Phylogenetic relationships, historical biogeography and character evolution of fig-pollinating wasps

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Nucleotide sequences from the cytochrome oxidase I (COI) gene were used to reconstruct phylogenetic relationships among 15 genera of fig-pollinating wasps. We present evidence supporting broad-level coalescence with respect to most but not all of the corresponding groups of figs. Using fossil evidence for calibrating a molecular clock for these data, we estimated the origin of the fig–wasp mutualism to have occurred ca. 90 million years ago. The estimated divergence times among the pollinator genera and their current geographical distributions corresponded well with several features of the break-up of the southern continents during the Late Cretaceous period. We then explored the evolutionary trajectories of two characteristics that hold profound consequences for both partners in the mutualism: the breeding system of the host (monoeocious or dioecious) and pollination behaviour of the wasp (passive or active). The fig–wasp mutualism exhibits extraordinarily long-term evolutionary stability despite clearly identifiable conflicts of interest between the interactors, which are reflected by the very distinct variations found on the basic mutualistic theme.

Keywords: fig wasp; pollination; biogeography; coevolution; Gondwana; mutualism

1. INTRODUCTION

The interaction between figs (Ficus: Moraceae) and fig-pollinating wasps (Agaonidae, Chalcidoidea) represents perhaps the most specialized case of obligate pollination mutualism known (Corner 1952, 1988; Ramirez 1970; Janzen 1979; Wiebes 1979; Herre et al. 1996; Herre 1999). Host fig species are generally pollinated by species-specific pollinator wasp species. With ca. 750 described fig species showing a pantropical distribution and a variety of growth habits, both fig and wasp species are remarkably diverse. Moreover, even within this diversity, multiple variants on the basic themes of the interaction exist. This variation profoundly affects the nature of the costs and benefits that each member derives from the mutualism.

Approximately half of all Ficus species are functionally monoecious with individual inflorescences performing both female (seed production and dispersal) and male (pollen production and dispersal) functions. In these systems, mated, pollen-bearing, female fig wasps (foundresses) enter the enclosed fig inflorescences (syconia), pollinate the uniovulate flowers inside, lay eggs in some of them and die. Their offspring develop by consuming the contents of one potential seed each, emerge later and mate. The female offspring then gather pollen from male flowers within the syconia and fly off in order to attempt to find a receptive fig tree and begin the cycle anew (Corner 1952, 1988; Galil & Eisikowitch 1968; Ramirez 1970; Herre 1989, 1999). The remaining Ficus species are gynodioecious, but functionally dioecious. In these species, some individuals produce only seed-bearing fruit and are functionally female, while others produce only pollen and pollen-carrying wasp progeny and are functionally male (Janzen 1979; Wiebes 1979; Kjellberg et al. 1987; Patel & Hossaert-McKey 2000).

The different breeding systems impose profoundly different reproductive consequences on both the host fig and the pollinator wasp. In the monoecious case, individual female foundresses fertilize the flowers using the pollen from their natal tree, thereby realizing male fitness for their own natal fig. Yet they then reproduce at the cost of some of those potential seeds, inflicting costs in both natal and receptive trees (Herre 1989, 1999; West & Herre 1994; Herre & West 1997). In the dioecious case, sexual functions in the trees are separated. Here, if the foundresses enter a ‘female’ inflorescence, they realize fitness for their natal tree by pollinating flowers that will develop seeds, but do not reproduce themselves. Alternatively, if individual foundresses enter a ‘male’ inflorescence, they are able to reproduce themselves, yet will produce no seeds with the pollen of their natal tree (Wiebes 1979; Kjellberg et al. 1987; Grafen & Godfray 1991; Anstett et al. 1997; Patel & Hossaert-McKey 2000).

Furthermore, both active and passive pollination occur across different species of wasps. These different pollination syndromes are associated with distinctive morphological adaptations in both the wasp and the fig. In species with active pollination, the wasps possess specialized structures for carrying pollen in the external part of the thorax and the front legs (Ramirez 1969) and show distinctive behaviours for collecting and depositing pollen (Frank 1984). The male flowers in actively pollinated figs are relatively small and less numerous (Galil & Meiri 1981). In contrast, wasps that passively pollinate their hosts lack or present a significant reduction in the size of...
the specialized structures found in active pollinators and the wasps show no pollination behaviour (Galil & Neeman 1977). Passively pollinated figs have relatively higher ratios of anthers to female flowers, produce much more pollen per syconium than actively pollinated figs and their mature anthers tend to deliquesce naturally, thereby facilitating the passive 'collection' of pollen by their pollinators (pollen adheres to various parts of the body surface) (Ramírez 1969; Galil & Eistikowitch 1971; Galil & Neeman 1977, Galil & Meiri 1981; Ramírez & Malavasi 1997).

The observation that related species of figs generally pollinate related species of figs has led to the proposal of strict-sense coevolution between the two groups (Ramírez 1974; Wiebes 1979, 1982; Berg & Wiebes 1992). However, existing classifications of figs and their pollinators are based on morphological characters that are often intimately involved in the interactions between the two mutualists (e.g. the breeding system of the figs and the characters involved in the pollination behaviour of the wasps). Therefore, the apparent congruence observed in their current classifications might simply reflect reciprocal adaptations leading to convergent evolution (e.g. Van Noort & Compton 1996). Fortunately, molecular data can provide independent characters for reconstructing phylogenies and rigorously testing evolutionary hypotheses concerning figs and their pollinators. For example, molecular studies of figs and wasps have been conducted at both fine (between species within a pollinator genus and their associated hosts) and broad (across genera of figs and their hosts) taxonomic scales and the data appear consistent with strict-sense coevolution (Herre et al. 1996). However, the sampling of taxa in those studies was limited and support for many of the proposed relationships was weak. Recent studies have increased the number of taxa sampled, but have focused on dioecious figs and their pollinators (Weihlen 1999, 2000, 2001).

In this paper, we present a more extensive study of the phylogenetic relationships among fig-pollinating wasps using nucleotide sequences from the cytochrome oxidase I (COI) mitochondrial gene. First, we use the reconstructed phylogeny in describing the evolution of the major groups of pollinating wasps and then discuss the phylogeny with respect to the classification of the corresponding groups of figs. Second, we use fossil data for calibrating a molecular clock and estimating the times of divergence of the pollinator genera and then use those times and the geographical distribution of Ficus in discussing the historical biogeography of the mutualism. Finally, we use the wasp phylogeny for studying the evolutionary transitions of the two important characteristics of the fig-pollinator interaction: the breeding system of the host (monoecious or dioecious) and pollination behaviour of the wasp (passive or active).

2. MATERIAL AND METHODS

(a) Fig and fig wasp taxonomy

The genus Ficus has generally been considered as comprising four subgenera (Pharmacosycea, Urostigma, Sycomorus and Ficus), which are further subdivided into numerous sections (Corner 1963, 1985; Berg 1989). Recently, Berg & Wiebes (1992) divided the subgenus Ficus into two subgenera (Ficus and Sycidium) and incorporated three sections of Ficus (Syrocappus, Neomphle and Adenosperma) into the subgenus Sycomorus. Here we follow their classification with the modification that the palaeotropical section Oreosyce is incorporated into the subgenus Urostigma as suggested by molecular evidence (Herre et al. 1996).

There are 20 recognized genera of fig-pollinating wasps, all belonging to the family Agaonidae sensu Rasplus (Rasplus et al. 1998) within the superfamily Chalcidoidea (Wiebes 1982, 1994; Boucek 1988; Berg & Wiebes 1992). With the exception of wasps from the genera Ceratosolen, Platyscapa and Wieshea, each genus is restricted to a single subgenus and section of fig.

(b) DNA methods

Genomic DNA was extracted from 32 individual wasps representing 15 out of the 20 genera of pollinating wasps (table 1) using Chelex 100 (Walsh et al. 1991). The five genera not included in this study (Agaon, Allotriazon, Derilagaon, Nigeriella and Paragaon) are all associated with African figs of the monoecious subgenus Urostigma. Sequences of 816 nucleotides were collected from the 3'-end of the mitochondrial COI gene (positions 2191–3007 of the Drosophila yakuba mitochondrial genome) (Clary & Wolstenholme 1985) using conserved insect polymerase chain reaction primers and standard manual and automated sequencing protocols (Simon et al. 1994; Machado et al. 1996; Machado 1998). Sequences have been deposited in GenBank (accession numbers AF302052–AF302056 and AY014964–AY014995).

(c) Phylogenetic analyses

All analyses were carried out with version 4.0b1 of PAUP* (Swofford 1998). Four species from two different subfamilies of non-pollinating fig wasps (Sycophaginae, Sycoctinae) were used as outgroups (table 1). Phylogenies were reconstructed using the maximum-likelihood (ML) optimality criterion. The most appropriate nucleotide substitution model for explaining the process of nucleotide substitution in the data was chosen by comparing three models that consider unequal base composition: HKY85 (Hasegawa et al. 1985), TN93 (Tamura & Nei 1993) and REV (Yang 1994). A likelihood ratio test (Goldman 1993) showed that the general reversible model with rate heterogeneity (REV + Γ) (Yang 1994a,b) was the most appropriate model for analysing the data. Five replications of the heuristic search algorithm with branch swapping (tree bisection–reconnection) were used for finding the ML tree. Alternative topologies representing different hypotheses of fig wasp relationships were compared using the Kishino–Hasegawa test (Kishino & Hasegawa 1989).

(d) Molecular clock calibrations

The hypothesis of rate constancy among taxa was tested by comparing the likelihoods of the data given the ML tree topology under the REV + Γ model with and without the constraint of a molecular clock using a likelihood ratio test (Felsenstein 1988). If the hypothesis of rate constancy was rejected, the two-cluster and branch length tests were applied using the ML tree topology and the complete data set as input to the programs from the package Lintre (Takezaki et al. 1995). The taxa identified by the tests as evolving at significantly different rates from the rest were eliminated from the data set and pruned from the ML tree. The likelihood ratio test was applied again to the smaller data set and pruned topology; if the test was significant, the two-cluster and branch length tests were applied again. The process was repeated until the hypothesis of
rate constancy was not rejected by the likelihood ratio test. Times of divergence were then estimated by multiplying the ML branch lengths estimated under the constraint of a molecular clock by the ratio of divergence time to branch length of a reference node for which fossil data were available. Confidence intervals for the estimated times of divergence were defined as plus or minus twice the standard error of the branch length multiplied by the rate of substitution. The branch lengths and their standard errors were obtained using the program PAML, v. 2.0a (Yang 1997).

Fossil wasps unambiguously identified as species of the neotropical genus *Pegoscapus* occur in Dominican Republic amber (Poinar 1993; Wiebes 1995). Therefore, the genus is at least 20 million years (Myr) old (Iturralde-Vinent & Macphee 1996). The molecular clock was then calibrated by using 15 extant *Pegoscapus* species for estimating the genetic distance to the deepest node in the phylogeny (Machado 1998), which was assumed to correspond to the origin of the genus. However, any substitution rate that estimates the deepest node of *Pegoscapus* at 20 Myr or more could appear consistent with the fossil evidence. Alternatively, it is possible that the most common recent ancestor of the extant species of *Pegoscapus* is younger than the fossil wasp. Therefore, we compared the implications of our rate estimates with both fossil and molecular evidence from other groups of insects.

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**Table 1. List of fig-pollinating wasps sampled in this study**

(n/a, not available.)

<table>
<thead>
<tr>
<th>pollinator species</th>
<th>Ficus host</th>
<th>locality</th>
<th>host subgenus</th>
<th>host section</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alfonsoellalongiscopa</em></td>
<td><em>Ficus thoningii</em></td>
<td>Harare Botanical Gardens, Zimbabwe</td>
<td><em>Urostigma</em></td>
<td><em>Galoglychia</em></td>
</tr>
<tr>
<td><em>Blatophaga (B) nipponica</em></td>
<td><em>Ficus erecta</em></td>
<td>Japan</td>
<td><em>Ficus</em></td>
<td><em>Ficus</em></td>
</tr>
<tr>
<td><em>Ceratosolen (C) arabicus</em></td>
<td><em>Ficus sycomorus</em></td>
<td>Harare Botanical Gardens, Zimbabwe</td>
<td><em>Sycomorus</em></td>
<td><em>Sycomorus</em></td>
</tr>
<tr>
<td><em>Ceratosolen (C) arabicus</em> sp. 2</td>
<td><em>Ficus sycomorus</em></td>
<td>Mpalal Ranch, Kenya</td>
<td><em>Sycomorus</em></td>
<td><em>Sycomorus</em></td>
</tr>
<tr>
<td><em>Ceratosolen (C) bisulcatus</em></td>
<td><em>Ficus septica</em></td>
<td>Lana Grant Quezon, Philippines</td>
<td><em>Sycomorus</em></td>
<td><em>Sycomopus</em></td>
</tr>
<tr>
<td><em>Ceratosolen (C) cophensis</em></td>
<td><em>Ficus sur</em></td>
<td>Cape Town Gardens, South Africa</td>
<td><em>Sycomorus</em></td>
<td><em>Sycomorus</em></td>
</tr>
<tr>
<td><em>Ceratosolen (C) constrictus</em></td>
<td><em>Ficus fistulosa</em></td>
<td>Rakata, Indonesia</td>
<td><em>Sycomorus</em></td>
<td><em>Sycomopus</em></td>
</tr>
<tr>
<td><em>Ceratosolen (C) gigli</em></td>
<td><em>Ficus sycomorus</em></td>
<td>Gonorazehoua, Zimbabwe</td>
<td><em>Sycomorus</em></td>
<td><em>Sycomorus</em></td>
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<td><em>Ceratosolen (R) notus</em></td>
<td><em>Ficus nota</em></td>
<td>UPLB campus, Philippines</td>
<td><em>Sycomorus</em></td>
<td><em>Sycomopus</em></td>
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<tr>
<td><em>Ceratosolen (R) pilipes</em></td>
<td><em>Ficus cericarpa</em></td>
<td>Lambir Hills National Park, Sarawak, Malaysia</td>
<td><em>Sycomorus</em></td>
<td><em>Sycomopus</em></td>
</tr>
<tr>
<td><em>Ceratosolen (C) solans</em></td>
<td><em>Ficus hiophila</em></td>
<td>Rakata, Indonesia</td>
<td><em>Sycomorus</em></td>
<td><em>Sycomopus</em></td>
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<tr>
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<td><em>Ficus schwarzii</em></td>
<td>Lambir Hills National Park, Sarawak, Malaysia</td>
<td><em>Sycomorus</em></td>
<td><em>Sycomopus</em></td>
</tr>
<tr>
<td><em>Courtella armata</em></td>
<td><em>Ficus sansbarica</em></td>
<td>Harare Botanical Gardens, Zimbabwe</td>
<td><em>Urostigma</em></td>
<td><em>Galoglychia</em></td>
</tr>
<tr>
<td><em>Courtella bekkelinsisi</em></td>
<td><em>Ficus polita</em></td>
<td>n/a</td>
<td><em>Urostigma</em></td>
<td><em>Galoglychia</em></td>
</tr>
<tr>
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<td><em>Ficus polita</em></td>
<td>n/a</td>
<td><em>Urostigma</em></td>
<td><em>Oreoxycea</em></td>
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<td><em>Ficus burt-davysi</em></td>
<td>South Africa</td>
<td><em>Urostigma</em></td>
<td><em>Galoglychia</em></td>
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<td><em>Urostigma</em></td>
<td><em>Galoglychia</em></td>
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<td><em>Ficus microcapta</em></td>
<td>Tunez</td>
<td><em>Urostigma</em></td>
<td><em>Conosycea</em></td>
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<td><em>Ficus capreolata</em></td>
<td>Gonorazehoua, Zimbabwe</td>
<td><em>Sycomorus</em></td>
<td><em>Sycomopus</em></td>
</tr>
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<td><em>Lapoharalaum tentacularis</em></td>
<td><em>Ficus montana</em></td>
<td>Rakata, Indonesia</td>
<td><em>Sycomorus</em></td>
<td><em>Sycomopus</em></td>
</tr>
<tr>
<td><em>Pegoscapus hoffmeyeri</em></td>
<td><em>Ficus obtusifolia</em></td>
<td>Barro Colorado Island, Panama</td>
<td><em>Urostigma</em></td>
<td><em>Americana</em></td>
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<tr>
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<td><em>Ficus obtusifolia</em></td>
<td>Barro Colorado Island, Panama</td>
<td><em>Urostigma</em></td>
<td><em>Americana</em></td>
</tr>
<tr>
<td><em>Pegoscapus lopezi</em></td>
<td><em>Ficus near trigonata</em></td>
<td>Barro Colorado Island, Panama</td>
<td><em>Urostigma</em></td>
<td><em>Americana</em></td>
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<tr>
<td><em>Platysycea soraria</em></td>
<td><em>Ficus ingens</em></td>
<td>Grahamstown, South Africa</td>
<td><em>Urostigma</em></td>
<td><em>Urostigma</em></td>
</tr>
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<td><em>Pleistodontes fougatti</em></td>
<td><em>Ficus rubiginosa</em></td>
<td>Australia</td>
<td><em>Urostigma</em></td>
<td><em>Malacantha</em></td>
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<td>Australia</td>
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<td><em>Malacantha</em></td>
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<tr>
<td><em>Tetrapus americanus</em></td>
<td><em>Ficus maxima</em></td>
<td>Barro Colorado Island, Panama</td>
<td><em>Pharmacosycea</em></td>
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<td><em>Tetrapus costaricanus</em></td>
<td><em>Ficus insipida</em></td>
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<td><em>Urostigma</em></td>
<td><em>Conosycea</em></td>
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<td><em>Waterstoniella sp.</em></td>
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<td><em>Ficus pumila</em></td>
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<td><em>Ficus</em></td>
<td><em>Rhzoeclados</em></td>
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<td><em>Ficus</em></td>
<td><em>Kalousyce</em></td>
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</table>

<table>
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<td><em>Ficus glutosa</em></td>
<td>Tanzania</td>
<td><em>Urostigma</em></td>
<td><em>Galoglychia</em></td>
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<td><em>Seres solaezienensis</em></td>
<td><em>Ficus sansbarica</em></td>
<td>Tanzania</td>
<td><em>Urostigma</em></td>
<td><em>Galoglychia</em></td>
</tr>
<tr>
<td><em>Cristagaster sp. 9A</em></td>
<td><em>Ficus maxima</em></td>
<td>Barro Colorado Island, Panama</td>
<td><em>Pharmacosycea</em></td>
<td><em>Pharmacosycea</em></td>
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<tr>
<td><em>Cristagaster sp. 10A</em></td>
<td><em>Ficus insipida</em></td>
<td>Barro Colorado Island, Panama</td>
<td><em>Pharmacosycea</em></td>
<td><em>Pharmacosycea</em></td>
</tr>
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* Collected in a light trap.
A second fossil fig-pollinating wasp from the Oligocene period, 34.5 Myr ago (from Florissant, CO, USA) (Brues 1910), seems to correspond to another modern group of New World pollinators (*Tetrapus*), suggesting that several of the modern genera are quite old. Further, several lines of evidence have suggested that the origin of the superfamily Chalcidoidea occurred in the Late Jurassic period, 144–163 Myr ago (Yoshimoto 1975; Roskam 1992) and that the Diptera–Hymenoptera divergence occurred at least 300 Myr ago (Carpenter & Burnham 1983; Labandeira & Sepkoski 1993). Following results from previous molecular studies (Machado et al. 1996; Machado 1998; Rasplus et al. 1998), we rooted the agaonids at the origin of Chalcidoidea. We then used different *Drosophila* sequences as the outgroup to the chalcids. The estimated substitution rate calibrated from dating the origin of *Pegasopus* at 28 Myr or more agrees with the proposed dates for the origin of Chalcidoidea (145 Myr), although it probably underestimates the Diptera–Hymenoptera split (246 Myr) (C. A. Machado and others, unpublished data). This calibration suggests a transversional substitution rate for the COI and COII genes of 0.21 and 0.22% Tt Myr⁻¹, which falls within the range of estimated rates for *Drosophila* (Beckenbach et al. 1993). Further, this calibration suggests a silent substitution rate of 1.9 × 10⁻⁸ per site per year for the COI gene of fig-pollinating wasps, which is similar to the silent substitution rate of the COI gene in *Drosophila* (2.0–2.9 × 10⁻⁸).

### (e) Character evolution

The breeding system of the host plants (monoecious or dioecious) was obtained from the literature (Corner 1965; Berg 1989). Pollination behaviour was obtained from the literature (Wiebesia 1994) and by observation of pollen pockets, coxal combs of and direct observation of the behaviour (E. Jousselin, F. Kjellberg and E. A. Herre, unpublished data). Passive pollination occurs in all known species of the genera *Tetrapus*, *Waterstoniella* and *Delagamon*. Pollination behaviour is polymorphic among species in *Pleistodontes*, *Blastophaga*, *Platyscapa* (several active and passive species), *Ceratosolen* (one non-pollinating ‘parasitic’ species, *Ceratosolen galili*) and *Wiebesia* (passive, with at least one species active). According to existing data the remaining genera are exclusively active. For the special case of *C. galili*, the pollination behaviour was coded as ‘absent’ because that species does not pollinate its host fig (Galil & Eisikowitch 1969; Compton et al. 1991). Characters were mapped on the ML phylogeny using the program MacClade, v. 3.0 (Maddison & Maddison 1992).

### 3. RESULTS

#### (a) Phylogenetic analyses

The ML phylogeny that was reconstructed with the REV + Γ model (−log(ℓ) = 10789.36331 and α = 0.755) is shown in figure 1. The neighbour-joining (NJ) tree has an almost identical topology (not shown). *Tetrapus* appears as the basal group of pollinators, although its position is weakly supported. However, the basal position of *Tetrapus* is independently well supported by a long A + T-rich insertion at the 3’-end of the COI gene, which is absent in all non-pollinating wasps and *Tetragastris*, but present in the rest of the pollinator genera (Herre et al. 1996; Machado 1998). The pollinators of the subgenera *Sycidium*, *Ficus* and *Sycomorus* appear in a basal position but with weak support (not shown) in the maximum-parsimony (MP) tree (length = 2374 and consistency index (CI) = 0.336). The ML and MP topologies are significantly different under the ML criterion (Δ = 43.43576 and p = 0.02) but not under the MP criterion (Δ(length) = 32, t = 1.8820 and p = 0.06).

The pollinators of the pantropical, monocious subgenus *Urostigma* form a well-supported monophyletic group. The Australasian genus *Pleistodontes* is well supported as the basal genus and the neotropical genus *Pegasopus* appears as the second group to branch within this clade. In addition to the analyses of the COI gene sequences, these two genera lack a two-amino-acid insertion in the COII gene that is only found in the rest of the *Urostigma* pollinators (Machado 1998). The relationships among the remaining genera that pollinate *Urostigma* are not well resolved, consistent with a rapid radiation.

Although their relationships with the other groups of wasps are poorly resolved, the pollinators of the dioecious subgenus *Ficus* (*Wiebesia* and *Blastophaga*) appear to branch just above the base of the pollinator phylogeny. The pollinators of the subgenus *Sycidium* (*Krattibia* and *Liporhopalum*) appear to be derived from the pollinators of the African subgenus *Sycomorus* (*Ceratosolen*) (figure 1). The alternative placement of *Krattibia* and *Liporhopalum* as sister taxa to *Ceratosolen* is not rejected by the data under the ML criterion (Δ = 7.106 91 and p = 0.61) or MP criteria (Δ(length) = 19, t = 1.3418 and p = 0.18). However, the hypothesis that the *Sycidium* pollinators are more closely related to the *Ficus* pollinators is nearly rejected under the ML criterion (Δ = 5.086 88 and p = 0.08) and is rejected under the MP criterion (Δ(length) = 32, t = 2.4192 and p = 0.01). Furthermore, additional data from the COII gene (Machado 1998) and two other recent molecular studies (Yokohama 1995; Weiblen 1999, 2001) have suggested that the *Ficus* pollinators are more closely related to the pollinators of *Urostigma* than to the pollinators of *Sycomorus* or *Sycidium*.

Using the currently recognized relationships between the figs (Berg & Wiebes 1992), the pollinators and their host figs show a general pattern of co-cladogenesis at a coarse taxonomic level (figure 1). However, while figs in the subgenus *Sycidium* appear to be sister taxa to those in the subgenus *Ficus* (Corner 1965; Berg & Wiebes 1992; Weiblen 2000; E. Jousselin, unpublished data), their pollinators do not appear to be sister taxa (figure 1). These results suggest a breakdown in strict-sense cospeciation at this broad taxonomic level.

#### (b) Times of divergence among pollinator genera

The molecular clock hypothesis was rejected for the complete data set (2Δ = log(ℓ) = 325.3643, p < 0.001 and d.f. = 34). A relative rate test for two lineages with multiple taxa (Li & Bousquet 1992) showed that the sequences from all the pollinators of the subgenera *Sycidium* and *Sycomorus* and from the *Ficus* pollinator *Wiebesia pumilae* have evolved significantly faster than the sequences from the rest of the pollinators (Σ(2) = 9.292 and p < 0.001). After removing the fast-evolving lineage of pollinators from the data set, the hypothesis of rate constancy was still rejected (2Δ = log(ℓ) = 75.7974, p < 0.001 and d.f. = 21). The branch length test identified sequences from six taxa as having different substitution
Figure 1. Phylogeny of fig-pollinating wasps (Agaonidae) showing the fig host associations on the right. The ML tree is shown. Numbers above branches are the bootstrap values (> 50%) (500 replications) for the same nodes of the NJ tree reconstructed using transversion distances only. The two samples of C. arabicus collected in Zimbabwe and Kenya (table 1) are quite different at the sequence level (6.1%) despite no clear morphological differences (S. van Noort, personal communication). The large sequence divergence between them suggests that they represent two different species.
4. DISCUSSION

(a) **Pattern of co-cladogenesis in the fig-pollinator mutualism**

This study corroborates others in showing that fig pollinators are monophyletic and that the fig-wasp mutualism was established once (Machado et al. 1996; Machado 1998; Rasplus et al. 1998). The passively pollinating genus *Tetrapus* appears as the most ancient pollinator genus and is associated with the monoecious neotropical subgenus *Pharmacosycea*. Both morphological and molecular data support *Pharmacosycea* as being the most primitive of the existing figs (Berg & Wiebes 1992; Herre et al. 1996; Weiblen 2000). Therefore, the primitive conditions in the mutualism appear to be a monoecious breeding system in the figs and passive pollination in the pollinators, which agrees with the preponderance of monoecy and wind pollination in the Urticales (Berg 1990a,b; Sakai et al. 2000). The first divergence event leading to *Tetrapus* was then followed by a less-well-resolved branching between the pollinators of the four remaining subgenera (*Urostigma*, *Sycomorus*, *Sycidium* and *Ficus*).

Consistent with previous studies, co-cladogenesis predominates at the scale of wasp genera and associated fig subgenera and sections. However, co-cladogenesis does not appear to be the case for the wasps that pollinate the figs in the subgenera *Ficus* and *Sycidium*. While both morphological (Berg 1989; Berg & Wiebes 1992) and molecular (Herre et al. 1996; Weiblen 2000; E. Jousselin, unpublished data) studies support the status of *Ficus* and *Sycidium* as sister groups, previous studies (Machado 1998; Weiblen 1999, 2001) and the data presented here suggest that their respective pollinators are not, with the pollinators of *Ficus* being more closely related to the pollinators of *Urostigma*. As figs from the subgenera *Ficus* and *Sycidium* are also supported to be more closely related to *Sycomorus*, the most likely scenario is that the ancestors of *Ficus* figs were colonized by ancestors of wasps currently associated with *Urostigma* figs and this new combination then jointly diversified.

In order to assess the evidence for cospeciation properly, it is important to consider that several cases of successful colonization of figs by 'wrong wasps' have been documented (Ramirez 1970; Michaloud et al. 1983; Compton 1990; Berg & Wiebes 1992; Ware & Compton 1992; Wiebes 1994). Furthermore, we have direct genetic evidence of both pollinator species in the New World genus *Pegoscapus* successfully reproducing in novel hosts.
and of genetic introgression in the host figs that is the likely result of such colonizations (E. A. Herre and C. A. Machado, unpublished data). In cases in which introgression has occurred, it will often be necessary to analyse the phylogenetic patterns based on several different loci in the figs, both nuclear and cytoplasmically inherited, in order to detect and describe it. Therefore, although co-cladogenesis appears to be the predominant pattern, a more complete understanding of the relative importance of co-cladogenesis and colonization events both between and within genera of wasps and their associated figs will emerge as robust phylogenies of a wider sample of figs and wasps become available.

(b) Historical biogeography of the mutualism

The current distributions of the pollinating wasp genera suggest a Gondwanan (Southern Hemisphere) origin of the mutualism (Corner 1958; Murray 1985). Our estimated times of divergence not only corroborate this view, but also show a very good match between the current estimates for the timing of the break-up of the different continental land masses and the dates of origin of the wasp groups that currently inhabit them. Reconstructions of Gondwana’s fragmentation during the Late Cretaceous period (Barron 1987; Hay et al. 1999) suggest that, ca. 100 Myr ago, Africa had already separated from South America, becoming a relatively isolated oceanic island. The land mass composed of South America, Antarctica, Australia and India–Madagascar remained joined for most of the Late Cretaceous period, with India linked to Antarctica through the Kerguelen Plateau until at least 80 Myr ago (Hay et al. 1999). The fig–wasp mutualism was established during this period. The ancestor of neotropical *Pharmacosycea* figs and *Tetrapus* wasps probably arose at the origin of the fig–wasp mutualism ca. 90 Myr ago in the South America–Antarctica–Australia–India–Madagascar continental block. Approximately 80 Myr ago or less India and Madagascar finally separated from the rest of the southern continents. This event coincides with the timing of the next radiation of groups of pollinating wasps associated with the subgenera *Urostigma*, *Sycidium*, *Ficus* and *Sycomorus* (81.7 ± 11.7 Myr). The ancestors of the pollinators of the current palaeotropical subgenera *Ficus*, *Sycomorus* and *Sycidium* probably dispersed to the rest of the Old World either by drifting with the Indian subcontinent and/or by colonizing Africa. Such a scenario is consistent with palaeontological data (Sahni 1984; Briggs 1987) and with palaeogeographical reconstructions of the position of the Indian subcontinent during the Late Cretaceous period (Chatterjee & Hotton 1986; Chatterjee 1992; Hay et al. 1999).

South America, Antarctica and Australia remained linked until the Palaeocene or Eocene period, when Australia–New Guinea separated from Antarctica (Audley-Charles et al. 1981; Barron 1987). This event corresponds to the split of the lineage leading to the Australasian genus *Pleistodontes* (64.4 ± 9.8 Myr). Later in the Eocene period, South America detached from Antarctica (Audley-Charles et al. 1981; Barron 1987), which corresponds to the split of the lineage leading to the neotropical genus *Pegasopus* (53.1 ± 9.0 Myr). The remaining *Urostigma* pollinators radiated during the Eocene and Oligocene periods (48.3 ± 8.2 Myr), dispersing to the rest of the palaeotropics.
The increased rates of substitution in the pollinators of *Ficus*, *Sycomorus* and *Sycidium* limit our ability to make more detailed inferences about their biogeographical history. Further tests of the relative likelihood of different biogeographical scenarios will depend on appropriate sampling and calibration of the relevant wasp lineages. For example, estimates of the divergence time between the lineages of *Ceratosolen* wasps pollinating figs from the almost exclusively African section *Sycomorus* and those that occur almost exclusively in Asia (section *Sycocarpus*) are expected to correspond to the separation of Madagascar from India.

**Conflicts of interest and the evolution of the fig--wasp mutualism**

The breeding system of the fig affects the form of the conflict between sexual functions within the fig and the form of the conflict of interest between the fig and the wasp, thereby holding a series of important reproductive consequences for both mutualists (Kjellberg *et al*. 1987; Herre 1989, 1999; Grafen & Godfray 1991; Anstett *et al*. 1997). In monoecious figs, all wasps that enter any fig syconium can potentially reproduce. Furthermore, in all cases they can potentially induce seed production by fertilizing the receptive flowers with pollen from their natal tree. However, in dioecious figs, the form of the relationship and its consequent rewards and risks to each partner are much different. Wasps entering female figs will not reproduce, although they cause the production of seeds with the pollen from their natal fig, while wasps that enter male figs reproduce themselves, but produce no seeds.

Moreover, in monoecious figs, studies of the factors that affect seed (female function) and wasp production (male function) have shown that, in many cases, one sexual function trades off against the other, strongly suggesting inefficiencies for a single fig to engage in both functions simultaneously (Herre 1989, 1999). In particular, in cases of high host fig density and foundress number, the specialization in sexual function that is characteristic of dioecy may be favoured by those conflicts (Herre 1989; Anstett *et al*. 1997). Therefore, many aspects of dioecy appear favourable for the fig, while a monoecious breeding system generally appears more favourable for the wasp. The reversals between breeding systems suggest different resolutions to the ongoing tensions generated within the mutualism.

Those tensions are also revealed by the multiple changes in mode of pollination. Pollination is the central service that the wasp provides to the fig and the stability of the mutualism depends in part on this service. Some authors have suggested that increased pollination capacity gives a direct positive benefit to the pollinator, either by reducing larval mortalities (Galil & Eiskowitz 1971) or by incrementing larval nutrition (Verkerke 1989). A recent study employing careful statistical analyses has shown that pollinators have the highest reproductive success in the fruits that show the highest seed production (Herre & West 1997). To the extent to which these increases in seed production are linked to increased pollination, the wasp’s reproductive success is tied to its capacity to pollinate (Herre 1999). Furthermore, fig species that are actively pollinated show relatively lower investment in the production of pollen than passively pollinated species (Galil & Neeman 1977; Galil & Meiri 1981). Therefore, the active pollination syndrome appears to be beneficial for both mutualists and the single inferred change from passive to active pollination was probably favoured by selection in both partners.

However, multiple reversals from active to passive pollination have occurred during the history of the mutualism. For the fig, the loss of pollination behaviour in the wasp does not cause a total loss of pollination and in some cases fig wasps are able to develop even in the absence of pollination (Galil & Eiskowitz 1971; Compton *et al*. 1991; Jousselin & Kjellberg 2001). Together, these observations imply that selection for wasps to pollinate actively can be relaxed and that there are costs associated with being an active pollinator.

In contrast to cases in which the mutualism is preserved despite the loss of active pollination, the case of *Ceratosolen galili* represents an unequivocal transition from active pollination to parasitism. *C. galili* is associated with *Ficus sycomorus*, a fig that is normally pollinated by *Ceratosolen arabicus*, an active pollinator. Although *C. galili* has pollen pockets, suggesting an origin as an active pollinator, it has lost the behaviour associated with active pollination (Galil & Eiskowitz 1968; Compton *et al*. 1991). Our phylogeny supports previous studies and clearly indicates that the parasitic *C. galili* is not the closest relative of the mutualistic *C. arabicus* (Galil & Eiskowitz 1968, 1969; Compton *et al*. 1991; Herre *et al*. 1996; Kerdelhue *et al*. 1999). This suggests that *C. galili* (or its ancestor) successfully colonized *F. sycomorus* and became a parasitic ‘cuckoo’. The ‘colonization by a non-sister taxa’ scenario mirrors the patterns observed among mutualistic and parasitic lineages of yucca moths (Pellmyr *et al*. 1996). These known cases of breakdown in a pollination mutualism support theoretical predictions that colonization events are the most likely scenario for establishing parasitic species within otherwise mutualistic lineages (Pellmyr *et al*. 1996; Herre 1999).

Our estimates suggest that the origin of the fig--wasp mutualism substantially pre-dates the earliest fossils of *Ficus*, which are known from Early Eocene period deposits (50 Myr) (Collinson 1989). Similarly, molecular estimates suggest that the actual age of the yucca--yucca moth mutualism (40 Myr) substantially pre-dates the fossil evidence of the host plant (14 Myr) (Pellmyr & Leebens-Mack 1999). The longevity of these obligate plant--insect associations emphasizes the fact that mutualisms, even those with clear conflicts of interest, can nonetheless be evolutionarily stable over vast expanses of time (Anstett *et al*. 1997; Herre & West 1997; Herre *et al*. 1999).

In the case of figs and fig wasps, those conflicts appear to have led to fairly distinct outcomes that hold a series of advantages and disadvantages for each partner. The observed shifts in the breeding system of the fig, the changes in mode of pollination and the breakdown of the mutualism (C. galili) illustrate the ongoing conflicts of interest and their diverse resolutions over a remarkable span of evolutionary time.

Stuart A. West, Koos Wiebes, William Ramirez, James Cook, I-Fang Sun, Rhett Harrison and Simon van Noort kindly provided some of the wasp samples used for this study.
comments of S. A. West, T. Parrish, T. P. Young, C. C. Labandeira, S. L. Wing, R. Lande and E. Leigh greatly improved the manuscript. We dedicate this work to the memory of J. T. Wiches who was instrumental in shaping our current understanding of fig wasps and an inspiration to us all. This work was partially supported by the Scholarly Studies Program of the Smithsonian Institute and by a Howard Hughes Medical Institute predoctoral fellowship to CA.M.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.