNA was based on secondary structure models (Gillespie et al., 2006), following Cruaud et al. (2010). Phylogenetic trees were estimated using both maximum

likelihood (ML) and Bayesian methods. We selected separate models of molecular evolution for different genomic regions including mitochondrial genes, rRNA stems, rRNA loops + regions of ambiguous alignment, and individual nuclear genes using the Akaike information criterion implemented in MrAIC.pl 1.4.3 (Nylander, 2004).

For each dataset, we performed ML analyses and associated bootstrapping (1,000 replicates) using the MPI-parallelized RAxML 7.0.4 software (Stamatakis, 2006b). GTRCAT approximation of models (Stamatakis, 2006a) was used for ML boostrapping (1,000 replicates). Bootstrap percentage (BP) > 70% was considered as strong support (Felsenstein and Kishino, 1993). Bayesian analyses (BA) were conducted using a parallel version of MrBayes 3.1.1. (Huelsenbeck and Ronquist, 2001). We assumed across-partition heterogeneity in model parameters by considering the parameter m (Nylander et al., 2004 ; Marshall et al., 2006; McGuire et al., 2007). Parameter values for the model were initiated with default uniform priors and branch lengths were estimated using default exponential priors.

To improve mixing of the cold chain and avoid it converging on local optima, we used Metropolis-coupled Markov chain Monte Carlo (MCMCMC) simulation with each run including a cold chain and three incrementally heated chains. The heating parameter was set to 0.02 in order to allow swap frequencies from 20% to 70%. For both figs and pollinators, we ran two independent runs of 30 million generations. All the values were sampled every 3,000 generations. For the initial determination of burn-in, we examined the plot of overall model likelihood against generation number to find the point where the likelihood started to fluctuate around a constant value. Convergence of the chains was evaluated using the online application AWTY (Nylander et al., 2008) and the results were based on the pooled samples from the stationary phases of the two independent runs. Given that posterior probabilities (PP) may overestimate clade support, for reasons discussed elsewhere (Suzuki et al., 2002;

Cummings et al., 2003; Erixon et al., 2003; Simmons et al., 2004), only clades with PP > 0.95 were considered strongly supported. All analyses were conducted on a 150core Linux Cluster at CBGP, Montferrier-sur-Lez, France.

Test of alternative hypotheses. — To assess whether certain alternative relationships among recovered clades could be statistically rejected, we performed AU (Shimodaira, 2002) and SH (Shimodaira and Hasegawa, 1999) tests in the CONSEL package (Shimodaira and Hasegawa, 2001). The program *makermt* was used to generate K=10 sets of bootstrap replicates (r_1=0.5, r_2=0.6, r_3=0.7, r_4=0.8, r_5=0.9, r_6=1, r_7=1.1, r_8=1.2 r_9=1.3, r_{10}=1.4). Each set consisted of 100,000 replicates of the row sums (10 times the default number of replicates). RAxML was used to compute the per-site log likelihoods for all topologies tested. To assess the relative support for competing phylogenetic hypotheses, we also conducted AU and SH tests on recently published datasets (Cruaud et al., 2010; Lopez-Vaamonde et al. 2009), which placed *Tetrapus* as sister to all other agaonids with strong support (PP=0.99 and BP=55/59; PP=1.00 respectively).

Effects of missing data. — There is debate in the literature as to the effect of missing data on the accuracy of phylogenetic analyses. Simulation results based on limited numbers of characters (Lemmon et al., 2009) indicated that non-random distributions of missing data can result in strong support for nodes that share no supporting characters. However, other empirical and simulation studies have concluded that taxa with extensive missing data can be accurately placed in phylogenetic analyses, and that adding characters with missing data is generally beneficial, if the overall number of characters is large and data are analyzed with appropriate methods (see Wiens and Morrill, 2011). To assess the impact of missing data on our analyses, we performed two sets of additional analyses.

First, we built new ("complete species") trees using only the more completely sequenced taxa (figs with more than three genes; wasps with more than five genes). Then we used AU and SH tests to compare the full (all species and genes) tree, pruned of incompletely sequenced taxa, with the matching "complete species" tree. Second, we built new ("complete genes") trees by removing gene fragments for which less than 60% of the taxa were available. We then used AU and SH tests to test if the full tree differed significantly from the "complete genes" tree. Taxa were pruned from the combined ML tree using the APE package (Paradis et al., 2004) in R 2.14.0 (http://www.R-project.org).

Bayesian Relative Rate tests and Long-branch attraction artifact. —We tested constancy of evolutionary rates among agaonid species using both BEAST 1.5.3 (Drummond and Rambaut, 2007) (coefficient of variation statistic and average rate for each branch of the chronogram, see Molecular Dating section) and a Bayesian relative rate (BRR) test (Wilcox et al., 2004). For the BBR test, the posterior probability distributions of lengths for all branches from the most recent common ancestor (MRCA) of the ingroup to each of the terminal taxa were based on 1,000 randomly chosen post-burn-in trees from the Bayesian analyses of the mitochondrial (mtDNA), nuclear (nuDNA) and combined datasets respectively. Following Wilcox et al. (2004), we considered rates of evolution significantly different between two taxa if the 95% confidence interval of the posterior probability distribution of the summed branch length did not overlap. Branch length estimates were compiled using Cadence v1.08b (Wilcox et al., 2004).

Long-branch attraction (LBA) artifacts can be difficult to detect, but methods have been proposed and we applied these to our data (Bergsten, 2005). For computation time reasons, all additional analyses were conducted using RAxML only.

Removing first and third codon positions, which are fast evolving, may be a way to

reduce LBA. However, this can also compromise tree resolution (Källersjö et al., 1999; Savolainen et al., 2002; Stefanović et al., 2004). The RY-coding strategy (Woese et al., 1991), by discarding fast-evolving transitions and reducing compositional bias, constitutes a better approach (Phillips and Penny, 2003; Philippe et al., 2005). We therefore compared the topologies obtained with or without RY-coding of 1) the third (nt3) and 2) first (nt1) and third mtDNA codon positions.

Long-branch extraction (LBE) is another approach advocated for cases where LBA is suspected (Pol and Siddall, 2001). Since LBA to the outgroups is the most frequent problem, analyses were conducted without the outgroups (Bergsten, 2005).

Finally, the different sensitivity of parsimony and ML methods can help to detect if LBA is playing a major role (Brinkmann et al., 2005). We therefore performed parsimony analysis on our dataset to detect potential shifts in position of agaonid groups. Parsimony analyses were conducted with TNT version 1.1 (Goloboff et al., 2008), using New Technology Search: 1,000 replicates of random addition sequences (RAS), followed by random sectorial searches with default options, 100 cycles of ratchet and three rounds of tree-fusing. All substitutions were equally weighted and gaps treated as missing data. Robustness of topologies was assessed by bootstrap procedures using 1,000 replicates.

Co-phylogenetic Analyses

We tested the congruence between fig and wasp phylogenies using both distance and event/topology-based methods. The former generate patristic distance matrices between species in each phylogeny and then test for correlations between the two matrices. In contrast, event-based methods use evolutionary events (co-speciation, duplication, host-shifts, lineage sorting and "failure to diverge" (Page and Charleston, 1998; Charleston and Perkins, 2006))

to map the associate phylogeny to the host one. A cost is assigned to each event type and we seek to find mappings that minimize the total cost. Statistical analyses can be performed by comparing the best costs found for the host-parasite dataset against those of randomized instances.

We used the distance-based method, ParaFit, developed by Legendre et al. (2002) and implemented in the program CopyCat (Meier-Kolthoff et al., 2007). ParaFit evaluates the global hypothesis of host-associate co-speciation with a matrix permutation test of co-divergence. This test combines three types of information: the associate phylogeny and the host phylogeny both described by their respective matrices of patristic distances, and the observed host-associate links. Each matrix representing associates and hosts is transformed into a matrix of principal coordinates. The association is then described by a new matrix, which includes both matrices of principal coordinates and the matrix of association. Patristic distances were computed from fig and wasp ML-phylogenetic trees. Tests of random association (null hypothesis) were performed using 9,999 permutations globally across both phylogenetic trees. Although the distance-based approach is computationally simple, it only yields a measure of overall phylogenetic congruence and no information on the relative distribution of underlying evolutionary events that might have produced the pattern.

Event-based methods have the advantage of modeling evolutionary processes directly, but are computationally intensive (Charleston, 2009). The problem of finding a mapping (event-based reconstruction) of minimum total cost has been shown to be computationally intractable ("NP-complete") (Ovadia et al., 2011). Some existing software packages e.g. TREEMAP 1.0; (Page, 1994) and 2.02; (Charleston and Page, 2002), use exhaustive searches, which are prohibitively slow and also permit only limited numbers of species. Other programs use heuristics (Merkle and Middendorf, 2005), which are fast but may converge on suboptimal or invalid solutions (e.g. ancestral speciation inferred to have occurred *after*

speciation of descendants nodes). For this reason, our analyses used a genetic algorithm to search a sample of the possible solution space with a dynamic programming step that efficiently evaluates the cost of each such sample. This approach, which finds solutions of near-optimal cost, was first implemented in the Jane software package (Conow et al., 2010) and was validated using a number of existing datasets in the literature (Libeskind-Hadas and Charleston, 2009). However, the sheer size of our datasets put the analysis far beyond the computational limits of the original version of Jane, which also lacks support for randomization tests. We therefore substantially optimized and improved the existing Jane co-phylogeny software package, resulting in a new system, Jane 2, which is capable of performing event-based analyses of very large datasets. Jane 2 and its tutorial are freely available for research and educational purposes at http://www.cs.hmc.edu/~hadas/jane/index.html.

We used Jane 2 with the following parameter values: the number of "generations" (iterations of the algorithm) was set to 40 and the "population" (number of samples per generation) was set to 1,000. We explored three different cost models, each with two types of randomization test. The first model set costs per event as cospeciation=0 and all other events = 1. This correponds to the TreeMap cost scheme so that a duplication event actually contributes 2 to the total cost because each of the two daughter lineages contributes one duplication event. The cost of the best solution was compared to the costs found in 100 randomizations in which the tip mappings were permuted at random, a method advocated by Aldous (2001). The second randomisation involved 100 randomly generated pollinator trees, of the same size as the actual wasp pollinator tree, with random tip mappings. The random pollinator trees were constructed using the Yule model with beta parameter equal to -1.

In the second model, we used costs of 0 for cospeciation, 1 for each duplication, 1 for each host switch, and 2 for each loss event. In the third model, we set the co-speciation cost at

-1 and all other costs to 0, where a negative cost maximizes the number of inferred co-speciations. For the second and third cost models we used the same two randomization tests described for the first model.

All the analyses were performed at Harvey Mudd College (Claremont, CA, USA) on a heterogenous cluster of commodity computers comprising a total of 168 cores. On a single commodity computer (e.g. a dual core Macintosh), a single fig/wasp tree required approximately three hours of computation and thus 100 randomized trials required several hours on our cluster.

Molecular Dating

We used the uncorrelated log-normal relaxed clock method implemented in BEAST 1.5.3 (Drummond and Rambaut, 2007) and the same modelling strategies as for MrBayes and RAxML analyses. We assumed a Yule tree prior and we used default priors for all other parameters. We used two runs of 60 million generations with sampling every 6,000 generations for figs, and two runs of 240 million generations with sampling every 24,000 generations for wasps. The two separate runs were then combined using LogCombiner 1.5.3. We ensured convergence using TRACER 1.5 (Drummond and Rambaut, 2007). Following the removal of 10% burn-in, the sampled posterior trees were summarized using TreeAnnotator 1.5.3 to generate a maximum clade credibility tree and calculate the mean ages, 95% highest posterior density intervals (95% HPD) and posterior probabilities (PP). We used independent calibration points to estimate divergence ages of the main *Ficus* and agaonid clades. Following Rønsted et al. (2005), crown-group *Ficus* was assigned a uniform prior distribution with a minimum age of 60 Ma based on fossilized achenes (Collinson, 1989) and a maximum age of 198 Ma based on converging molecular estimates for the origin

of the angiosperms (Bell et al., 2005). Given uncertainties over the age of Dominican amber (Grimaldi, 1994; Iturralde-Vinent and MacPhee, 1996; 1999), crown-group *Pegoscapus* and *Tetrapus* were assigned uniform prior distributions with minimum ages of 15 Ma and maximum ages of 60 Ma based on Dominican amber fossil (Poinar, 1993; Penalver et al., 2006). For both fig and wasp phylogenies, nodes including taxa endemic to La Réunion were modelled with a normal distribution with a mean of 8 Ma and SD of 1 Ma based on the proposed age for the Mascarene archipelago (McDougall and Chamalaun, 1969; McDougall, 1971).

Ancestral Area Reconstructions and Evolution of Pollination Mode

We inferred the evolution of pollination mode and the ancestral areas for figs and their pollinators using both ML and parsimony approaches implemented in Mesquite 2.73 (Maddison and Maddison, 2008). Pollination modes and ancestral areas were inferred on the ML topologies. For ML optimization, we used a stochastic Markov model of evolution (Mk1). The Likelihood Decision Threshold was set to two log-likelihood units. Character data for *Ficus* and Agaonidae were obtained both from the literature (Kjellberg et al., 2001; Berg and Corner, 2005) and from our examination of flowers, pollen pockets and coxal combs. Following Lopez-Vaamonde et al. (2009), current species distributions were categorized into four character states: (0) Afrotropics, (1) Australasia, (2) Neotropics, (3) Eurasia. However, because several taxa occur in both Eurasian and Australasian regions and a couple of taxa occur in both Eurasian and Afrotropical regions, and Mesquite requires unique character states, we also defined two other states: (4) Australasia + Eurasia and (5) Afrotropics + Eurasia. We took into account all published geographic localities for *Ficus* and agaonids, museum specimens and about 3,000 samples of fig wasp communities that we collected over

the last 15 years. We also used the dispersal-extinction-cladogenesis model implemented in Lagrange (Ree and Smith, 2008), using the same raw data and four character states. Dispersal rate between all areas was set to 1 during the whole period considered (data available upon request).

RESULTS

DNA Sequence Data

The completeness of taxa in the combined data matrices is different for fig and wasps (Appendices 1-2 and Table S1 in the Supplementary Material Online). On average, wasps have sequences from 77% of the six genes and 67% of the species were sequenced for at least five gene regions. On average, figs have sequences from 60% of the five genes and 70% of the species were sequenced for at least three regions. Plastid regions provide little phylogenetic information within *Ficus,* enforcing the use of more informative single copy nuclear regions. These are known to be notoriously difficult to amplify from plants in general (Rønsted et al., 2007) and this was also the case for *Ficus* in the present study. Indeed, *ncpGS* and *waxy* matrices only include 24% and 23% of the taxa respectively. Models chosen by MrAIC for each partition were as follows. *Ficus* dataset: GTR + Γ (ETS, ITS, *ncpGS* and *waxy*), GTR + I + Γ (*G3pdh*); Agaonid dataset: GTR + Γ (mtDNA), GTR + I + Γ (*EF1a, Wg, rRNA stems*), HKY + I + Γ (rRNA loops). Given that α and the proportion of invariable sites can not be optimized independently from each other (Gu, 1995) and following Stamatakis' personal recommendations (RAxML manual), we used GTR + Γ with four discrete rate

categories for all partitions. As RAxML does not implement the HKY model, we used GTR instead.

Wasp Phylogeny

Our phylogenetic trees (Figs. 2, S1), reconstructed using ML and Bayesian approaches provide several new insights into the systematics of fig wasps.

Monophyly of the genera and intergeneric relationships. — Fifteen agaonid genera are recovered as monophyletic with strong support (*Agaon, Alfonsiella, Allotriozoon Ceratosolen, Courtella, Deilagaon, Elisabethiella, Eupristina, Kradibia, Nigeriella, Pegoscapus, Pleistodontes, Tetrapus, Valisia* and *Waterstoniella*). In contrast, *Platyscapa* is polyphyletic, and *Dolichoris* is paraphyletic with respect to *Blastophaga psenes* (the pollinator of *F. carica* and type species of the genus *Blastophaga*), indicating the need for taxonomic rearrangements (Cruaud et al., 2012).

The relationships among the major clades are unclear (Fig. 2). BEAST analysis places *Ceratosolen* + *Kradibia* (subfamily Kradibiinae) as the sister group to the remaining Agaonidae with strong support (PP_{BEAST} =0.98), but this position is not strongly supported by MrBayes ($PP_{MrBayes}$=0.88) and ML analyses (BP=43). Bayesian analyses place *Tetrapus* (monogeneric subfamily Tetrapusinae) nested within the Agaonidae with strong support ($PP_{MrBayes}$=1.00, PP_{BEAST}=1.00), although this position is only moderately supported by ML analyses (BP=67).

Phylogenetic placement of the genus Tetrapus. — By not placing *Tetrapus* as sister to all other agaonids, our topology challenges all previous molecular studies by ourselves and

others (Herre et al., 1996; Machado et al., 1996; Machado et al., 2001; Lopez-Vaamonde et al., 2009; Cruaud et al., 2010, Table S3). This result deserves further examination, so we have conducted additional analyses on not only our current dataset, but also previously published datasets. We provide here a summary of the main results (see the Appendix S3 for further details):

1) Both AU and SH tests fail to reject alternative topologies in which either *Tetrapus,* or the clade of pollinators associated with subgenus *Synoecia* and subsection *Frustescentiae* (corresponding to Group 4 in Cruaud et al., 2010), is constrained to be the sister group to all other Agaonidae (Table S2). Furthermore, AU and SH tests also fail to reject alternative positions of *Tetrapus* using two previously published datasets (Lopez-Vaamonde et al., 2009; Cruaud et al., 2010) that recover *Tetrapus* as sister to all other Agaonidae (Table S2).

2) *Tetrapus* is recovered nested within the Agaonidae in all the analyses conducted to assess the impact of missing data on the accuracy of our phylogeny. AU and SH tests showed that phylogenetic trees pruned of incompletely sequenced taxa and trees built only on gene fragments for which at least 60% of the taxa were available, were not signifiantly different from the orginial trees including all available data (Fig S2B&C, Table S2). Therefore, our analyses show that missing data are not responsible for the position of the genus *Tetrapus.*

3) Examination of branch lengths (mtDNA, nuDNA and combined trees, Figs. S1, S3) indicates considerable variation in rates of molecular evolution among agaonid lineages This result is confirmed by the Bayesian relative rate (BRR) tests (Figs S4, S5) and the BEAST outputs (95% credible interval for the coefficient of variation of rates is not abutting against zero for each partition and covariance values span zero). Furthermore, a long branch leading to *Tetrapus*, is visible in both the nuDNA tree (Fig S3b) and ML and Bayesian combined trees (Fig S1). BRR tests and branch-specific rates inferred by BEAST reveal a lineage-

specific increase in nucleotide substitution rates on this branch, and this is also the case for the branch leading to the outgroups (Fig. S4).

4) RY-coding of first and third mtDNA codon positions does not result in significant topological changes, but increases support for *Tetrapus* nested within the Agaonidae (Fig S2D&E, Table S2).

5) Unrooted and rooted topologies appeared congruent (Fig S2J), showing that rooting does not alter the ingroup topology. Furthermore, the unrooted topologies from Lopez-Vaamonde et al. (2009) and Cruaud et al. (2010) do not show conflicts with the topology presented here (Fig S7b&c).

6) Parsimony analysis of the combined dataset recovers *Tetrapus* as sister to the remaining Agaonidae (BP=64) (Fig S6).

We conclude that neither our study nor previous ones have a strong basis for inferring which group is sister to all other agaonids. Accordingly, the placement of *Tetrapus* remains unresolved. However, we suggest that the repeated recovery of *Tetrapus* as sister to all other agaonids in previous studies may be due to long branch attraction to the outgroups and we await further studies.

Ficus Phylogeny

The *Ficus* phylogenetic trees (Figs. 2, S9) are globally congruent with previous hypotheses (Herre et al., 1996; Weiblen, 2000; Jousselin et al., 2003; Rønsted et al., 2005; Rønsted et al., 2008; Cruaud et al., 2011a; Xu et al., 2011) (Table S5).

Monophyly of the subgenera and infrageneric relationships — Several moderately to strongly supported clades broadly correspond to currently recognized sections or subsections

based on previous molecular phylogenetic studies (see Rønsted et al., 2008) and morphology (sections *Pharmacosycea, Oreosycea, Americana, Galoglychia, Adenosperma s.l., Sycomorus s.l., Sycocarpus, Eriosycea*, and subsections *Malvanthera, Conosycea, Urostigma, Ficus* and *Frutescentiae*). Only three of the six *Ficus* subgenera currently recognized based on morphology (Berg and Corner, 2005) are recovered as monophyletic with strong support. These are: *Sycomorus* (BP=71, $PP_{MrBayes}$=0.75, PP_{BEAST}=1.00); *Sycidium* (BP=100, $PP_{MrBayes}$=1.00, PP_{BEAST}=1.00) and *Synoecia* (BP=100, $PP_{MrBayes}$=1.00, PP_{BEAST}=1.00). Relationships of deeper nodes are not strongly supported. The first split within *Ficus* is between section *Pharmacosycea* (BP=100, $PP_{MrBayes}$=1.00, PP_{BEAST}=1.00) and the remainder of *Ficus* (BP=39, $PP_{MrBayes}$=0.88, PP_{BEAST}=0.85). The next split is between a clade with all members of subgenus *Urostigma* except subsection *Urostigma* (BP=100, $PP_{MrBayes}$=1.00, PP_{BEAST}=1.00) and a clade with members of subsection *Urostigma*, subgenus *Sycomorus* and all other dioecious figs (BP=66, $PP_{MrBayes}$=0.95, PP_{BEAST}=1.00).

Exploration of bias in the Ficus phylogenetic trees — Previous molecular studies are similar in recovering section *Pharmacosycea* (pollinated by the genus *Tetrapus*) as sister to the other *Ficus* species. However, with the exception of the BEAST analysis by Xu et al. (2011), this relationship is supported by parsimony only (Table S5). The difference in likelihood scores between our best ML tree and the trees from analyses constrained to place either subgenus *Sycomorus* or a clade of subgenera (*Sycomorus*, *Sycidium*, *Ficus* and *Synoecia*) sister to the remaining *Ficus* were not significant (Table S2). This confirms that relationships within *Ficus* are unstable along the backbone of the tree and should be regarded as uncertain.

Analyses conducted to assess the impact of missing data on the accuracy of our phylogeny resulted in topologies that were congruent with the topology estimated from the

global dataset (Table S2). It is noteworthy that using only *Ficus* species for which at least three gene regions were available slightly increases node support, but deeper nodes remain unresolved (not shown).

Co-phylogenetic Comparisons

All our analyses rejected the null hypothesis of no correlation between fig and wasp phylogenies. Using distance-based methods, the global test of co-speciation (Parafit) rejected a random association between host and pollinator taxa (ParaFitGlobal = 1.37866, $P \leq 0.01$). Further, 176 of the 200 tests of individual host-associate pairs resulted in significant associations between figs and their agaonid pollinators ($P \leq 0.01$) (Table S6).

In event-based analyses, exact results depend on the weights assigned to different speciation events. Under the classic TreeMap cost-model of zero for co-speciation and one for other events (Charleston and Page, 2002), Jane 2 inferred 198 co-speciation events, 204 duplications, 102 host shifts and 61 losses between fig and wasp phylogenies, accounting for an optimal cost of 367. Whatever the cost model used, the number of co-speciation events inferred by Jane 2 was always significantly greater than expected by chance (Fig. S10).

This topological correlation suggests co-diversification, but does not establish a time line, so we next used independent relaxed molecular-clock dating techniques to test for contemporaneous divergence (Percy et al., 2004). We found strong temporal congruence between both stem and crown mean ages of most partner clades and between the ages of inferred co-speciation events (Fig. 3). Co-diversification test results were not sensistive to the order of deep branching in the phylogenies.

Evolution of Pollination Modes

Parsimony and likelihood reconstruction on the wasp topology both inferred the ancestral pollination mode as ambiguous. Parsimony inferred passive and active pollination as equiprobable ancestral conditions. Similarly, the likelihood difference between the two states was not significant (proportional likelihoods of 0.53 and 0.47, respectively) (Fig. S11). Using the *Ficus* topology, parsimony again inferred active and passive pollination as equibrobable. However, likelihood favours active pollination as the ancestral condition (proportional likelihood of 0.91 versus 0.09 for passive pollination). Overall, the reconstructions reveal that pollination modes are homoplastic with several independent shifts between states (passive / active) along both phylogenies (Fig. S11).

Biogeographic Analyses

Our dating analyses indicate that the current pantropical distribution of the mutualism cannot have resulted simply from vicariance following the break-up of Gondwanaland. Instead, our ancestral area reconstructions suggest that figs and their pollinators arose simultaneously in Eurasia (Mesquite proportional likelihood = 0.72 for figs and 0.97 for wasps, Fig. S12) during the Late Cretaceous about 75 Ma (74.9 Ma for figs and 75.1 Ma for wasps, Figs. 2, S13, and Table 1). Mesquite and Lagrange results were similar, indicating that fig wasps most probably arose in Eurasia. However, Lagrange reconstructions for the fig phylogeny were equivocal due to a basal polytomy. The Eurasian region was proposed as the ancestral area of origin for *Ficus* in one of the alternative reconstructions that fall within two log-likelihood units of the optimal scenario (data not shown, but available upon request).

Although the concordance in means crown ages is striking, the posterior probability density around the mean estimate is quite wide (101.9-60.0 for figs and 94.9-56.2 for wasps, Table 1).

Overall, our analyses favour an Eurasian origin for both *Ficus* and their pollinators. Indeed, in most Eurasian clades, Sino-Himalayan figs and their associated pollinators appear sister to the rest of the species (Fig. 2, grey rhombus). The overall biogeographical signal was similar across the different methods used and showed instances of dispersal resulting in southward range expansion. The major lineages of figs and pollinators split during the Tertiary and it appears that they then spread southwards from Eurasia (Fig. 4), as reflected by the branching order of several clades (Fig. 2, grey arrows). The major lineages subsequently diversified within the Paleotropics and Neotropics during the Miocene.

DISCUSSION

Co-Diversification

Our analyses provide both topological and temporal lines of evidence to indicate that figs and fig wasps may represent the first significant case of long-term (ca. 75 myr) co-diversification in an insect-plant association. The existence of mutualism *per se* appears insufficient for co-diversification, because speciation in other intimate and sophisticated insect pollination mutualisms (e.g. Yuccas and *Yucca* moths, *Glochidion* and *Epicephala* moths) seems to be driven by host shifting and host tracking rather than co-speciation (Smith et al., 2008; Kawakita and Kato, 2009). A plausible explanation for the significant pattern of

co-speciation in the fig-fig wasp mutualism is the unusually strong phenotypic co-adaptation of key traits, such as the specificity of the chemical mediation between partners (Grison-Pige et al., 2002), the lock-and-key shapes of fig ostioles and wasp heads (van Noort and Compton, 1996; Kjellberg et al., 2001).

Despite a history dominated by co-diversification, there are also some clear mismatches between fig and wasp phylogenies (Fig. 2). Our analyses support some ancient host-shifts (e.g. by the pollinators of *Eriosycea*, *Conosycea* and *F. carica*), implying that co-adapted pollinators are sometimes replaced by other wasp species without collapse of the mutualism. Finally, several host shifts occur at shallow nodes, such as between *Ficus* species in the section *Americana* (Fig. S10).

Overall, our tree reconciliation analyses suggest that fig-pollinator history includes numerous species duplications and host shifts, as well as co-speciation events. However, most host shifts are inferred to have occurred between relatively closely-related fig species, consistent with observations of extant wasp species occasionally sharing two closely related fig species (Molbo et al., 2003; Erasmus et al., 2007). If more distant host shifts were common, the congruence of fig and wasp phylogenies would be eroded rapidly, even if co-speciation remained common (Machado et al., 2005; Cook and Segar, 2010). Considering the uncertainty of the sister to all other fig-pollinating wasps, it should be noted that an alternative topology with *Tetrapus* as sister to all other fig-pollinating wasps would mirror the position of *Ficus* section *Pharmacosycea* as sister to all other figs and should therefore increase cophylogenetic signal.

Biological observations and phylogenetic trees show that pollinators of figs are clustered into groups that are consistently associated to *Ficus* sections, subsections, and even to some species groups of figs. These inter- and intra-generic wasp clusters are highly diverged and relatively old and groups of wasps rarely experience shifts to other groups of

figs. Considering only resolved nodes of both phylogenies (Fig 2, white boxes), we observed only four shift events between fig subgenera (*Blastophaga psenes*, *Wiebesia* cf *callida*, three pollinators of *Frutescentiae* (*Wiebesia pumilae*, *W. quadrupes* and *W.* sp. ex *F. oleifolia*) and *Valisia* spp.) and five shift events between fig sections (*Platyscapa bergi* and *P.* sp. (ex *F. glaberrima*) to *Conosycea*, *Ceratosolen vissali* and *Ceratosolen* sp. (ex *F. semivestita*) to *Sycocarpus* and *Adenosperma* respectively, and *K. subulatae* and *K. sessilis* to *Palaeomorphe*). This could be explained by 1) the allopatry of many fig and agaonid groups, 2) the differences between habitats of their host figs (e.g. forest canopy versus savannah), and 3) their host specificity due to intricate co-adaptation of phenotypes, including key traits involved in their reproduction. Consequently, we hypothesize that these figs and associated wasp groups evolve largely independently as closed systems (see also Machado et al., 2005; Cook and Segar, 2010) and rarely exchange genes or pollinator species, a kind of higher level of lineage sorting.

Recent analyses suggest that the stability of fig/pollinator associations can be erratic before complete lineage sorting has occurred, or before ecological/geographical isolation of fig groups (Machado et al., 2005; Jackson et al., 2008; Jousselin et al., 2008; Renoult et al., 2009; Cornille et al., in press). During that period of time, pollinator duplication, extinction and hosts shifts within local groups of related figs sharing similar phenotypic traits may occur frequently. However, these events should not disrupt long-term phylogenetic correlations, if lineages sort over evolutionary time (Cook and Segar, 2010). Future work should also seek to understand the patterns and processes of cospeciation and other processes between closely related figs and wasps such as within fig sections. Previous studies at this level have focused on sections *Americana* (e.g. Machado et al., 2005; Jackson et al., 2008), *Galoglychia* (e.g. Jousselin et al., 2008) and *Sycomorus* s.l. (Weiblen and Bush, 2002) and future studies should

focus on adding also more dioecious and Eurasian clades and to explain why the degree of cospeciation appears to vary between clades.

Wasp and Fig Systematics

Our phylogenetic trees provide several new insights into the systematics of figs and fig wasps and a sound evolutionary framework for future studies in community and behavioural ecology. The question of which groups of wasps and figs are sister to the rest of agaonids and figs respectively remains open. Statistical support for the deeper nodes of the phylogeny is low and precludes us from drawing any definite conclusion. Our additional analyses show that there is little support for *Tetrapus* as sister to all other Agaonidae based on molecular data (see Appendix S3 for details). Instead, it appears that Kradibiinae (*Ceratosolen* + *Kradibia*) or Group 4 (most *Wiebesia* species and pollinators of subsection *Frustescentiae*) are good candidates for the sister taxon to all other agaonids. We raise the possibility that an LBA artifact may have confounded all previous molecular analyses resulting in the inference of *Tetrapus* as sister to all other agaonids (Appendix S3).

One notable difference between this and previous studies concerns taxon sampling, which can be critical in phylogenetic analyses (e.g. Philippe et al., 2005). By including a larger number of species and using sampling that reflects the known diversity of each group, we may counteract potential problems with long branches (e.g. Bergsten, 2005). Increasing taxonomic sampling to break up long branches has been applied repeatedly, often with the conclusion that earlier studies were misled (e.g. Soltis and Soltis, 2004; Stefanović et al., 2004; Leebens-Mack et al., 2005; Phillips et al., 2010; Philippe et al., 2011).

To further investigate *Tetrapus* placement we analysed several morphological characters used in the past to assess the relationships between agaonid genera (Ramirez, 1978;

Wiebes, 1982b; see Appendix S3 and Fig S14 for details). We show that there is no evidence from these morphological characters to support *Tetrapus* as sister to all other agaonids. On the contrary, several independent morphological characters support *Tetrapus* as nested within the family and closely related to *Dolichoris* (Appendix S3).

Further studies using more genes and increased taxonomic sampling of both ingroups and outgroups of figs and wasps are still needed and should contribute to resolving the higher taxonomic group relationships with more confidence and determine the earliest divergence among the figs and their pollinating wasps.

Pollination

A number of authors (including some coauthors of this study) have previously proposed passive pollination as the ancestral mode for the mutualism, followed by a single shift to active pollination and several independant reversions to passive (Machado et al., 2001 ; Jousselin et al., 2003 ; Herre et al., 2008 ; Jandér and Herre, 2010). This hypothesis has intutive appeal as most other insects that pollinate do so passively, but it based primarily on the fact that *Tetrapus* wasps are passive pollinators and appeared as the sister of all other pollinators in previous phylogenetic analyses. Similarly, their host figs (*Pharmacosycea)* appeared as sister to all other figs. However, our new phylogenetic trees supports a different phylogenetic position for *Tetrapus.*

Consequently, the issue of ancestral pollination mode must be revisited. The phylogenetic tree itself is in question, but we also highlight a key issue about the interpretation of a given phylogeny. The previous conclusion that passive pollination is ancestral relies on the assumption that “basal” branches of the trees are more informative about ancestral character states. However, there is no reason to assume that traits found in

Tetrapus/Pharmacosycea are “more primitive” or represent traits of the common ancestors of both sister groups (Krell and Cranston, 2004 ; Crisp and Cook, 2005 ; Lamm and Redelings, 2009). At the present time, the ancestral pollination mode should be considered as equivocal and our analyses imply that it remains so.

Of our four reconstructions, three find the ancestral state equivocal, whereas one (ML on fig phylogeny) favours active pollination. This indicates that further studies are needed to infer the ancestral pollination mode with more confidence. Importantly, these results were established on fully bifurcating trees, but in reality the backbones of both trees are not strongly supported and may change in future studies. Recent advances in our understanding of the morphological evolution of Moraceae, and in particular of an expanded tribe Castilleae, the figs closest relatives, may also shed new light on the evolution of the mutualism (Clement and Weiblen, 2009).

Co-Biogeography

Molecular divergence time estimates point to a Cretaceous origin for the mutualism, but differ with respect to biogeographic scenarios (Tables S3&S5). Previous biogeographic analyses of fig-wasps have argued in favour of Gondwanan vicariance (Machado et al., 2001). However, a previous study by Lopez-Vaamonde et al. (2009) reconstructed ancestral areas of fig-pollinating wasps using a phylogenetic tree with *Tetrapus* as sister to the remainder of the fig-pollinating wasps. These authors concluded that the most recent common ancestor of all extant fig-pollinating wasps was most likely Asian, although a southern Gondwanan origin could not be rejected. A Laurasian origin with subsequent dispersal has been proposed for figs and their nearest relatives (Zerega et al., 2005).

Our analyses indicate that the fig-wasp mutualism was already in existence circa 75 Ma in Eurasia and our independently derived mean date estimates for figs (75.1 ± 19.4 Ma) and wasps (74.9 ± 21.0 Ma) crown groups are remarkably similar, although the size of the confidence intervals introduces a degree of uncertainty. Despite differences in sampling and dating algorithms, the dates obtained correspond well with most previous estimates (Tables S3&S5).

In addition, the hypothesis of an Eurasian origin of the mutualism is supported by several other lines of evidence: 1) the presence in Asia of 70% of the major *Ficus* clades; 2) the early divergence of Sino-Himalayan fig and wasp species in most Eurasian clades (e.g. *F. henryi, F. sarmentosa, F. tikoua, F. nervosa*); 3) the fact that pollinators of the subsection *Frustescentiae* are found only in Continental Asia (Fig. 2); 4) the age estimates for Moraceae in general and Ficeae (Dorstenieae and Castilleae) in particular (Zerega et al., 2005) and 5) the fact that the oldest fig and wasp fossils are known only from the Northern Hemisphere (Collinson, 1989; Compton et al., 2010) (see our review of the literature on fig fossils in Appendix S4.) Finally, Burnham and Graham (1999), analysing the origin of the tropical component in northern Latin American vegetation also suggest that *Ficus* arrived from the north. Accordingly, current data support the conclusion that the mutualism probably originated in the tropical forests of Eurasia (Otto-Bliesner and Upchurch, 1997).

Pharmacosycea and *Tetrapus* divergence is dated to 74.9 - 62.1 Ma (mean stem figs-mean stem age wasp; Table 1), before South America split from Antarctica. However, rather than explaining the South American colonisation of *Pharmacosycea* / *Tetrapus* by trans-antarctic routes, we propose that both lineages might have reached the New World across North Atlantic land bridges (Tiffney, 1985), dispersing through the evergreen woodland and tropical forest belts of Eurasia (Fig. 4). South America may have been colonised later via "stepping-stone" volcanic islands. Indeed, most *Pharmacosycea* species inhabit the Northern

Andes and there are none in Chile and Patagonia, which have vegetation similar to late Cretaceous Antarctica (Poole et al., 2003). Since figs are also absent from the exceptionally good fossil flora of Patagonia (Wilf et al., 2003), trans-Antarctic dispersal seems unlikely but cannot be completely ruled out. Although the Laguna del Hunco flora is not considered a tropical flora, this Patagonian flora hosts one *Papuacedrus* species very closely related to extent tropical Papuan species (Wilf et al., 2009). In Papua New Guinea and in Papua Barat (Indonesia), this conifer is found in the same mountainous habitats as *Malvanthera* fig tree species at altitudes above 2000m (for example in the Arfak mountains, Kebar Valley, Bulolo-Wau, Mt Kerewa). Accordingly, despite not being considered a tropical flora, the Laguna del Hunco flora could have also have included *Ficus* and the fact that *Ficus* appears absent from this exceptionally good flora, supports the later arrival of *Ficus* from Eurasia.

Based on our biogeographic analyses, the major lineages of figs and pollinators split during the Tertiary and spread southwards from Eurasia (Fig. 4), possibly in response to the cooling climate (Davis et al., 2002). Subsequent diversification occurred within continents during the warmer Miocene epoch (Zachos et al., 2001).

The general scenario of fig-wasp co-diversification is illustrated by the charismatic hemi-epiphytic or 'strangler' figs (the subgenus *Urostigma* clade, Fig. 4), which evolved about 52 - 50.3 Ma, during a period of global warming. A first clade, probably living west of the Turgai straits (Akhmetiev and Beniamovski, 2009), dispersed southwards into Africa to form section *Galoglychia* and into South America to form section *Americana*, some 32.3 - 38.2 Ma. Another clade, probably occurring in east Eurasia, spread to India and Sundaland to form section *Conosycea* and to Australasia to form section *Malvanthera*, ca. 50.3 - 43.4 Ma. This latter dispersal was probably via stepping-stones through the Ninety East Ridge (Carpenter et al., 2010), since direct dispersal from Sundaland to Australia was impossible before 25 Ma (Hall, 2002). Today, each tropical continent has its own major endemic

radiation of strangler figs, stemming from these ancient dispersal processes. Interestingly, pollinator biogeography shows a few discrepancies with this scenario for fig dispersal. Indeed, the genus *Pleistodontes* pollinating *Malvanthera* figs is sister to all other *Urostigma* pollinators. Therefore we propose that *Conosycea* was colonised by a host shift of an ancestral *Galoglychia/Americana* pollinator in southern Eurasia before spreading to southern Sunda.

CONCLUSION

Based on multiple lines of evidence (fossils, Moraceae history, branching pattern and ancestral area reconstructions), we infer an Eurasian origin for the fig/pollinator mutualism. We show that the mutualism arose about 75 Ma, confirming previous estimates (Tables S3&5). Since that time, the insects and plants have diversified together leaving a strong long-term signal of phylogenetic congruence, confirming previous studies based on smaller datasets (Tables S3&5). This is not due to strict co-speciation alone, but reflects a history with large amounts of co-speciation and insufficient host shifts to alter the marked phylogenetic matching. This is the only known example of long-term insect/plant co-diversification and we are not aware of other candidates for such a pattern. Other insect/plant pollination mutualisms do not appear to be characterized by phylogenetic congruence, and we propose that strong co-diversification of figs and their pollinators is driven by their unusually high level of phenotypic trait matching. Figs and their pollinators have spread across the globe to occupy all tropical continents, where they play important ecological roles in forests and savannahs. Their numerous interactions with other species, such as vertebrate frugivores, mean that the evolution of entire tropical ecosystems has been influenced strongly by this unique strong pattern of co-diversification between fig trees and their pollinating insects.

SUPPLEMENTARY MATERIAL

Supplementary material, including data files and/or online-only appendices, can be found in the Dryad data repository at http://datadryad.org, doi: 10.5061/dryad.hr620. Matrices are also available in TreeBASE (No. xxxxxx) at http://www.treebase.org/.

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AUTHORS CONTRIBUTION

JYR and VS designed the research. AsC, BhC, WC, JMC, RDH, EJ, CK, FK, MHM, SvN, RASP, PYQ, JYR, RU, CLV, GDW and DRY provided material or data. SvN, RU and JYR identified the fig wasps. FK, JYR, GDW and NR identified the figs. GG, RJZ and AC performed and coordinated fig wasp DNA sequencing. NR and WC performed and coordinated fig DNA sequencing. AsC, NR and JYR performed the analyses. JYR examined fig wasp morphological characters and reviewed the literature about fig fossils. RLH, AY, TS, JP and BC implemented statistical tests and performed analyses with Jane2. AsC, NR, JYR, JMC, GDW and VS wrote the manuscript, with major comments from RH, CK and FK. All authors commented on the manuscript.

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Figure Captions

FIGURE 1. Classification and worldwide distribution of *Ficus*. The numbers of species per subgenus is represented as a proportion of total *Ficus* species richness. Breeding systems are indicated as either monoecious (M) or dioecious (D) and modes of pollination are indicated as passive (P) or active (A).

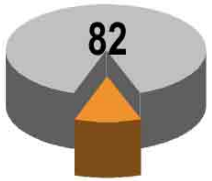
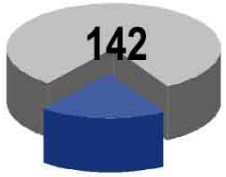
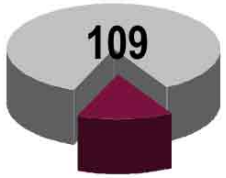
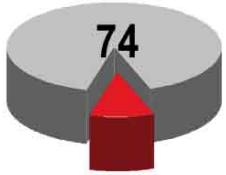
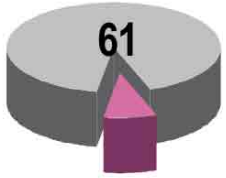
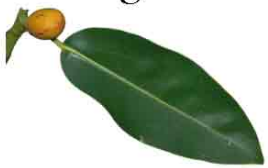
* *Agaon*, *Alfonsiella*, *Allotriozoon*, *Courtella*, *Elisabethiella*, *Nigeriella* and *Paragaon*

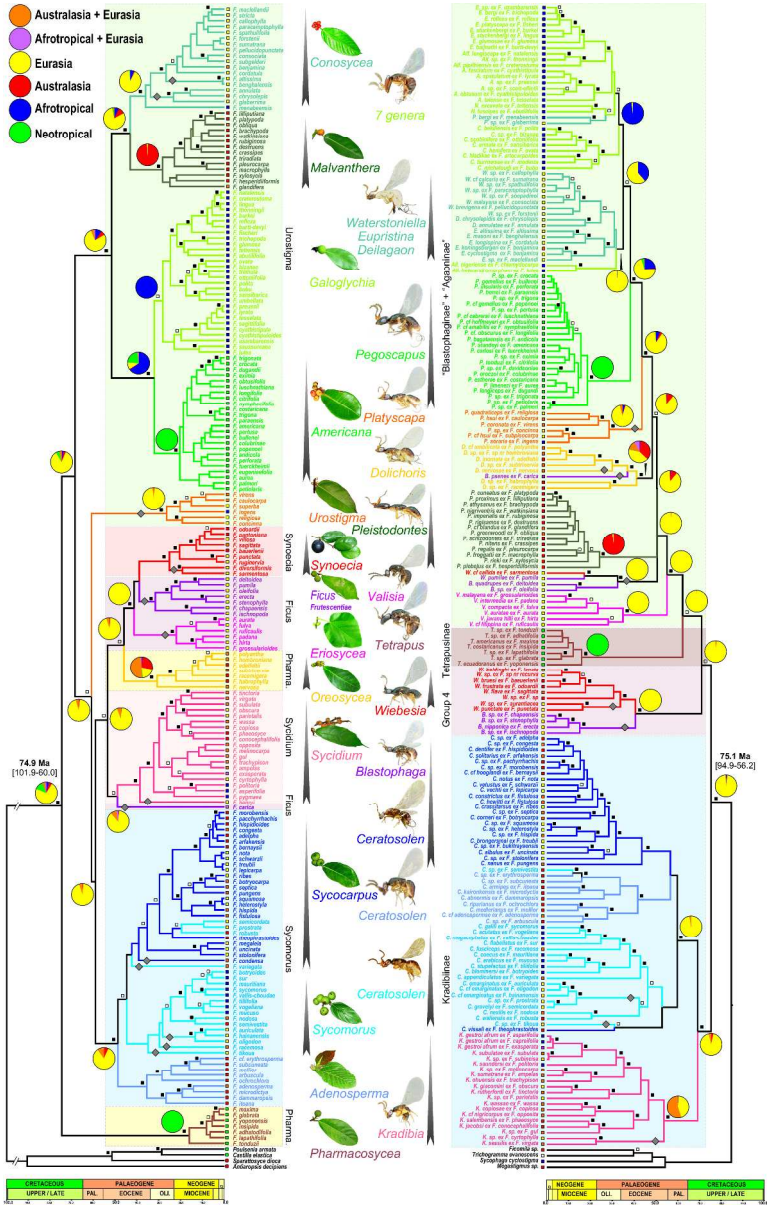
** *Deilagaon* and *Waterstoniella*

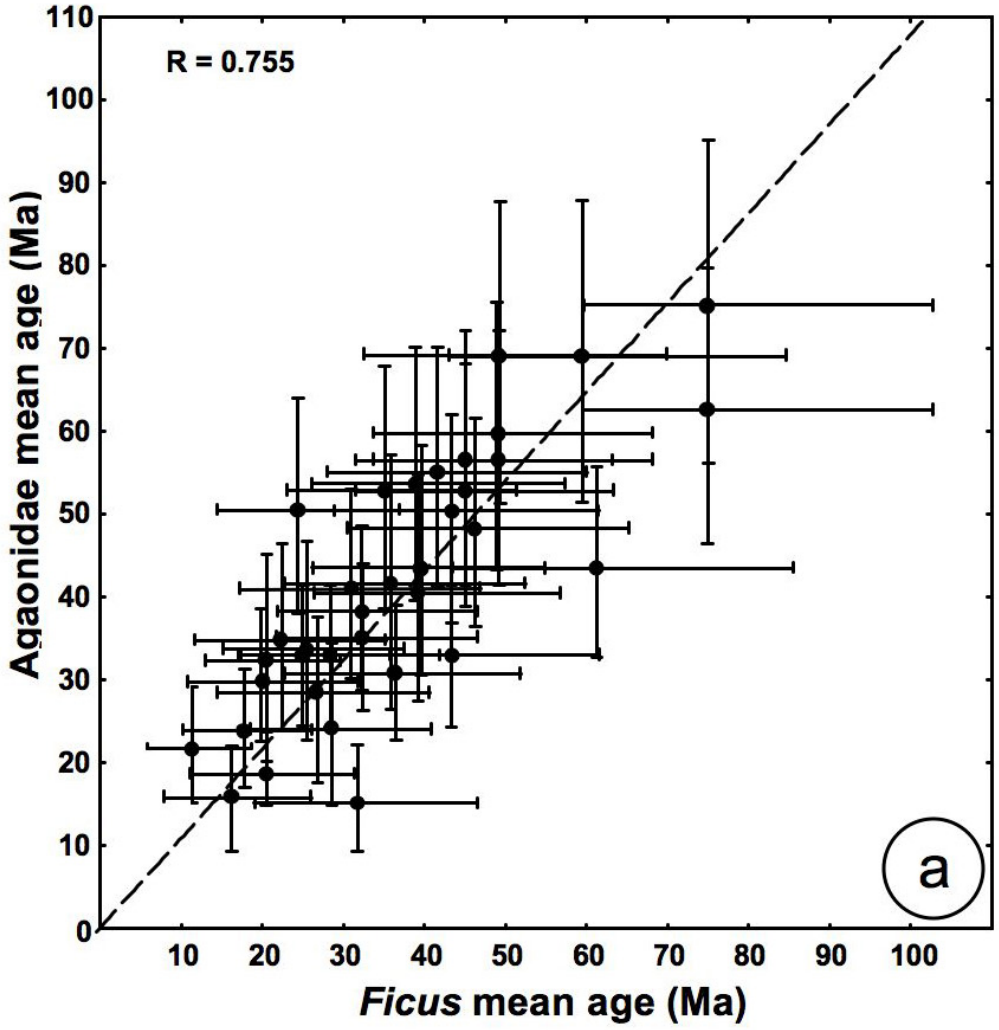
FIGURE 2. BEAST chronograms of the evolutionary history of figs and fig wasps. Groups of figs and their associated genera of pollinators are represented using the same colour. *Ficus* subgenera and Agaonidae subfamilies according to current classifications are delimited by colored rectangles (Pharma. for *Ficus* subgenus *Pharmacosycea*). Pie charts at main nodes show the likelihood of different geographic areas of origin as inferred by Mesquite (see Methods). Grey rhombuses show clades of fig species from Continental Asia, while grey arrows indicate hypothesised southward migration of clades. Squares correspond to node supports: Black square: BP > 70% and $PP_{MrBayes}$ or PP_{BEAST} > 0.95; white square: BP > 70% or $PP_{MrBayes}$ or PP_{BEAST} > 0.95.

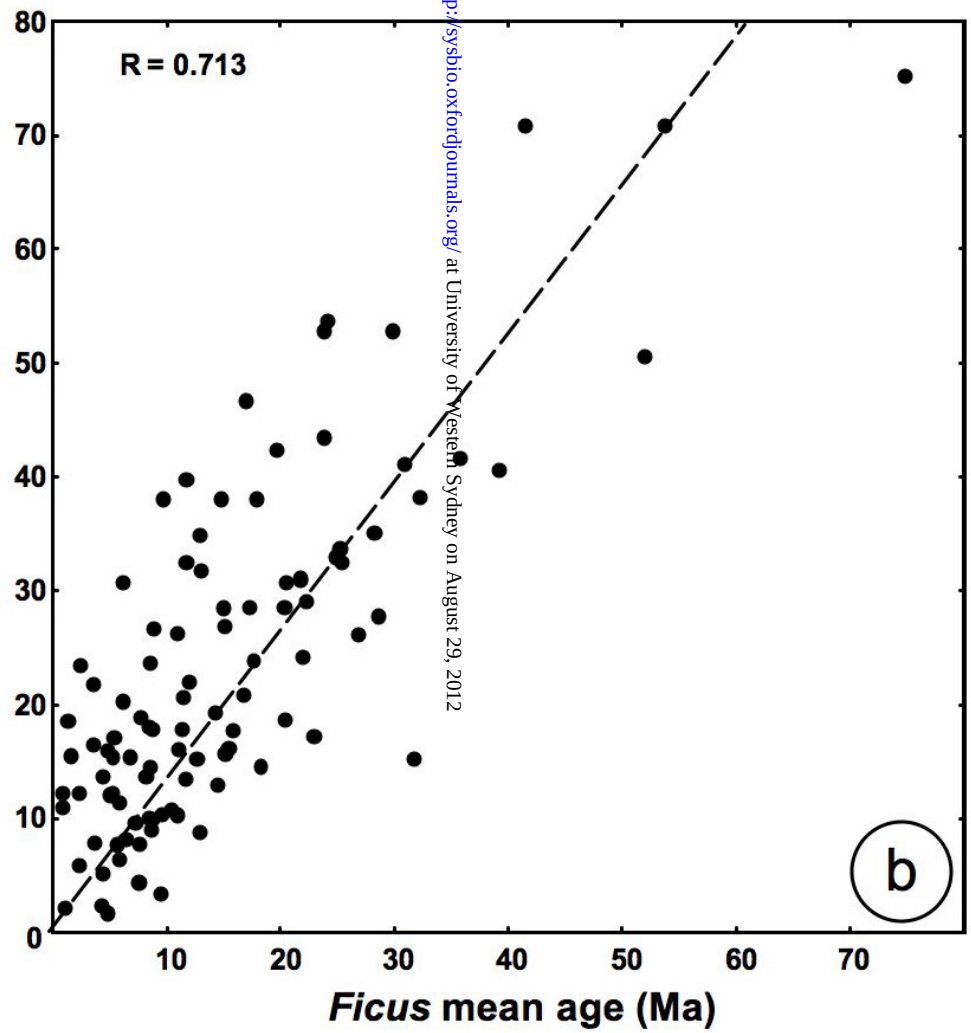
FIGURE 3. Temporal evidence for fig and fig wasp co-divergence. a) Correlation between stem and crown mean ages of major fig and wasp groups (with 95% HPD). b) Temporal congruence of the 198 co-speciation events inferred by Jane 2.

FIGURE 4. Hypothetical biogeographical scenario of mutualism diversification. Scenarios are presented on four different maps for clarity. Solid arrows: figs; dashed arrows: wasps. Nodes with BP < 70% or PP < 0.95 are collapsed.

Subgenera	Diversity	Section	Oecy	Distribution	Pollinator	Mode
Pharmacosycea 		*Pharmacosycea* *Oreosycea*	M M		*Tetrapus* *Dolichoris*	P A
Sycomorus 		*Sycocarpus* *Sycomorus* *Adenosperma*	D MD D		*Ceratosolen*	A
Sycidium 		*Sycidium* *Palaeomorphe*	D D		*Kradibia*	A
Synoecia 		*Kissosycea* *Rhizocladus*	D D		*Wiebesia*	AP
Ficus 		*Ficus* *Eriosycea*	D D		*Blastophaga* *Valisia*	P A
Urostigma 		*Americana* *Galoglychia* *Malvanthera* *Urostigma* *Conosycea*	M M M M M		*Pegoscapus* 7 genera* *Pleistodontes* *Platyscapa* *Eupristina* & 2 genera**	AP A AP AP AP P







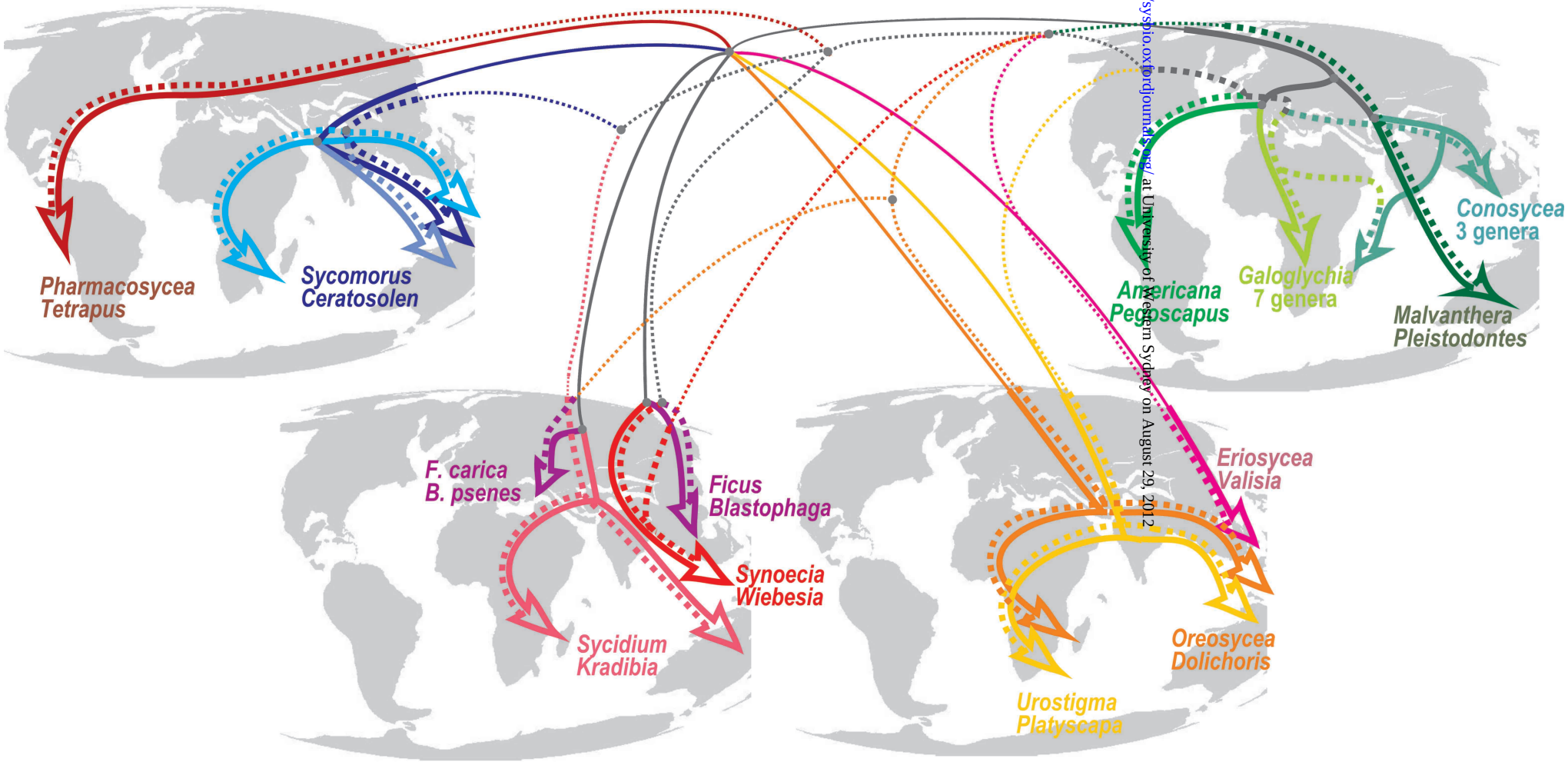


TABLE 1. Comparison of mean age estimates (Ma) for selected nodes in the fig and wasp phylogenies.

Nodes	*Ficus* Mean age Ma (95% HPD)	Agaonidae Mean age Ma (95% HPD)
Crown *Ficus* / Crown Agaonidae	74.9 (101.9-60.0)	75.1 (94.9-56.2)
Crown *Pharmacosycea* / *Tetrapus**	16.2 (25.7-8.2)	15.9 (22.0-9.3)
Stem *Pharmacosycea* / *Tetrapus**	74.9 (101.9-60.0)	62.1 (79.0-45.2)
Crown *Sycomorus* / *Ceratosolen*	49.1 (67.4-34.0)	59.7 (75.4-43.3)
Stem *Sycomorus* / *Ceratosolen*	59.4 (83.9-43.5)	69.0 (87.6-51.5)
Crown sect. *Adenospermae* / sg. *Strepitus*	35.8 (51.9-23.1)	41.6 (57.1-26.7)
Crown sect. *Sycocarpus* / sg. *Rothropus*	39.5 (54.3-26.5)	43.4 (58.2-30.6)
Crown sect. *Sycomorus* / sg. *Ceratosolen*	35.1 (50.7-23.3)	52.7 (67.8-38.8)
Stem sect. *Adenospermae* / sg. *Strepitus*	49.1 (67.4-34.0)	56.5 (72.0-41.5)
Stem sect. *Sycocarpus* / sg. *Rothropus*	45.0 (62.8-31.9)	56.5 (72.0-41.5)
Stem sect. *Sycomorus* / sg. *Ceratosolen*	45.0 (62.8-31.9)	52.7 (67.8-38.8)
Crown *Sycidium* / *Kradibia*	38.9 (56.6-26.3)	53.6 (70.0-39.7)
Stem *Sycidium* / *Kradibia*	49.2 (69.3-33.0)	69.0 (87.6-51.5)
Crown *Synoecia* / *Wiebesia*	25.4 (37.0-15.3)	33.6 (46.4-22.7)
Stem *Synoecia* / *Wiebesia*	39.2 (56.1-26.7)	40.5 (54.7-27.6)
Crown *Frutescentiae* / *Blastophaga*	31.8 (46.0-19.4)	15.1 (22.0-9.3)
Crown *Eriosycea* / *Valisia*	22.2 (34.8-11.9)	34.8 (46.5-24.2)
Stem *Eriosycea* / *Valisia*	41.6 (59.5-28.4)	55.0 (70.0-41.4)
Crown "*F. pumila* group" / *Wiebesia***	20.4 (30.9-11.3)	32.4 (45.3-20.4)
Stem "*F. pumila* group" / *Wiebesia***	24.4 (36.4-14.6)	50.5 (63.9-38.1)
Crown *Malvanthera* / *Pleistodontes*	28.5 (41.7-17.8)	24.1 (34.4-14.9)
Stem *Malvanthera* / *Pleistodontes*	43.4 (61.0-29.4)	50.3 (62.0-37.1)
Crown *Oreosycea* / *Dolichoris*	31.0 (46.4-17.6)	41.0 (52.8-30.2)
Stem *Oreosycea* / *Dolichoris*	46.2 (64.6-31.0)	48.2 (61.5-36.5)
Crown *Urostigma* / *Platyscapa*	26.6 (40.2-14.8)	28.5 (37.6-17.9)

Stem *Urostigma* / *Platyscapa*	61.2 (84.7-43.9)	43.5 (55.5-32.7)
Crown *Americana* / *Pegoscapus**	20.5 (29.3-13.1)	18.6 (23.8-15.0)
Stem *Americana* / *Pegoscapus**	32.3 (46.1-22.1)	38.2 (48.5-28.8)
Crown *Galoglychia* / Afrotropical pollinators***	28.3 (40.3-18.6)	32.9 (41.4-24.5)
Stem *Galoglychia* / Afrotropical pollinators***	32.3 (46.1-22.1)	35.0 (44.0-26.4)
Crown *Conosycea* / (*Deilagaon*, *Eupristina*, *Waterstoniella*)	36.3 (51.3-23.1)	30.8 (39.1-22.9)
Stem *Conosycea* / (*Deilagaon*, *Eupristina*, *Waterstoniella*)	43.4 (61.0-29.4)	32.9 (41.4-24.5)
Crown *Cyathistipulae* / *Agaon*	11.3 (18.3-5.8)	21.6 (28.8-15.0)
Stem *Cyathistipulae* / *Agaon*	20.0 (31.4-11.0)	29.8 (38.5-22.7)
Crown *Caulocarpae* / *Courtella*	17.7 (25.8-10.4)	23.8 (31.0-16.9)
Stem *Caulocarpae* / *Courtella*	24.9 (35.3-17.2)	32.9 (41.4-24.5)

95% lower and upper highest posterior distribution inferred by BEAST is reported between parentheses. *Crown-group *Pegoscapus* and *Tetrapus* were assigned uniform prior distributions with minimum ages of 15 Ma and maximum ages of 60 Ma based on Dominican amber fossils. **"*F. pumila* group" refers to the clade including *F. pumila*, *F. oleifolia* and *F. deltoidea.* ****Allotriozoon* excepted.