

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 cladogenesis 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 (monoecious 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 pan-tropical 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

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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 dehisce naturally, thereby facilitating the passive 'collection' of pollen by their pollinators (pollen adheres to various parts of the body surface) (Ramirez 1969; Galil & Eisikowitch 1971; Galil & Neeman 1977; Galil & Meiri 1981; Ramirez & Malavasi 1997).

The observation that related species of wasps generally pollinate related species of figs has led to the proposal of strict-sense coevolution between the two groups (Ramirez 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 wasps 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 (Weiblen 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 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 1965, 1985; Berg 1989). Recently, Berg & Wiebes (1992) divided the subgenus *Ficus* into two subgenera (*Ficus* and *Sycidium*) and

incorporated three sections of *Ficus* (*Sycocarpus*, *Neomorphe* and *Adenosperma*) into the subgenus *Sycomorus*. Here we follow their classification with the modification that the palaeotropical section *Oreosycea* 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 *Wiebesia*, 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*, *Allotriozoon*, *Deilagaon*, *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, Sycoryctinae) 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 1994a). 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

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

(n/a, not available.)

pollinator species	<i>Ficus</i> host	locality	host subgenus	host section
<i>Alfonsiella longiscapa</i>	<i>Ficus thonningi</i>	Harare Botanical Gardens, Zimbabwe	<i>Urostigma</i>	<i>Galoglychia</i>
<i>Blastophaga (B) nipponica</i>	<i>Ficus erecta</i>	Japan	<i>Ficus</i>	<i>Ficus</i>
<i>Ceratosolen (C) arabicus</i>	<i>Ficus sycomorus</i>	Harare Botanical Gardens, Zimbabwe	<i>Sycomorus</i>	<i>Sycomorus</i>
<i>Ceratosolen (C) arabicus</i> sp. 2	<i>Ficus sycomorus</i>	Mpala Ranch, Kenya	<i>Sycomorus</i>	<i>Sycomorus</i>
<i>Ceratosolen (C) bisulcatus</i>	<i>Ficus septica</i>	Lana Grant Quezon, Philippines	<i>Sycomorus</i>	<i>Sycocarpus</i>
<i>Ceratosolen (C) capensis</i>	<i>Ficus sur</i>	Cape Town Gardens, South Africa	<i>Sycomorus</i>	<i>Sycomorus</i>
<i>Ceratosolen (C) constrictus</i>	<i>Ficus fistulosa</i>	Rakata, Indonesia	<i>Sycomorus</i>	<i>Sycocarpus</i>
<i>Ceratosolen (C) galili</i>	<i>Ficus sycomorus</i>	Gonarazdhoua, Zimbabwe	<i>Sycomorus</i>	<i>Sycomorus</i>
<i>Ceratosolen (R) notus</i>	<i>Ficus nota</i>	UPLB campus, Philippines	<i>Sycomorus</i>	<i>Sycocarpus</i>
<i>Ceratosolen (R) pilipes</i>	<i>Ficus cereicarpa</i>	Lambir Hills National Park, Sarawak, Malaysia	<i>Sycomorus</i>	<i>Sycocarpus</i>
<i>Ceratosolen (C) solmsi</i>	<i>Ficus hispida</i>	Rakata, Indonesia	<i>Sycomorus</i>	<i>Sycocarpus</i>
<i>Ceratosolen (R) vetustus</i>	<i>Ficus schwarzi</i>	Lambir Hills National Park, Sarawak, Malaysia	<i>Sycomorus</i>	<i>Sycocarpus</i>
<i>Courtella armata</i>	<i>Ficus sansibarica</i>	Harare Botanical Gardens, Zimbabwe	<i>Urostigma</i>	<i>Galoglychia</i>
<i>Courtella bekiliensis</i>	<i>Ficus polita</i>	n/a	<i>Urostigma</i>	<i>Galoglychia</i>
<i>Dolichoris</i> sp.	n/a ^a	Danum Valley Field Center, Sabah, Malaysia	<i>Urostigma</i>	<i>Oreosycea</i>
<i>Elisabethiella bajinathi</i>	<i>Ficus burtt-davyi</i>	South Africa	<i>Urostigma</i>	<i>Galoglychia</i>
<i>Elisabethiella glumosa</i>	<i>Ficus glumosa</i>	Tanzania	<i>Urostigma</i>	<i>Galoglychia</i>
<i>Eupristina verticillata</i>	<i>Ficus microcarpa</i>	Tunex	<i>Urostigma</i>	<i>Conosycea</i>
<i>Kradibia gestroi</i>	<i>Ficus capreifolia</i>	Gonarazdhoua, Zimbabwe	<i>Sycidium</i>	<i>Sycidium</i>
<i>Liporrhopalum tentacularis</i>	<i>Ficus montana</i>	Rakata, Indonesia	<i>Sycidium</i>	<i>Sycidium</i>
<i>Pegoscapus hoffmeyerii</i>	<i>Ficus obtusifolia</i>	Barro Colorado Island, Panama	<i>Urostigma</i>	<i>Americana</i>
<i>Pegoscapus gemellus</i>	<i>Ficus popenoi</i>	Barro Colorado Island, Panama	<i>Urostigma</i>	<i>Americana</i>
<i>Pegoscapus lopesi</i>	<i>Ficus near trigonata</i>	Barro Colorado Island, Panama	<i>Urostigma</i>	<i>Americana</i>
<i>Platyscapa soraria</i>	<i>Ficus ingens</i>	Grahamstown, South Africa	<i>Urostigma</i>	<i>Urostigma</i>
<i>Pleistodontes froggatti</i>	<i>Ficus rubiginosa</i>	Australia	<i>Urostigma</i>	<i>Malvanthera</i>
<i>Pleistodontes imperialis</i>	<i>Ficus rubiginosa</i>	Australia	<i>Urostigma</i>	<i>Malvanthera</i>
<i>Tetrapus americanus</i>	<i>Ficus maxima</i>	Barro Colorado Island, Panama	<i>Pharmacosycea</i>	<i>Pharmacosycea</i>
<i>Tetrapus costaricanus</i>	<i>Ficus insipida</i>	Barro Colorado Island, Panama	<i>Pharmacosycea</i>	<i>Pharmacosycea</i>
<i>Tetrapus</i> sp.	<i>Ficus glabrata</i>	Barro Colorado Island, Panama	<i>Pharmacosycea</i>	<i>Pharmacosycea</i>
<i>Waterstoniella</i> sp.	n/a ^a	Danum Valley Field Center, Sabah, Malaysia	<i>Urostigma</i>	<i>Conosycea</i>
<i>Wiebesia pumilae</i>	<i>Ficus pumila</i>	Kenting Uplifted Coral Reef National Park, Taiwan	<i>Ficus</i>	<i>Rhizocladus</i>
<i>Wiebesia punctata</i>	<i>Ficus punctata</i>	Danum Valley Field Center, Sabah, Malaysia	<i>Ficus</i>	<i>Kalosycea</i>
outgroups				
<i>Philocaenus warei</i>	<i>Ficus glumosa</i>	Tanzania	<i>Urostigma</i>	<i>Galoglychia</i>
<i>Seres solweziensis</i>	<i>Ficus sansibarica</i>	Tanzania	<i>Urostigma</i>	<i>Galoglychia</i>
<i>Critogaster</i> sp. 9A	<i>Ficus maxima</i>	Barro Colorado Island, Panama	<i>Pharmacosycea</i>	<i>Pharmacosycea</i>
<i>Critogaster</i> sp. 10A	<i>Ficus insipida</i>	Barro Colorado Island, Panama	<i>Pharmacosycea</i>	<i>Pharmacosycea</i>

^a Collected in a light trap.

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 & Macphée 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.

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 1985; 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 *Pegoscapus* 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 E. A. Herre, unpublished data). This calibration suggests a transversal substitution rate for the COI and COII genes of 0.21 and 0.22% Tv 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* ($2.0\text{--}2.9 \times 10^{-8}$).

(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 (Wiebes 1994) and by observation of pollen pockets, coxal combs or/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 *Deilagaon*. 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 ($-\log(L) = 10\,789.36351$ and $\alpha = 0.755$) 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 *Tetrapus*, but present in the rest of the pollinator genera (Herre *et al.* 1996; Machado 1998). The pollinators of the subgenera *Sycidium*, *Ficus* and *Sycomor* 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 ($\Delta - \log(L) = 43.43576$ and $p = 0.02$) but not under the MP criterion ($\Delta(\text{length}) = 32$, $t = 1.8820$ and $p = 0.06$).

The pollinators of the pantropical, monoecious subgenus *Urostigma* form a well-supported monophyletic group. The Australasian genus *Pleistodontes* is well supported as the basal genus and the neotropical genus *Pegoscapus* 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* (*Kradibia* and *Liporrhopalum*) appear to be derived from the pollinators of the African subgenus *Sycomor* (*Ceratosolen*) (figure 1). The alternative placement of *Kradibia* and *Liporrhopalum* as sister taxa to *Ceratosolen* is not rejected by the data under the ML ($\Delta - \log(L) = 7.10691$ and $p = 0.61$) or MP criteria ($\Delta(\text{length}) = 19$, $t = 1.3148$ 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 ($\Delta - \log(L) = 28.05688$ and $p = 0.08$) and is rejected under the MP criterion ($\Delta(\text{length}) = 32$, $t = 2.4192$ 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 *Sycomor* 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 ($2\Delta - \log(L) = 325.3643$, $p \ll 0.001$ 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 *Sycomor* and from the *Ficus* pollinator *Wiebesia pumilae* have evolved significantly faster than the sequences from the rest of the pollinators ($\zeta = 9.292$ and $p \ll 0.001$). After removing the fast-evolving lineage of pollinators from the data set, the hypothesis of rate constancy was still rejected ($2\Delta - \log(L) = 75.7974$, $p \ll 0.001$ and d.f. = 21). The branch length test identified sequences from six taxa as having different substitution

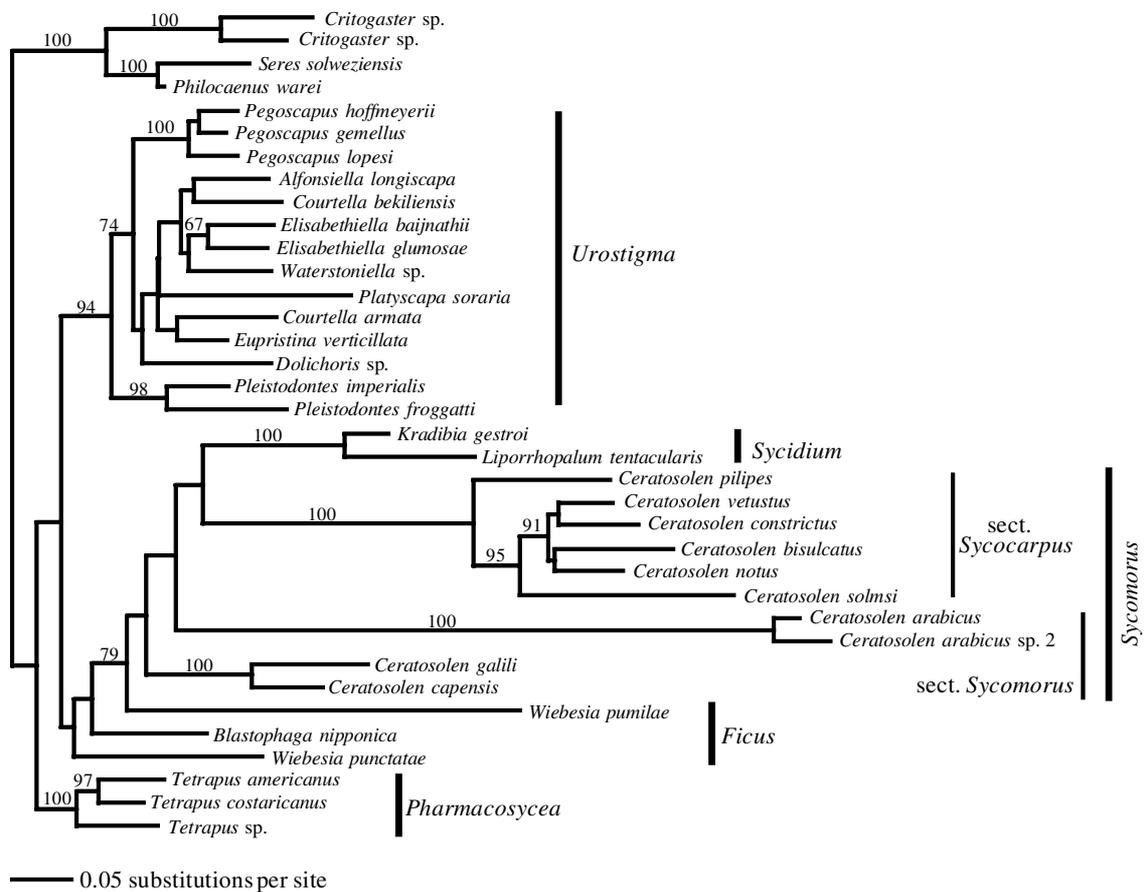


Figure 1. Phylogeny of fig-pollinating wasps (Agaonidae) showing the fig host associations on the right. The ML tree is shown. Numbers above branches are the bootstrap values ($> 50\%$) (500 replications) for the same nodes of the NJ tree reconstructed using transversion distances only. The two samples 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.

rates from the average: *Philoaenus warei*, *Dolichoris* sp., *Eupristina verticillata*, *Platyscapha soraria*, *Tetrapus costaricanus* and *Pegoscapus lopesi*. After pruning those six sequences, the hypothesis of rate constancy was not rejected ($2\Delta - \log(L) = 21.80114$, $p = 0.11$ and d.f. = 15). Based on a basal date for *Pegoscapus* of 28 Myr, the gene copies of *Pegoscapus hoffmeyerii* and *Pegoscapus gemellus* shared a common ancestor 21 ± 6.5 Myr ago. The age of that node was then used to estimate the times of divergence among pollinating genera (figure 2). The estimated dates suggest that the mutualism is very old and arose during the Late Cretaceous period (87.5 ± 12.8 Myr), pre-dating all the available fossil evidence for *Ficus* by over 30 Myr.

(c) Character evolution

Figure 3a shows the reconstruction of the host breeding system in the pollinator phylogeny. The figure suggests that monoecy was the primitive state and that dioecy arose twice: first in the ancestor of the *Ficus*–*Sycidium*–*Sycomorus* clade and then in the ancestor of *Sycidium* and the section *Sycocarpus* after the reversal to monoecy in the ancestor of the section *Sycomorus*. Two additional reversals to monoecy within the section *Sycocarpus* appear likely, as neither the two monoecious species in this section (*Ficus pritchardii* and *Ficus microdictya*) (Corner 1970) nor their pollinators (Wiebes 1994) appear to be closely related. Although these reconstructions assume that relationships

between host figs mirror those of their pollinators at this coarse scale, the possible breakdown of cospeciation between the wasps and the hosts of the subgenera *Sycidium* and *Ficus* does not affect these inferences.

More conservative conclusions are reached if the host breeding system is reconstructed in a tree with the alternative placement of *Kradibia* and *Liporrhopalum* as sister taxa to *Ceratosolen* ($\Delta - \log(L) = 7.10691$ and $p = 0.61$). That reconstruction suggests a single origin of dioecy in the ancestor of the *Ficus*–*Sycidium*–*Sycomorus* clade and the same three reversals to monoecy in the subgenus *Sycomorus*.

Figure 3b shows the most parsimonious reconstruction of the evolution of pollination behaviour. The reconstruction suggests that passive pollination was the ancestral state and that active pollination arose once in the ancestor of all non-*Tetrapus* pollinators. Reversals to passive pollination seem to have occurred independently in at least five different lineages of pollinators (*Waterstoniella*, *Pleistodontes*, *Blastophaga*, *Platyscapha* and *Wiebesia*). Passive pollinators from those lineages show vestigial or reduced pollen pockets (E. Joussetin and F. Kjellberg, unpublished data), suggesting that the inference of multiple transitions from active to passive pollination is correct. In addition, there has been one transition from active to no pollination in *C. galili*, which effectively represents a transformation of the mutualism into parasitism (Galil & Eisikowitch 1968, 1969; Compton *et al.* 1991).

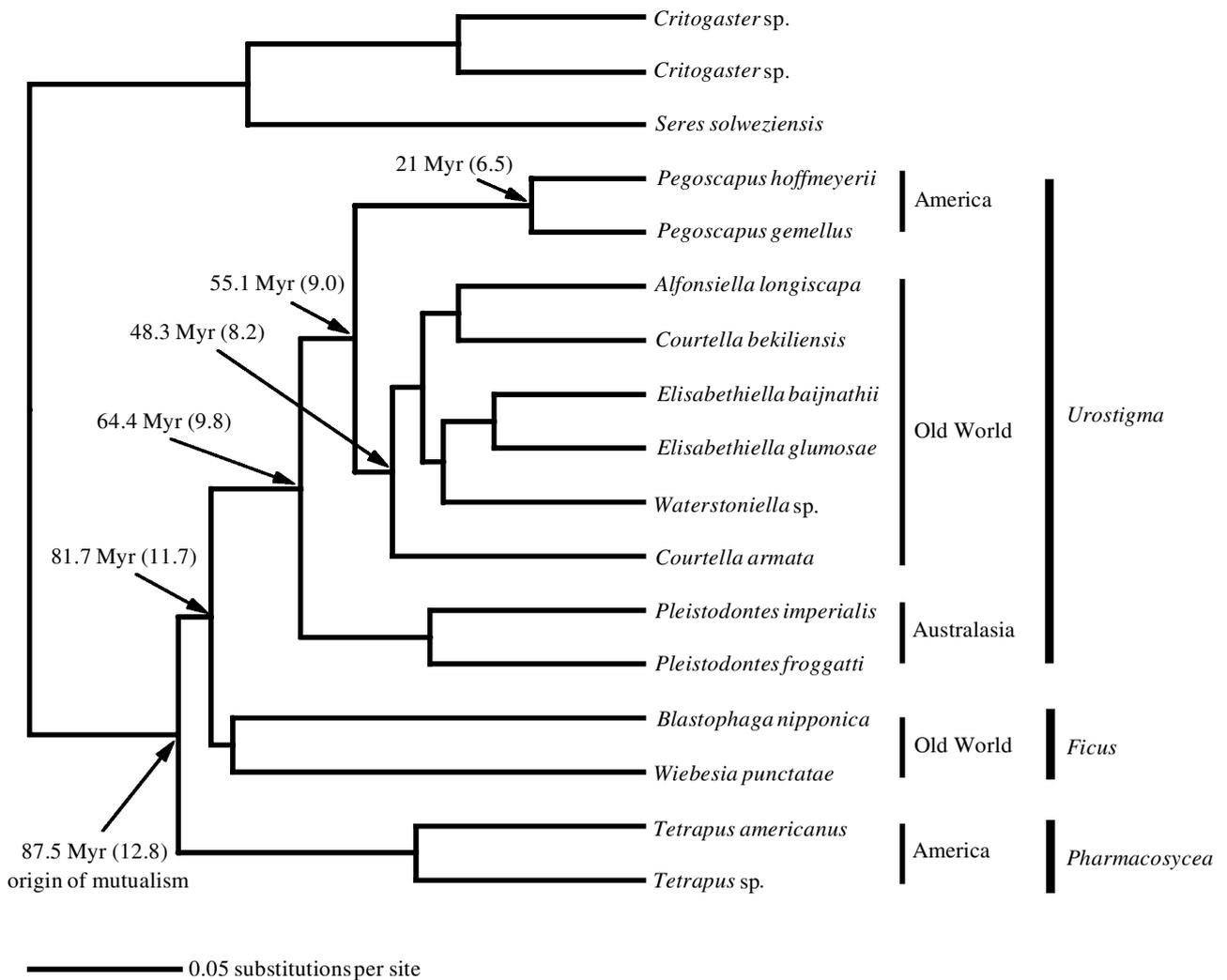


Figure 2. ML tree with molecular clock assumption of the constantly evolving COI gene sequences of fig-pollinating wasps. The estimated times of divergence are shown (\pm s.e. in parentheses). The estimated divergence times among pollinator taxa correspond well with geological estimates of the break-up of the continents that these groups currently inhabit (see text).

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-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 1990*a,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. Jouselin, 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.* 1985; 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

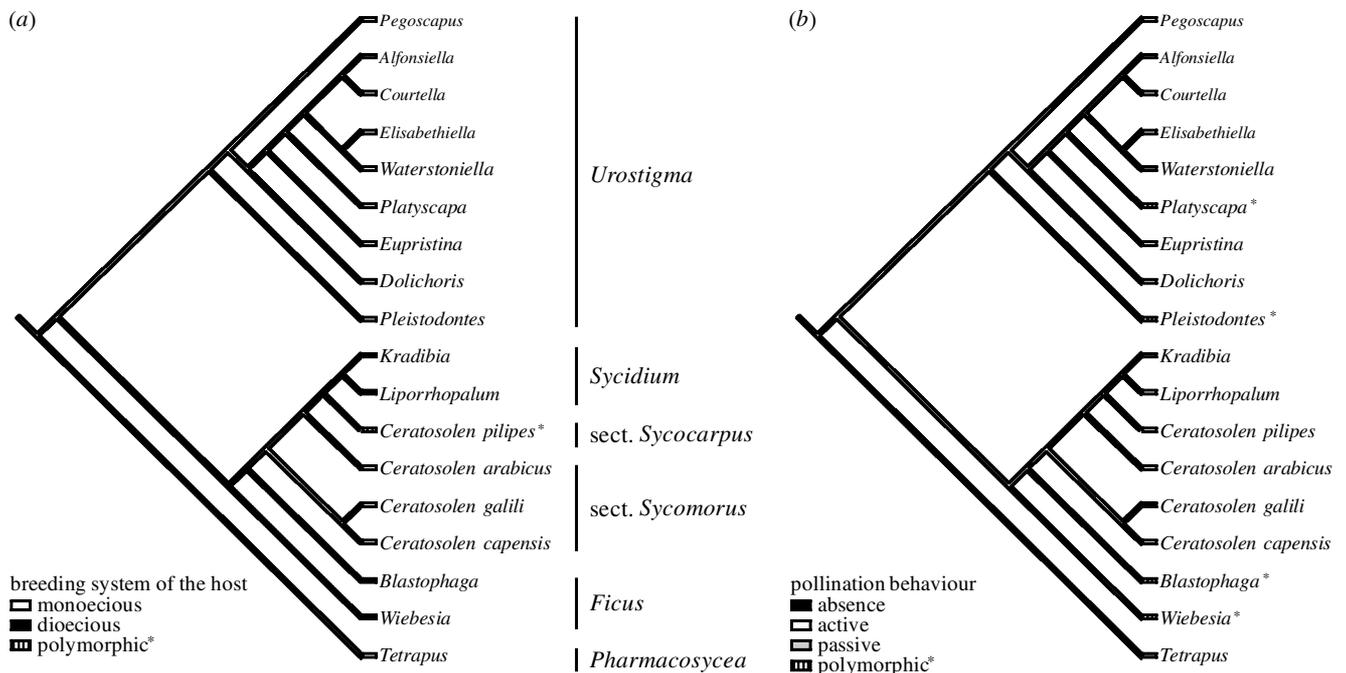


Figure 3. Most parsimonious reconstructions of the evolution of two important characters of the mutualism in the ML phylogeny: (a) the breeding system of the host and (b) pollination behaviour. Branch shadings represent the most parsimonious reconstructions of character evolution and boxes beside taxon names represent the character state for each genus. Polymorphic genera are also marked with an asterisk. Only one species per genus was used except for *Ceratosolen*, which is associated with both monoecious and dioecious figs and which has the only known reversion to seed parasitism (*C. galili*). *Ceratosolen pilipes* itself is not associated with a host polymorphic for its breeding system. It simply represents wasp taxa associated with a group of figs (section *Sycocarpus*) that is polymorphic (mainly dioecious with two monoecious species).

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*, *Sycomor*, *Ficus* and *Sycidium* (81.7 ± 11.7 Myr). The ancestors of the pollinators of the current palaeotropical subgenera *Ficus*, *Sycomor* 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 *Pegoscapus* (55.1 ± 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.

(c) *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 & Eisikowitch 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 & Eisikowitch 1971; Compton *et al.* 1991; Jusselin & 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 & Eisikowitch 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 & Eisikowitch 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 distinct 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. The

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. Wiebes 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 Institution and by a Howard Hughes Medical Institute predoctoral fellowship to C.A.M.

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