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Molecular phylogenies of fig pollinating and non-pollinating wasps and the implications for the origin and evolution of the fig–fig wasp mutualism

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Abstract. Figs host three ecologically distinct groups of wasps: pollinators, non-pollinators (parasitic wasps) and parasitoids. Both pollinators and non-pollinators complete their life cycles using fig tissue, while parasitoids appear to attack some groups of non-pollinators. We used nucleotide sequence data to address a series of questions concerning genealogical associations, host specificities and degree of strict-sense co-evolution exhibited by members of these groups. We used the relatively conserved 12S rRNA gene of the mitochondria to estimate high level relationships among pollinator, parasitic and parasitoid taxa by sampling species collected from host figs representing five sections (three subgenera) from Asia, Africa, Europe and Central America. We found that all pollinators formed a clear monophyletic group. However, we could not resolve whether or not all of the non-parasitoid wasps associated with figs (Agaonidae, *sensu* Bouček) formed a single monophyletic

group. Further, we used the more variable COII mitochondrial gene to attempt to determine relationships among closely related species of pollinators within two New World genera. Using sequences from the same gene we estimated the phylogenetic relationships among the parasites collected from the same New World host fig species and compared them with those of the pollinators. At fine taxonomic scale, we found that for both pollinator and parasites, species were generally specific to a given fig host. Moreover, the phylogenies of the non-pollinators are largely congruent with those of the pollinators, suggesting the predominance of strict-sense co-evolution on shared host fig species. The implications of these findings and opportunities for future research are discussed.

Key words. Agaonidae, co-evolution, COII, mitochondrial DNA, molecular phylogeny, mutualism, 12S rRNA.

INTRODUCTION

Figs (*Ficus*, Moraceae) and their pollinating wasps (Agaoninae, Agaonidae, Chalcidoidea, Hymenoptera) constitute one of the classic examples of pollination mutualism and appear to present one of the best cases of co-evolution known (Janzen, 1979; Wiebes, 1979, 1982; Berg, 1989; Herre, this issue). Figs are completely dependent on their mutualist pollinator wasps for pollen dispersal and the production of viable seeds. For their part, the fig pollinating wasps are completely dependent on the fig for the completion of their life cycle (Corner, 1940; Ramirez, 1974; Galil, 1977; Janzen, 1979; Herre, 1989). In most cases studied, one species of wasp pollinates one species of fig. Further, most authors agree that there is a general congruence among proposed phylogenies of both pollinators and host figs, implying a predominance of strict-sense co-evolution between the two groups, although the degree of

match is in debate (Ramirez, 1974; Wiebes, 1979, 1982; Corner, 1985; Berg, 1989; Berg & Wiebes, 1992).

In addition to the pollinating wasps, a diverse community of non-pollinating (parasitic) wasps also exploit figs (Gordh, 1975; Hamilton, 1979; Janzen, 1979; Bouček, Watsham & Wiebes, 1981; Godfray, 1988; Murray, 1989; Bronstein, 1991; Compton & Hawkins, 1992; Compton & van Noort, 1992; Hawkins & Compton, 1992; Bouček, 1988, 1993; West & Herre, 1994; West *et al.*, this volume; Cook, this issue). Individual host fig species may harbour many different species of non-pollinating wasps, and related host figs may possess very similar assemblages of these species. Although the basic biology of most of these non-pollinator wasps is unknown, recent work has begun to describe some of their ecologies and indicates that these species often reduce the fig's production of pollinators and seeds without providing any obvious benefit (Compton, 1993; West & Herre, 1994; Herre *et al.*, 1996; West *et al.*, this issue). Different authors have suggested classifications and phylogenies for some groups of these wasps, some of which suggest that host specificity has also developed in the non-pollinating wasps (Gordh, 1975; Bouček *et al.*, 1981; Ulenberg, 1985; Bouček,

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1988 & 1993; van Noort, 1991; van Noort, this issue). Nevertheless, the extent to which there has been co-evolution between these wasps and their fig or wasp hosts is not clear, and the question is open.

Despite (and because of) these ambiguities, figs and their associated wasps present one of the richest natural systems for studying a wide spectrum of coevolutionary questions, from both adaptive and phylogenetic perspectives. Indeed, there is a tremendous range in the basic ecology and life cycles of pollinator and non-pollinator fig wasp species (Compton & Hawkins, 1992; Hawkins & Compton, 1992; West & Herre, 1994; West *et al.*, this issue). For example, the pollination interaction itself varies dramatically across fig and wasp taxa, with active pollination the rule in some and passive pollination the rule in others (Ramirez, 1974; Wiebes, 1979; Frank, 1984; Corner, 1985; Berg, 1989; Kjellberg *et al.*, this issue). Further, the morphology and mating behaviour of males varies tremendously across pollinator and non-pollinator taxa and has been used to test several ideas concerning the forms that reproductive competition might take (Hamilton, 1979; Murray, 1989). Moreover, in addition to providing an important test of sex allocation theory (Hamilton, 1967, 1979; Kjellberg, 1983; Frank, 1985; Herre 1985, 1987), the sex ratio of the pollinators varies widely across species, and influences the reproductive success of the fig (Herre, 1989). Interestingly, the patterns observed in brood sex ratio of the different wasp species appear to correlate with several other characteristics of their particular host fig species, implying causal linkages (Herre, this issue). In all of these cases, the function of the characteristics appears to be understood and attendant adaptive arguments have been supported. However, both the strength and the scope of these arguments is limited by the absence of knowledge of the phylogenetic relationships among the organisms that have been studied.

Beyond questions that pertain to the workings of the fig-wasp system itself, the extent of co-evolution in figs and their associates is particularly interesting because there are few well documented cases of widespread co-evolution (Hafner & Nadler, 1988; Hafner *et al.*, 1994; Moran & Baumann, 1994). In many cases co-evolution is assumed to have occurred because of the functional nature of the interaction. Unfortunately, those hypotheses are only rarely tested from an historical, phylogenetic perspective.

For the case of figs and their pollinators, there have been some attempts to take this historical, phylogenetic approach and use it to analyse character evolution (Ramirez, 1974; Wiebes, 1982; Berg & Wiebes, 1992). However, the existing classifications of figs and their pollinators are both incomplete and controversial. The classifications that exist are based on morphological characters involved in the interaction. The general phylogenetic congruence between wasps and figs suggests a functional relationship of the characters used to produce the phylogenies but does not provide good evidence for assessing historical relationships because of the non-independence of the characters utilized. The same pattern could be observed if processes of colonization are followed by rapid morphological convergence, and in fact the latter does not seem to be an unlikely event (van Noort, this issue). For this reason it is

desirable to have independent evidence that could support the suggestion of strict sense co-evolution from an historical, phylogenetic perspective.

Nucleic acid sequences constitute a useful set of independent characters that have found widespread use in the estimation of organismal phylogenies. Mitochondrial DNA, in particular has been widely utilized owing to the secure comparison of homologous sequences across taxa. This work is the first attempt to use nucleic acid sequences to reconstruct the phylogeny of species belonging to the major groups of pollinating and non-pollinating fig wasps (family Agaonidae, *sensu* Bouček, 1988) from different parts of the world. We have included species of pollinators (subfamily Agaoninae, *sensu* Bouček, 1988) from the genera *Elisabethiella*, *Pegoscapus* and *Tetrapus*; and species of non-pollinators from the genera *Critogaster*, *Sycoscapter*, *Philotrypesis* (subfamily Sycoryctinae), *Idarnes* (subfamily Sycophaginae) and *Aepocerus* (subfamily Otitesellinae). Additionally, we included one species of non-pollinator parasitoid from the family Torymidae (genus *Physothorax*). Further, we have used nucleotide sequence data to determine the degree of congruence between the phylogeny of pollinator and non-pollinator wasps associated with eight species of *Ficus* trees growing in Panamá.

NATURAL HISTORY AND TAXONOMY

The fig-pollinating wasps all have very similar life histories. In monoecious species, mated, pollen-bearing female pollinating wasps arrive at the tree, enter the syconium (the enclosed inflorescence that defines the genus *Ficus*) through the ostiole, pollinate the uniovulate flowers, probe the flowers with their ovipositors and lay eggs in the ovaries. The foundress wasps generally die inside the fruit after pollinating and laying eggs (but see Kjellberg *et al.*, this issue). As the fruit ripens, pollen-bearing male flowers grow inside the fruit. Just before final ripening takes place, the wingless male wasps chew their way out of the seeds in which they have developed and crawl around the interior of the fruit searching for seeds with female wasps inside them. The pollinating wasp males chew open these seeds and mate with the females. The females of the pollinating wasp then emerge from their seeds, collect pollen, leave through a hole cut in the fig wall by their males and fly off in search of a receptive conspecific fig (Herre, 1989). The life cycles of both wasp and fig are somewhat more complicated in the dioecious figs (Wiebes, 1979; Kjellberg *et al.*, 1987).

The non-pollinating fig wasps form a very diverse taxonomic group. Until recently, the basic ecology of most of these wasps had been largely unknown. However, recent studies are beginning to clarify the details of the diverse ecologies of many groups and indicate that most of these wasps negatively affect the fig and pollinating wasp's reproductive interest (Compton & Hawkins, 1992; Hawkins & Compton, 1992; West & Herre, 1994; Cook, this issue; West *et al.*, this issue). With the exception of all members of the subfamily Sycoecinae and of the genus *Sycophaga* from the subfamily Sycophaginae, which are only found in the Old World, all non-pollinator species oviposit from the

outside of the fruit. In the New World, these externally ovipositing wasps seem to comprise three very ecologically distinct groups: (1) a group of wasps similar in size to the pollinators and which appear to compete with them for the same resources for larval development (e.g. *Critogaster*, and some groups of *Idarnes* (*carne* and *flavicollis*)); (2) a group of relatively larger wasps that lay their eggs in the flowers or fruit walls, inducing the formation of large galls in which the larvae develop and which seem not to require the presence of the pollinators in order to utilize the syconium (E.A.H., pers. obs.) (e.g. *Aepocerus*, *Heterandrium* and *Idarnes* (*incerta*)); and (3) a group of true parasitoids of the larger gall-formers (e.g. *Physothorax*) (West & Herre, 1994; West *et al.*, this issue).

All chalcidoid wasps that depend on the tissues of the syconium for completing their life cycles (all the pollinators and the majority of non-pollinators) have been recently grouped in the family Agaonidae (Bouček, 1988). According to this classification, all pollinating fig-wasp genera belong to the subfamily Agaoninae, while non-pollinators are grouped in different subfamilies (Epichrysomallinae, Otitesellinae, Sycoricetinae, Sycophaginae and Sycoecinae). Other groups of non-pollinators that appear to be parasitoids of some agaonids are placed in different chalcidoid families (Torymidae, Eurytomidae and Pteromalidae).

Two pollinator wasp genera are endemic to the neotropics: *Pegoscapus* and *Tetrapus*. They pollinate, respectively, figs of the section Americana from subgenus Urostigma (mostly stranglers) and section Pharmacosycea from the subgenus of the same name (Ramirez, 1974; Berg, 1989; Bouček, 1993). Fig species (subgenus Urostigma) pollinated by *Pegoscapus* are parasitized by wasps of the genus *Idarnes* (subfamily Sycophaginae) (Gordh, 1975; Bouček, 1993). *Idarnes* includes some species (*carne* and *flavicollis* groups) that are roughly the size of the *Pegoscapus* pollinators and appear to compete with them for oviposition sites (West & Herre, 1994), as well as relatively larger species (*incerta* group) that form distinctive galls (Bouček, 1993; West *et al.*, this issue). Additionally, they also differ in male morphologies (West *et al.*, this issue). Fig species (subgenus Pharmacosycea) pollinated by *Tetrapus* are parasitized by wasps of the genus *Critogaster* (subfamily Sycoricetinae). These wasps appear to correspond to the smaller *Idarnes* species in their ecology and effect on the fig (West *et al.*, this issue). Interestingly, we encountered several differently coloured *Critogaster* species in each host fig species. Our analyses are based on samples from species with yellow bodies (*Critogaster* Mayr, *flavescens* group. See also West *et al.*, this issue). Additionally, New World Urostigma figs also host different species of non-pollinator wasps from the subfamily Otitesellinae (genera *Heterandrium* and *Aepocerus*).

MATERIALS AND METHODS

Collections

Panamanian wasps were collected in the vicinity of the Panamá Canal from eight native fig species (Table 2).

Pollinator and non-pollinator wasps were collected, respectively, from single foundress broods and single fruits, in order to maximize the probability of obtaining single mitochondrial lineages. Wasps were randomly collected from different trees and different crops. Thus we were able to assess levels of intraspecific variation and check the specificity of the association from a molecular perspective. Care was taken to avoid the presence of mites or nematodes in the samples. The fruits were collected late in the fruiting cycle before any wasps had abandoned the fruit. Each fruit was opened and put into Petri dishes. All wasps emerged in the Petri dishes and we collected pollinators and non-pollinators separately. The wasps were stored in 70% ethanol at 4°C or immediately utilized for genomic DNA extraction. The majority of the Panamanian pollinating wasps from the genus *Pegoscapus* have been recently described by Wiebes (1995). The Panamanian non-pollinating wasp species included in this study have not been described yet and we will refer to them only as nominal species. To avoid confusion we have provided our own codes for referring to each species (see Table 2). Ethanol preserved specimens of wasps from Old World groups of agaonids were obtained from different researchers (S. G. Compton (*Elisabethiella*), F. Kjellberg (*Phylotrypesis*), L. Chou (*Sycoscapter*) and were collected during the last 10 years (Table 1).

DNA protocols

In order to determine relationships among the major groups of pollinators and non-pollinators we used sequences from the III domain of the small ribosomal mitochondrial subunit (12S rRNA), known to evolve at relatively slow rates and hence useful for determining relationships at higher taxonomic levels (Simon *et al.*, 1989). For studying fine scale relationships, we utilized sequences from the cytochrome oxidase subunit II of the mitochondrial DNA. This gene has been shown to evolve at high enough rates to resolve relationships among closely related species (Beckenbach, Wei & Liu, 1993).

Genomic DNA was extracted from either ethanol preserved specimens or newborn individuals. DNA was isolated from one to ten individual wasps using standard phenol-chloroform extractions (Sambrook, Fritsch & Maniatis, 1989). Using the polymerase chain reaction (PCR) (Saiki *et al.*, 1988), the full COII gene (684 bp) was amplified for the different species of *Pegoscapus*, *Tetrapus*, *Idarnes* and *Critogaster*, as well as 320 bp of the 12S rRNA from all major groups of pollinator and non-pollinator fig wasps (Tables 1,2). Primers StLeu, A3389, COII Hope and AtAsp, were utilized for amplifying and sequencing the COII gene. The Leu tRNA primer was slightly modified from a similar primer developed by Liu & Beckenbach (1992); sequence for the A3389 primer was obtained from the laboratory of R. Harrison (Cornell University) and corresponds to a conserved region in the COII gene of *Drosophila yakuba* (Clary & Wolstenholme, 1985) and *Apis mellifera* (Crozier, Crozier & Mackinlay, 1989); COII Hope was designed using the insect COII sequences from Liu & Beckenbach (1992); AtAsp was formerly designed by Willis, Winston & Honda (1992). Primer sequences for the 12S region were adapted

TABLE 1. Taxonomic position, ecological role, *Ficus* host and country of collection of the ten species of fig-associated wasps from which 12S mt rRNA nucleotide sequences were obtained. Taxonomic positions of the wasps are from Bouček (1988, 1993). Taxonomic positions of the figs are from Berg (1989). Ecology of the wasps from Joseph (1957), Ramirez (1974), West & Herre (1994) and West *et al.* (this issue). Abbreviations: pol. = pollinator; com. = competitor; gall. = galler; par. = parasitoid.

| Species | Ecology | <i>Ficus</i> host | <i>Ficus</i> subgenus | Location |
|---------------------------------|---------|--------------------------|-----------------------|--------------|
| Family Agaonidae | | | | |
| Subfamily Agaoninae | | | | |
| <i>Pegoscapus silvestrii</i> | pol. | <i>Ficus pertusa</i> | Urostigma | Panama |
| <i>Elisabethiella baijnathi</i> | pol. | <i>Ficus burtt-davyi</i> | Urostigma | South Africa |
| <i>Tetrapus ecuadoranus</i> | pol. | <i>Ficus yoponensis</i> | Pharmacosycea | Panama |
| Subfamily Sycophaginae | | | | |
| <i>Idarnes</i> sp. | com. | <i>Ficus dugandii</i> | Urostigma | Panama |
| <i>Idarnes</i> sp. (incerta) | gall. | <i>Ficus trigonata</i> | Urostigma | Panama |
| Subfamily Sycoryctinae | | | | |
| <i>Critogaster</i> sp. | com. | <i>Ficus maxima</i> | Pharmacosycea | Panama |
| <i>Phylotrypesis caricae</i> | com.? | <i>Ficus carica</i> | Ficus | France |
| <i>Sycoscapter gajimaru</i> | com.? | <i>Ficus microcarpa</i> | Urostigma | Taiwan |
| Subfamily Otitesellinae | | | | |
| <i>Aepocerus</i> sp. | gall. | <i>Ficus obtusifolia</i> | Urostigma | Panama |
| Family Torymidae | | | | |
| <i>Physothorax</i> sp. | par. | <i>Ficus dugandii</i> | Urostigma | Panama |

from Kocher *et al.* (1989) and Simon *et al.* (1989). The primer sequences are presented in Table 3. Single stranded templates for sequencing were generated following the λ -Exonuclease protocol of Higuchi & Ochman (1989) and were sequenced using the dideoxynucleotide chain-termination method (Sanger, Nicklen & Coulson, 1977). Aligned sequences are available from the first author by request.

Sequence alignment and cladistic analysis

Sequences were read from both strands with 50–90% of overlap. Initial alignments for the COII region were done using the program MacVector (International Biotechnologies, Inc.; Version 4.1). 12S sequences were aligned using CLUSTAL V (Higgins & Sharp, 1988). Final alignments were made by eye. The aligned sequences were analysed by maximum parsimony using the program PAUP (Version 3.1; Swofford, 1991). Only phylogenetically informative sites were considered in the analyses. Sequences from P2 (*Idarnes*) and W2 (*Pegoscapus*) were used as outgroups for the phylogenetic analysis of the COII sequences of pollinating and non-pollinating wasps, respectively (Table 2). Analyses with different outgroups recovered the same tree topologies. Most parsimonious trees were obtained using the exhaustive search option in PAUP. Additionally, neighbour-joining (Saitou & Nei, 1987) was used as implemented in the program MEGA (Kumar, Tamura & Nei, 1993), utilizing Tamura's correction for multiple substitutions (Tamura, 1992). 12S sequences were analysed using parsimony, neighbour-joining and the maximum likelihood method as implemented in the dnaml program from PHYLIP (Felsenstein, 1993). For the analysis of the 12S data, a sequence from the homologous region in one species of *Braconidae*: *Doryctinae* was used as outgroup. Each node of the recovered trees was analysed looking at the unambiguous changes supporting it using the program MacClade (Version 3.0; Maddison & Maddison, 1992). The

reliability of each node in the obtained phylogenies was examined using bootstrap resampling procedures (Felsenstein, 1985). Different hypotheses of fig wasp evolution were tested using Kishino & Hasegawa's (1989) maximum likelihood test as implemented in PHYLIP (Felsenstein, 1993). In order to determine the fit between the estimated phylogeny of *Pegoscapus* and *Tetrapus* pollinators with the phylogeny of *Idarnes* and *Critogaster* non-pollinators, the cladograms were compared using the program COMPONENT (Version 2.0; Page, 1993a).

RESULTS

Phylogeny of fig pollinator and non-pollinator wasps

First, we consider the results of the reconstruction of the high level phylogenetic relationships among major groups of pollinators and non-pollinators. Of the 238 bp of the 12S gene considered for the analyses, 159 sites were variable and 100 phylogenetically informative. Only the phylogenetically informative characters were included in the analyses. Using the sequence from the Braconid as outgroup a single most parsimonious tree of 264 steps was recovered (Fig. 1A). The same tree was also recovered using the maximum likelihood method with different transition/transversion ratios. This tree places all parasitic wasps and *Physothorax* sp. (family Torymidae) as sister groups. The two next most parsimonious trees are also shown in Fig. 1. In all three phylogenies the pollinating wasps constitute a monophyletic group. Specially interesting is tree 1C, which places the pollinators (Agaoninae) and the non-pollinator wasps (Sycophaginae, Sycoryctinae, Otitesellinae) as a monophyletic group. This same tree was also recovered by neighbour-joining analysis. Using Kishino–Hasegawa's (1989) maximum likelihood test, trees 1B and 1C are not significantly worse than tree 1A. The internal nodes marked with an asterisk in Fig. 1 have low support and analyses of

TABLE 2. List of the species of neotropical fig pollinating and non-pollinating wasps for which COII nucleotide sequences were obtained. Codes for the species, number of sequenced individuals, their host figs and the taxonomic position of both figs and wasps are presented. Names of figs and their pollinators are from Herre (1989) and Wiebes (1995).

| Ficus species | Pollinator wasps | | | Parasitic wasps | | |
|-----------------------------|------------------|-------------------------------|-----------------------|-----------------|-------------------------|-----------------------|
| | Code | Species | Samples sequenced (n) | Code | Species | Samples sequenced (n) |
| Subgenus Urostigma | | Family Agaonidae | | | Family Agaonidae | |
| Section Americana | | Subfamily Agaoninae | | | Subfamily Sycophaginae | |
| <i>Ficus paraensis</i> | 1 | <i>Pegoscapus herrei</i> | 2 | W1 | <i>Idarnes</i> sp. | P1 |
| <i>Ficus obtusifolia</i> | 2 | <i>Pegoscapus hoffmeyerii</i> | 2 | W2 | <i>Idarnes</i> sp. | P2 |
| <i>Ficus trigonata</i> | 6 | <i>Pegoscapus grandii</i> | 2 | W6 | <i>Idarnes</i> sp. | P6 |
| <i>Ficus near trigonata</i> | 7 | <i>Pegoscapus lopesi</i> | 3 | W7 | <i>Idarnes</i> sp. | P7 |
| <i>Ficus dugandii</i> | 12 | <i>Pegoscapus longiceps</i> | 1 | W12 | <i>Idarnes</i> sp. | P12 |
| Subgenus Pharmacosycea | | | | | | |
| Section Pharmacosycea | | | | | Subfamily Sycortictinae | |
| <i>Ficus maxima</i> | 9 | <i>Tetrapus americanus</i> | 2 | W9 | <i>Critogaster</i> sp. | P9 |
| <i>Ficus insipida</i> | 10 | <i>Tetrapus costaricanus</i> | 2 | W10 | <i>Critogaster</i> sp. | P10 |
| <i>Ficus glabrata</i> | 11 | <i>Tetrapus</i> sp. | 2 | W11 | | P10.1** |
| | | | | | | P11 |
| | | | | | | P11.1** |
| | | | | | | P11.1** |

** Same species.

TABLE 3. List of the PCR primers utilized for amplifying the COII and 12S mitochondrial regions. A=antisense strand; B, S=sense strand. See text for references.

| Primer | Sequence | Mitochondrial region |
|-----------|---------------------------|-------------------------------|
| StLeu | ATGGCAGATTAGTGCAAT | tRNA LEU (UUR) 5' end of COII |
| A3389 | TCATAAGTTCARTATCATTG | COII (position 306–326) |
| COII Hope | TTCGTCCCTGGAACWGC/ARTC | COII (position 511–529) |
| AtAsp | GGCCGTCTGACAAACTAATGTTAT | tRNA ASP 3' end of COII |
| 12S A | AAACTGGGATTAGATACCCCACTAT | 12S rRNA |
| 12S B | GAGGGTGACGGGCGGTGTGT | 12S rRNA |
| 12S a | TAGGATTAGATACCCTATTA | 12S rRNA |
| 12S A1 | AAACTAGGATTAGATACCCT | 12S rRNA |
| 12S B2 | AAGAGCGACGGGCGAT | 12S rRNA |

bootstrap pseudoreplicates yields values lower than 40% for each of them. Fig. 2 shows the consensus tree of the three cladograms from fig. 1 together with bootstrap values for the well supported nodes.

The 12S-based phylogeny of the non-pollinators studied is in general agreement with the morphological classification of Bouček (Bouček, 1988, 1993). Members of the subfamily Sycophaginae (e.g. *Idarnes* sp.) form a group of their own. All species from the subfamily Sycoryctinae (*Sycoscapter*, *Critogaster*, *Philotrypesis*) form a single monophyletic group. However, one of the species considered to be in the subfamily Otitesellinae (*Aepocerus* sp.) appears to be close to *Critogaster* (subfamily Sycoryctinae). Additionally, we have obtained a partial sequence for another species classified in the subfamily Otitesellinae (*Heterandrium* sp. collected from *F. dugandii*). The analysis of the partial sequence (150 bp) places it in the lineage leading to *Idarnes*, and hence not together with the members of its own subfamily. These preliminary observations suggest that the subfamily Otitesellinae, as presently defined, is not a monophyletic group. Additionally, Bouček further hypothesized that the pollinators are a sister taxon to either the Sycophaginae or the Sycoecinae because these two subfamilies have members that enter the fig syconium to oviposit. Our data do not support the placement of Sycophaginae as sister taxa of the pollinators, and we do not yet have samples representing the Sycoecinae.

Species specificity and fine level phylogenetic congruence of neotropical pollinators and parasites

In order to determine the relationships among closely related species of neotropical pollinators and among their associated species of parasitic wasps, we collected nucleotide sequences from the mitochondrial COII gene. Sequences 684 base pairs in length were obtained for two 'individuals' (one to ten female wasps from single foundress broods) representing each of the species of agaonid pollinators (*Pegoscapus*, *Tetrapus*) and parasitic wasps (*Idarnes*, *Critogaster*) associated with eight species of *Ficus* from the New World (Table 2). The DNA sequence from each morphospecies was different. Each species of pollinating wasp was associated with a single species of host fig. Interspecific divergences using Tamura's correction ranged from 6.5% to 10% inside *Pegoscapus*, and from 7.2% to 10% inside

Tetrapus. Between-genera divergences ranged from 21% to 25%. In all cases levels of intraspecific variation were much lower than the levels of interspecific variation and all second samples sequenced showed few if any nucleotide substitutions. These results confirm the idea of fig-host specificity for the studied species of neotropical pollinators from a molecular perspective.

As is the case with the pollinators, host-free specificity was also generally observed for the species of non-pollinator wasps. However, there was one case in which two different *Idarnes* species were isolated from a single host (*F. trigonata*). We only have partial sequence of one of them, and for that reason have not included the second species in the analyses. The phylogenetic position of the two species is somewhat different, implying either occasional crossing over or the presence of at least two sympatric lineages of *Idarnes* associated with this species of fig. Further, one species of *Critogaster* (P10.1 = P11.1) was collected from two different hosts, although each host (*F. insipida* and *F. glabrata*) also had an apparently specific species of *Critogaster* associated with it, as well. Interspecific divergences ranged from 9.7% to 16% inside *Idarnes* and from 13% to 20% inside *Critogaster*. Divergences between the two genera ranged from 25% to 34%.

The COII sequences of the pollinators and P2 showed 299 variable sites and 173 phylogenetically informative sites. Phylogenetic analysis of these sequences yielded one most parsimonious tree of 335 steps (CI=0.693) (Fig. 3). A tree of 336 steps was identical to the tree recovered by NJ analysis, differing from the most parsimonious tree in the placement of W6 and W7 as sister taxa. Character analysis of unambiguous changes and bootstrap analysis showed that the clade W12–W7 was better supported than the clade W7–W6. For that reason, we consider the most parsimonious tree in Fig. 3 as the better hypothesis of relationships among these taxa based on the COII gene. Phylogenetic analysis of the different sequences from species of *Critogaster* and *Idarnes* gave one most parsimonious tree of 558 steps (CI=0.577) (346 and 229 variable and informative sites, respectively) (Fig. 4). This tree was identical to the one recovered by NJ analysis. Forcing the tree topology to match exactly that of their related pollinators adds nine steps to the total length of the tree.

Two kinds of evidence are normally considered necessary to document co-speciation among two groups of organisms:

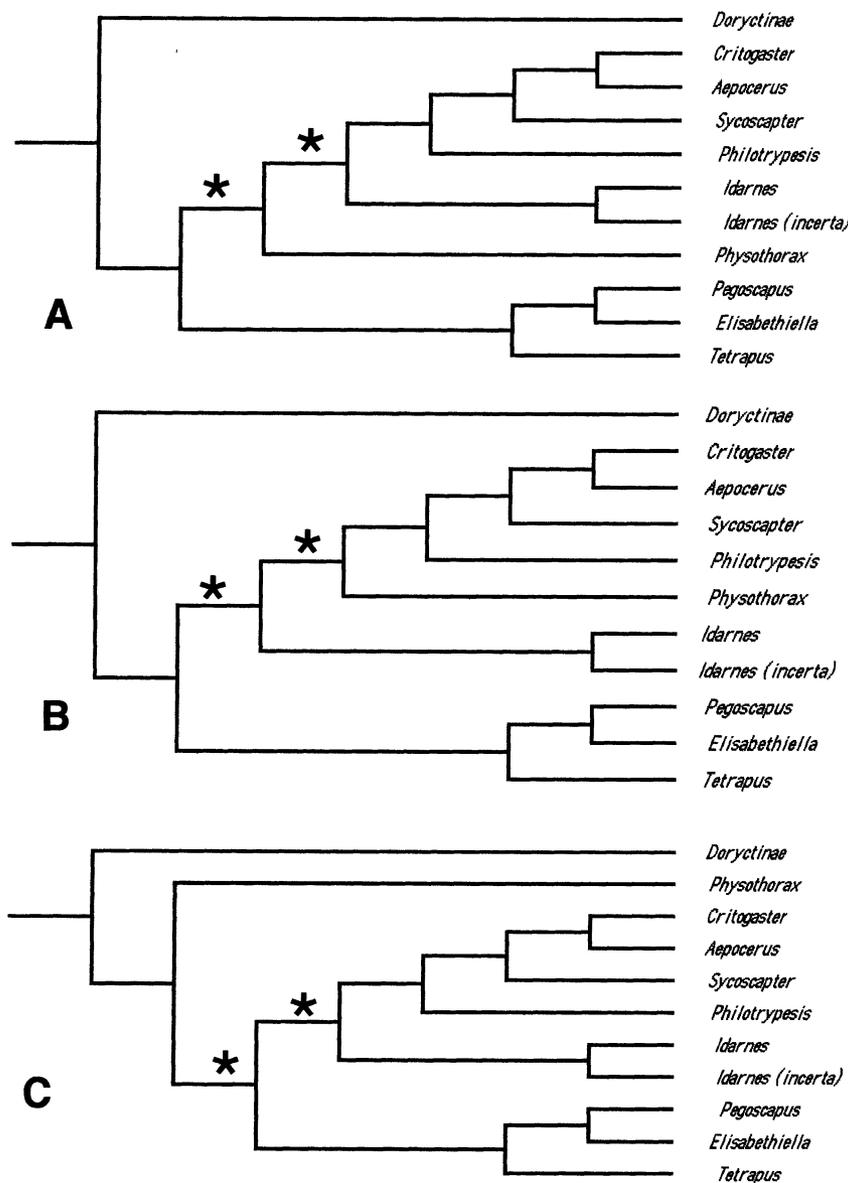


FIG. 1. The three most parsimonious trees estimating the phylogenetic relationships of species representing major groups of pollinator and non-pollinator fig associated wasps (see Table 1), generated by analysis of 12S mt rRNA nucleotide sequences. Gaps were treated as missing data. Tree lengths: A = 264, B = 266, C = 268 (CI = 0.57). Each internal branch is supported by at least seven unambiguous changes, with the exception of the ones marked with an asterisk. Tree A was also recovered by the maximum likelihood method using a diverse variety of transition/transversion ratios. Tree C was also recovered by neighbour-joining analysis. Trees B and C are not significantly worse than tree A, as determined using Kishino-Hasegawa's test (1989).

independence of the estimated phylogenies and statistical evidence that the degree of fit between the cladograms for both groups of organisms is higher than what would be expected by chance alone (Hafner & Nadler, 1990; Page 1993a, 1993b, 1994). We determined the fit between the parasitic and pollinator wasp phylogenies using the program COMPONENT (Version 2.0; Page, 1993a). The degree of fit between the trees was measured using two criteria: number of leaves added and minimum number of losses required to reconcile the trees (Page, 1993a, 1994). The measures of fit obtained from comparing the most

parsimonious tree of the pollinators with the most parsimonious tree of the parasitic wasps were then compared with the distribution of the same measures between the parasitic wasp phylogeny and a set of 1000 random pollinator trees, in order to determine whether the fit among the pollinator and parasite phylogenies was better than what would be expected by chance alone. Using both criteria of fit the comparison was found to be statistically significant ($P < 0.01$). Therefore, the probability of this degree of similarity occurring by chance is remote. This result strongly suggests co-speciation between non-pollinator and

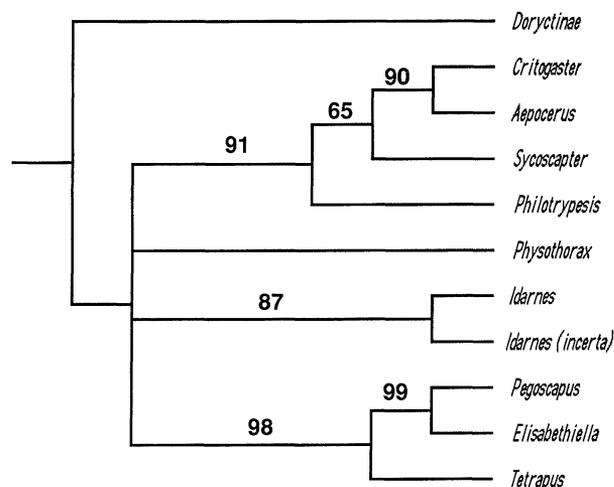


FIG. 2. Strict consensus cladogram of the phylogenetic trees from Fig. 1. Numbers above each branch are bootstrap values from 200 replicates. The internal branches joining each of the major groups of fig-associated wasps (marked with asterisks in Fig. 1) are supported by few informative sites and were recovered less than 50% of the time in the analyses of bootstrap pseudoreplicates.

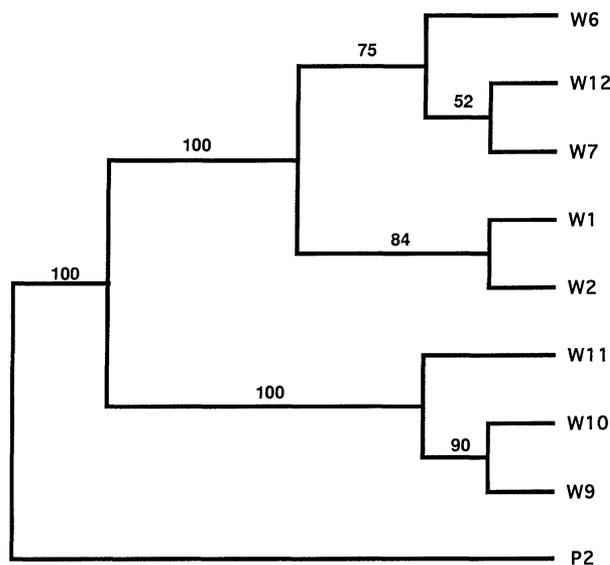


FIG. 3. The most parsimonious phylogenetic tree of the eight species of neotropical fig pollinating wasps (*Pegoscapus* and *Tetrapus*) generated by analyses of COII sequences using P2 as outgroup (see Table 2 for species names). Numbers above branches are bootstrap values from 200 replicates.

pollinator wasps on shared host fig species at this very fine level.

In Fig. 5 the two most parsimonious phylogenetic reconstructions of pollinator (Fig. 3) and parasitic wasps (Fig. 4) are compared. Assuming the pattern to have arisen by association by descent (i.e. no dispersal or host-switching events allowed) (Page, 1993a), two duplication events (presence of two sympatric lineages of parasitic wasps associated to one of the pollinating wasp ancestors, which later radiated independently but following the path of the

pollinators) is required to explain the incongruity between *Idarnes* and *Pegoscapus*. This arrangement implies six extinctions of parasitic wasp lineages or an incomplete sampling of species in order to explain the observed pattern of relationships. As mentioned before, we isolated another parasitic species from *F. trigonata* from which we only have a partial COII sequence. Interestingly, a phylogenetic analysis using that partial sequence places that species inside the group comprising P2, P7 and P12. This observation supports the possibility of the suggested lineage duplication in *Idarnes* and hence either the possibility of lineage extinctions in the clade containing P6 or the incomplete sampling of species of this same clade. For *Critogaster*, the presence of the generalist species P10.1/P11.1 can be explained (under the same initial assumptions) by two lineage duplications and three extinctions (or cases of incomplete sampling).

DISCUSSION

Three very distinct groups of wasps depend on figs for completing their life cycles: pollinators, non-pollinators (parasites) and parasitoids (Compton, 1993; West & Herre, 1994; West *et al.*, this issue). However, their phylogenetic associations are unclear. Resolving these association would be useful in understanding the origins of the pollinator–host mutualism, as well as in understanding the evolutionary history of the taxa that currently interact as components of the insect communities associated with figs. The results shown from the analyses of the mitochondrial 12S rRNA sequences clearly show that pollinators constitute a monophyletic group, strongly supporting a single origin of the pollinator syndrome. An interesting question to answer is from wasps with what sort of ecology did the mutualist pollinators arise?

Based on morphological and ecological characteristics, it has been proposed that pollinators and all the non-parasitoid fig-associated wasps form a monophyletic group (Bouček, 1988, 1993). However, the 12S data set we collected did not provide enough resolution to either reject or accept this attractive hypothesis (Figs 1 & 2). Specifically, the position of *Physothorax* (a parasitoid) with respect to pollinators and non-pollinators is not clear. This difficulty could be the result of lack of resolving power of the 12S region for deep branching events and/or limited sampling of taxa (both of outgroup and ingroup taxa). In order to solve this doubt about the phylogenetic position of *Physothorax* we are attempting to collect molecular information from a wider range of appropriate outgroups. Resolving this issue is very important because it could give enormous insights about the origin and evolution of the mutualism. Basically, the idea of monophyly of fig-associated wasps (e.g. having Torymids like *Physothorax* as outgroups) would strongly support the thesis of a single origin for the pre-agaonid. In essence, with the closure of the syconium a single type of wasp (pre-agaonid) diversified into two different ecological roles: mutualist and parasite. This initial diversification between pollinating and parasitizing wasps

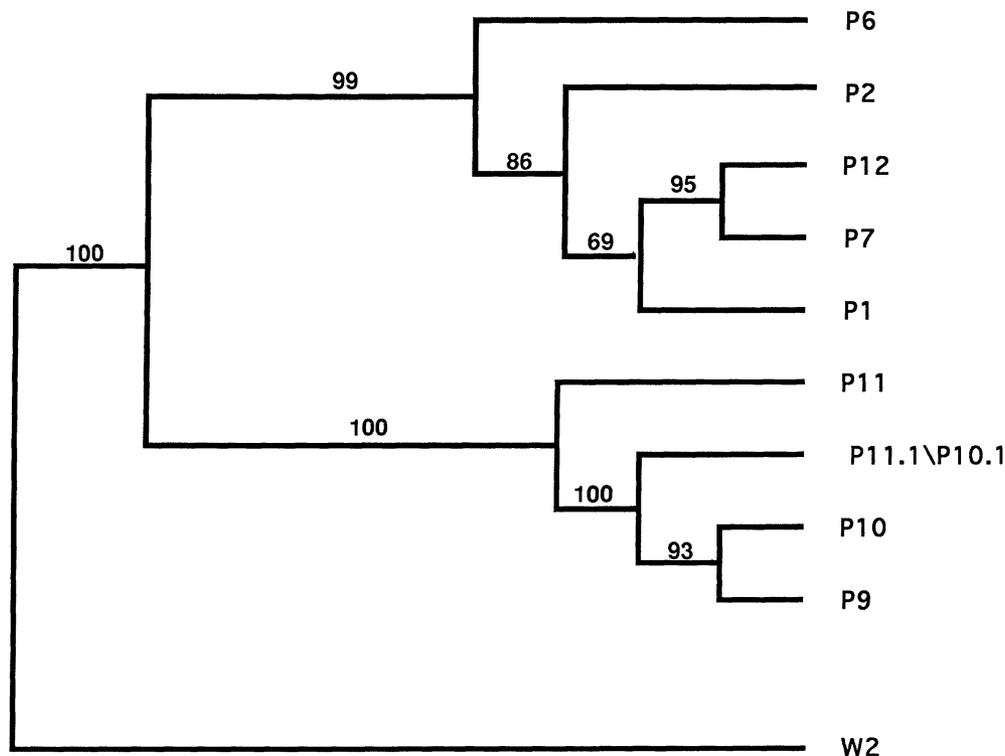


FIG. 4. The most parsimonious phylogenetic tree of the species of *Idarnes* and *Critogaster* non-pollinator group generated by analysis of COII sequences (see Table for species codes). W2 was used as outgroup. All non-informative characters were excluded. Numbers above branches are bootstrap values from 200 replicates.

was then followed by speciation and ecological radiation, particularly among the non-pollinators. Additionally, if pollinators and non-pollinators were in fact sister groups, an appropriately calibrated molecular clock could be used to estimate the time since initiation of the fig-wasp system as it is presently constituted (e.g. date the closure of the syconium).

With respect to the non-parasitoid fig wasp fauna, it is extremely important to obtain molecular evidence from the parasitic wasps of the subfamily Sycoecinae and from the genus *Sycophaga* (subfamily Sycophaginae). The fact that, among all non-pollinating wasps, only species in these Old World groups enter the fig syconium to oviposit suggests that they could constitute a sister group of the pollinators (Bouček, 1993). We are also collecting more data from wasps of the subfamily Otitesellinae (e.g. *Heterandrium*, *Aepocerus*) to re-evaluate its validity as a taxonomic group. In addition, looking at the association of ecological and morphological characteristics of the parasitic wasps with their phylogenies provides clear evidence of convergence and plasticity at both morphological and ecological levels. Also, at a high level the parasite clades do not correspond very well with the pollinator clades, suggesting the possibility of major colonization or host-switching events during the evolution of parasitic wasps (Machado *et al.*, this issue and unpublished results).

A striking result from these studies is the documentation of the predominance of host specificity at a fine taxonomic level. With few exceptions, individual species of both

pollinators and parasites are associated with specific species of host figs. The mechanism underlying that specificity is almost certainly based on the chemicals released by the fig, and is consistent with the notion that both groups of wasps are attracted by the same chemicals (see also van Noort, Ware & Compton, 1989; Ware *et al.*, 1993; Hossaert-McKey, Gibernau & Frey, 1994). If true, then it is likely that a fig tree cannot attract its pollinator wasps without also attracting its parasites. One interesting research opportunity would be the characterization of the attractant chemicals of the different fig species followed by comparison to the phylogenies of selected host figs and their pollinators. Depending on the taxa chosen, such a dataset could be used to test whether the cases of non-specificity that have been reported predominantly occur among species that use chemically similar attractants.

Further, the *Idarnes* and *Critogaster* species for which we have data appear to have co-specified with species of *Pegoscapus* and *Tetrapus* that share the same host figs, supporting suggestions of strict-sense co-evolution by other authors (Gordh, 1975; Ulenberg, 1985; van Noort, 1991). Beyond constituting one of the few supported cases of co-speciation, these results specifically demonstrate phylogenetic co-evolution among clades of competitor species. A host fig phylogeny based on protein electrophoretic data suggests that strict-sense co-speciation also predominates among this group of pollinators and their host figs (Herre *et al.*, this issue). Thus, phylogenetic congruence across at least three taxonomic levels suggest

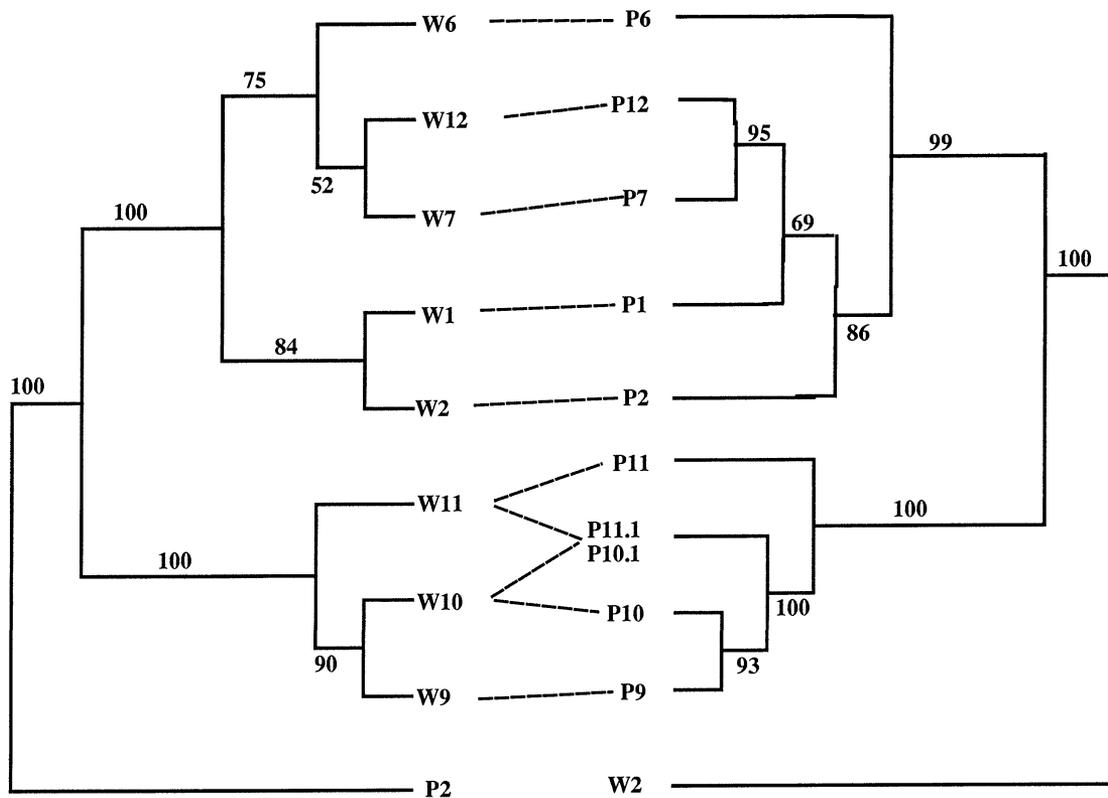


FIG. 5. Comparison of the pollinator and non-pollinator phylogenies (Figs 3 and 4, respectively). Four duplications and nine losses are required to explain the lack of congruence between the two phylogenies (see text).

that co-speciation has played a central role in the diversification of agaonid wasps.

Moreover, the parallel phylogenies of these organisms offers exciting opportunities to study factors that influence rates of molecular evolution. Each species of fig has several taxa or organisms associated with it: pollinators, several species of non-pollinating wasps, parasitic nematodes, mites, etc. (Corner, 1940). Because of their close ties to the ripening cycle of the fig fruit, most of these organisms share the same generation time. As mentioned above, the parallel phylogenies of the taxa thus far tested strongly suggest cospeciation (including nematodes, Machado *et al.*, in prep.). In those cases in which there has been cospeciation (e.g. pollinators and non-pollinators, wasps and nematodes, wasps and mites, etc.), the rates of change in homologous genes can be compared across distinct taxa which share the same generation time and have experienced the same history of divergence and speciation. Therefore, with generation time and divergence time constant across taxa, we will be able to determine whether there are rate differences in identical genes that correspond to phylum (e.g. wasps versus nematodes), to size and its correlates such as metabolic rate (e.g. different species of *Idarnes*), or life histories (e.g. the gallers and their parasitoids). Preliminary analyses strongly suggest that the nematode phylogenies are also largely congruent and that rate differences are

the rule rather than the exception (Machado *et al.*, in prep.).

Finally, although the genetical, ecological, and behavioural sampling and characterization of the fig and wasp taxa are incomplete and need to be augmented, our results strongly suggest the predominance of coupled, long-term evolutionary interactions among several different taxa (pollinator, parasite, nematode and host plant) with only occasional switching. It is therefore likely that the characters mentioned earlier (sex ratios, male morphologies and behaviour, fig characteristics, etc.) generally represent the outcome of selection within a relatively long-term co-evolutionary setting (Herre, this issue). Interestingly, we find that in some cases character combinations appear tightly linked with phylogeny (e.g. the wingless non-fighting males found in all the pollinators), while others are not. Indeed, various *Idarnes* species with very distinct ecologies, male morphologies and mating behaviours are placed together both by morphological and genetically based phylogenies. Moreover, very distantly related taxa often converge on very similar morphologies (van Noort, this issue), further supporting the idea that many characters can exhibit very high degrees of evolutionary plasticity and flexibility. In addition to expanding the taxa sampled and more in depth analyses of character evolution within co-speciating taxa, future work can be directed towards identifying those cases in which colonization of new hosts

(as opposed to co-speciation) has occurred and determining whether there have been detectable consequences for the evolution of some characters.

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REFERENCES

- Beckenbach, A.T., Wei, Y.W. & Liu, H. (1993) Relationships of the *Drosophila obscura* species group, inferred from mitochondrial cytochrome oxidase II sequences. *Mol. Biol. Evol.* **10**, 619–634.
- Berg, C.C. (1989) Classification and distribution of *Ficus*. *Experientia*, **45**, 605–611.
- Berg, C.C. & Wiebes, J.T. (1992) African fig trees and fig wasps. *Verhand. K. Ned. Akad. Wetensch.* **89**, 1–298.
- Bouček, Z. (1988) 6. Family Agaonidae. *Australasian Chalcidoidea (Hymenoptera). A biosystematic revision of genera of fourteen families, with a reclassification of species*, pp. 156–209. CAB International, Wallingford, U.K.
- Bouček, Z. (1993) The genera of chalcidoid wasps from *Ficus* fruit in the new world. *J. Nat. Hist.* **27**, 173–217.
- Bouček, Z., Watsham, A. & Wiebes, J.T. (1981) The fig wasp fauna of the receptacles of *Ficus thonningii*. *Tijdschr. Entomol.* **124**, 149–231.
- Bronstein, J.L. (1991) The non-pollinating wasp fauna of *Ficus pertusa*: exploitation of a mutualism? *Oikos*, **61**, 175–186.
- Clary, D.O. & Wolstenholme, D.R. (1985) The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. *J. Mol. Evol.* **22**, 252–271.
- Compton, S.G. (1993) One way to be a fig. *Afric. Entomol.* **1**, 151–158.
- Compton, S.G. & Hawkins, B.A. (1992) Determinants of species richness in southern African fig wasp assemblages. *Oecologia*, **91**, 68–74.
- Compton, S.G. & van Noort, S. (1992) Southern African fig wasps (Hymenoptera: Chalcidoidea): resource utilization and host relationships. *Proc. K. Ned. Akad. Wetensch.* **95**, 423–435.
- Corner, E.J.H. (1940) *Wayside Trees of Malaya*. Singapore Government Printing Office, Singapore.
- Corner, E.J.H. (1985) *Ficus* (Moraceae) and Hymenoptera (Chalcidoidea): figs and their pollinators. *Biol. J. Linn. Soc.* **25**, 187–195.
- Crozier, R.H., Crozier, Y.C. & Mackinlay, A.G. (1989) The COI and COII region of honey-bee mitochondrial DNA: evidence for variation in insect mitochondrial rates. *Mol. Biol. Evol.* **6**, 399–411.
- Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783–791.
- Felsenstein, J. (1993) PHYLIP, Phylogenetic Inference Package, Version 3.5. University of Washington, Seattle.
- Frank, S.A. (1984) The behavior and morphology of the fig wasps *Pegoscapus assuetus* and *P. jimenezii*: descriptions and suggested behaviors for phylogenetic studies. *Psyche*, **91**, 289–307.
- Frank, S. (1985) Hierarchical selection theory and sex ratios. II On applying the theory, and testing with fig wasps. *Evolution*, **39**, 949–964.
- Galil, J. (1977) Fig biology. *Endeavour*, **1**, 52–56.
- Godfray, H.C.J. (1988) Virginity in haplodiploid populations: a study on fig wasps. *Ecol. Entomol.* **13**, 283–291.
- Gordh, G. (1975) The comparative external morphology and systematics of the neotropical parasitic fig wasp genus *Idarnes* (Hymenoptera: Torymidae). *Univ. Kansas Sci. Bull.* **50**, 389–455.
- Hafner, M.S. & Nadler, S.A. (1988) Phylogenetic trees support the coevolution of parasites and their hosts. *Nature*, **332**, 258–259.
- Hafner, M.S. & Nadler, S.A. (1990) Cospeciation in host-parasite assemblages: comparative analysis of rates of evolution and timing of cospeciation events. *Syst. Zool.* **39**, 192–204.
- Hafner, M.S., Sudman, P.D., Villablanca, F.X., Spradling, T.A., Demastes, J.W. & Nadler, S.A. (1994) Disparate rates of molecular evolution in cospeciating hosts and parasites. *Science*, **265**, 1087–1090.
- Hamilton, W.D. (1967) Extraordinary sex ratios. *Science*, **156**, 477–488.
- Hamilton, W.D. (1979) Wingless and fighting males in fig wasps and other insects. *Sexual Selection and Reproductive Competition in Insects* (ed. by M.S. Blum and N.A. Blum), pp. 167–220. Academic Press, London.
- Hawkins, B.A. & Compton, S.G. (1992) African fig wasp communities: undersaturation and latitudinal gradients in species richness. *J. Anim. Ecol.* **61**, 361–372.
- Herre, E.A. (1985) Sex ratio adjustment in fig wasps. *Science*, **228**, 896–898.
- Herre, E.A. (1987) Optimality, Plasticity, and selective regime in fig wasp sex ratios. *Nature*, **329**, 627–629.
- Herre, E.A. (1989) Coevolution of reproductive characteristics in twelve species of new world figs and their pollinator wasps. *Experientia*, **45**, 637–647.
- Herre, E.A., West, S.A., Cook, J.M., Compton, S.G. & Kjellberg, F. (1996) Fig wasps: pollinators and parasites, sex ratio adjustment and male polymorphism, population structure and its consequences. In *Social Competition and Cooperation in Insects and Arachnids: I. Evolution of mating systems* (ed. by J. Choe and B. Crespi). Cambridge University Press, Cambridge.
- Higgins, D.G. & Sharp, P.M. (1988) CLUSTAL: a package for performing multiple sequence alignments on a microcomputer. *Gene*, **73**, 237–244.
- Higuchi, R.G. & Oehman, H. (1989) Production of single-stranded DNA templates by exonuclease digestion following the polymerase chain reaction. *Nucl. Ac. Res.* **17**, 5865.
- Hossaert-McKey, M., Gibernau, M. & Frey, J.E. (1994) Chemosensory attraction of fig wasps to substances produced by receptive figs. *Ent. Exp. Appl.* **70**, 185–191.
- Janzen, D.H. (1979) How to be a fig. *Ann. Rev. Ecol. Syst.* **10**, 13–51.
- Joseph, K.J. (1957) Contributions to our knowledge of fig insects (Chalcidoidea: Parasitic Hymenoptera) from India. VII. Descriptions of fifteen new and revision of some old species. *Bull. Soc. Ent. Fr.* **125**, 97–133.
- Kishino, H. & Hasegawa, M. (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *J. Mol. Evol.* **29**, 170–179.
- Kjellberg, F. (1983). La strategie reproductive du figuier (*Ficus carica*) et de son pollinisateur (*Blastophaga psenes* L.), un exemple de coevolution. PhD thesis, Institut Nationale Agronomique, France.
- Kjellberg, F., Gouyon, P.-H., Ibrahim, M. & Valdeyron, G. (1987) The stability of the symbiosis between dioecious figs and their

- pollinators: a study of *Ficus carica* L. and *Blastophaga psenes* L. *Evolution*, **41**, 693–704.
- Kocher, T.D., Thomas, W.K., Meyer, A., Pääbo, S.V., Villablanca, F.X. & Wilson, A.C. (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Natl Acad. Sci. U.S.A.* **86**, 6196–6200.
- Kumar, S., Tamura, K. & Nei, M. (1993) MEGA: Molecular Evolutionary Genetics Analysis, Version 1.0. The Pennsylvania State University, University Park, Pennsylvania.
- Liu, H.L. & Beckenbach, A.T. (1992) Evolution of the mitochondrial cytochrome oxidase II gene among 10 orders of insects. *Mol. Phylogenet. Evol.* **1**, 41–52.
- Maddison, W.P. & Maddison, D.R. (1992) *MacClade: Analysis of phylogeny and character evolution*, Version 3.0 Sinauer Associates, Sunderland, Massachusetts.
- Moran, N. & Baumann, P. (1994) Phylogenetics of cytoplasmically inherited microorganisms of arthropods. *Trends Ecol. Evol.* **9**, 15–20.
- Murray, M.G. (1989) Environmental constraints on fighting in flightless male fig wasps. *Anim. Behav.* **38**, 186–193.
- Page, R.D.M. (1993a) *COMPONENT*, Version 2.0. Natural History Museum, London.
- Page, R.D.M. (1993b) Maps between trees and cladistic analysis of historical associations among genes, organisms and areas. *Syst. Biol.* **43**, 58–77.
- Page, R.D.M. (1994) Genes, organisms and areas: the problem of multiple lineages. *Syst. Biol.* **42**, 77–84.
- Ramirez, W.B. (1974) Specificity of Agaonidae: the coevolution of *Ficus* and its pollinators. PhD thesis, University of Kansas.
- Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. & Ehrlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, **239**, 487–491.
- Saitou, N. & Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425.
- Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York.
- Sanger, F., Nicklen, S. & Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. U.S.A.* **74**, 5463–5467.
- Simon, C., Pääbo, S.V., Kocher, T.D. & Wilson, A.C. (1989) Evolution of mitochondrial ribosomal RNA in insects as shown by the polymerase chain reaction. *Molecular Evolution. UCLA Symposia on Molecular and Cellular Biology*. New series, vol. 122 (ed. by M. Clegg and S. Clark), pp 235–244. Alan R. Liss Inc., New York.
- Swofford, D. (1991) *PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1*. Illinois Natural History Survey, Champaign, Illinois.
- Tamura, K. (1992) Estimation of the number of nucleotide substitutions when there are strong transition–transversion and G + C-content biases. *Mol. Biol. Evol.* **9**, 678–687.
- Ulenberg, S.A. (1985) The phylogeny of the genus *Apocrypta Coquerl* in relation to its hosts, *Ceratosolen mayr* (Agaonidae) and *Ficus* L., *Verhand. K. Ned. Akad. Wetensch.* **83**, 149–176.
- van Noort, S. (1991) The systematics and phylogenetics of the Sycoecinae (Agaonidae, Chalcidoidea, Hymenoptera). PhD thesis, Rhodes University, Grahamstown, South Africa.
- van Noort, S., Ware, A.B. & Compton, S.G. (1989) Pollinator-specific volatile attractants released from the figs of *Ficus burtt-davyi*. *Sud.-Afrik. Tydskr. Wetens.* **85**, 323–324.
- Ware, A.B., Perry, T.K., Compton, S.G. & van Noort, S. (1993) Fig volatiles: their role in attracting pollinators and maintaining pollinator specificity. *Plant Syst. Evol.* **186**, 147–156.
- West, S. & Herre, E.A. (1994) The ecology of the New World fig parasitizing wasps *Idarnes* and implications for the evolution of the Fig–pollinator mutualism. *Proc. R. Soc. Lond. B*, **258**, 67–72.
- Wiebes, J.T. (1979) Co-evolution of figs and their insect pollinators. *Ann. Rev. Ecol. Syst.* **10**, 1–12.
- Wiebes, J.T. (1982) The phylogeny of the Agaonidae (Hymenoptera, Chalcidoidea). *Neth. J. Zool.* **32**, 395–411.
- Wiebes, J.T. (1995) Agaonidae (Hymenoptera, Chalcidoidea) and *Ficus* (Moraceae): fig wasps and their figs, xv (Meso-American *Pegoscapus*). *Proc. K. Ned. Akad. Wetensch.* **98**, 167–183.
- Willis, L.G., Winston, M.L. & Honda, B.M. (1992) Phylogenetic relationships in the honey bee (Genus *Apis*) as determined by the sequence of the cytochrome oxidase II region of mitochondrial DNA. *Mol. Phylogenet. Evol.* **1**, 169–178.

Note added in proof:

We have recently collected sequences from the 12S mtDNA region of three species of Old World non-pollinating wasps that enter the fig syconium to oviposit: two Sycoecinae (*Crossogaster* sp., *Philocaenus* sp.) and *Sycophaga sycomori* (Sycophaginae). Phylogenetic analyses of those sequences put the Sycoecinae together with members of the subfamily Sycoryctinae. *Sycophaga sycomori* falls inside the Sycophaginae. Therefore, the ability to enter the fig syconium has arisen at least two times during the evolution of the non-pollinating fig wasps. Moreover, the non-pollinating wasps that enter the fig to oviposit do not appear to be the closest relatives of the pollinators as was previously suggested.