

Breakdown of the one-to-one rule in Mexican fig-wasp associations inferred by molecular phylogenetic analysis

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Abstract

The interaction between figs (*Ficus* spp., Moraceae) and fig-pollinating wasps (Chalcidoidea, Agaonidae) is one of the most species-specific cases of mutualism, and is a model system for studying coevolution and cospeciation between insects and plants. To test the specificity-breakdown hypothesis, we performed a phylogenetic analysis using mitochondrial COI gene sequences of Mexican fig-pollinating wasps collected from each fig species at various localities. Phylogenetic analysis revealed a clear division of Mexican fig pollinators into two major groups: one pollinating the *Ficus* species of subgenus *Pharmacosycea*; and the other pollinating *Ficus* subgenus *Urostigma*. In some cases, wasps pollinating the same fig species do not form clades, and in others, pollinators of different fig species have identical gene sequences. Incorporation of COI sequences of Panamanian fig-pollinating wasps also showed inconsistency between pollinator phylogeny and host fig species. These results suggest: 1) the possible breakdown of the “one-to-one rule” in Mexican fig-wasp associations; 2) the absence of phylogenetic evidence for cospeciation between the *Ficus* species and its pollinators; and 3) host switching may occur frequently among these pollinating wasps. Our findings also suggest that the *Americana*-pollinating wasps have radiated into various lineages within a short time.

Keywords: *Ficus*, fig wasp, COI, phylogenetic tree, cospeciation, coevolution

1. Introduction

The genus *Ficus* (figs: family Moraceae) is composed of about 750 species, which are widely distributed in the tropical and subtropical regions of the world (Corner, 1965; Berg, 1989). Figs play an important role in tropical rain forests because of the year-round production of fruits that are essential for the maintenance of a large number of frugivores such as birds and mammals (McKey, 1989; Lambert and Marshall, 1991), and consequently ecological networks are centralized around the fig trees. To understand and conserve the biodiversity of terrestrial ecosystems in tropical and subtropical regions, it is therefore important to

study the mechanisms maintaining the interactions and the diversification in *Ficus* and their associated animals (Janzen, 1979; Frank, 1989; Nason et al., 1998; Serrato et al., 2004).

All figs have a closed, urn-shaped inflorescence (or syconium), which is lined with unisexual florets. Figs present a unique pollinating system with fig wasps (Hymenoptera: Chalcidoidea: Agaonidae), which carry pollen grains into the syconium through the ostiole, a narrow entrance protected by bracts. In this system, fig wasps also gain benefits from figs, as they lay eggs in some of the fig ovaries, and larval development is entirely completed within the fig.

The interaction between figs and fig-pollinating wasps is probably the most species-specific case of pollination mutualism known, and is a model system for investigating coevolution and cospeciation between insects and plants

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Table 1. List of the *Ficus* species from which the pollinating wasps were collected for this study.

<i>Ficus</i> species	Voucher	Sample	No.	Locality	Accession nos.
<i>F. yoponensis</i>	ASD 025	<i>Tetrapus ecuadoranus</i> M	09	Temazcal, Oaxaca	AB308322
<i>F. lapathifolia</i>	ASD 017	<i>T. sp.</i>	10	Matías Romero, Oaxaca	AB308323
	ASD 017b	<i>T. sp.</i>	29	Matías Romero, Oaxaca	AB308324
<i>F. maxima</i>	ASD 040	" <i>T. americanus</i> MA"	01a	Valle de Tehuacán-Cuicatlán, Oaxaca	AB308325
	ASD 040	" <i>T. americanus</i> MA"	01b	Valle de Tehuacán-Cuicatlán, Oaxaca	AB308326
	GIM w/n	" <i>T. americanus</i> MB"	23	Tuxtlas, Veracruz	AB308327
<i>F. insipida</i>	ASD 028	<i>T. costaricanus</i> M	15	Valle Nacional, Oaxaca	AB308328
	GIM w/n	<i>T. costaricanus</i> M	18	Chamela, Jalisco	AB308329
	GIM w/n	<i>T. costaricanus</i> M	47a	Chamela, Jalisco	AB308330
	GIM w/n	<i>T. costaricanus</i> M	47b	Chamela, Jalisco	AB308331
	GIM w/n	<i>T. costaricanus</i> M	50	Chamela, Jalisco	AB308332
	GIM w/n	<i>T. costaricanus</i> M	51	Chamela, Jalisco	AB308333
	ASD 015	<i>T. costaricanus</i> M	44	Matías Romero, Oaxaca	AB308334
<i>F. microcarpa</i>	ASD et al. 056	<i>T. costaricanus</i> M	57 (D)	La Cañada, Cuernavaca, Morelos	AB308329
	ASD 048	<i>Eupristina</i> sp.	06	Cd. Valles, San Luis Potosí	AB308335
	ASD 023b	" <i>Pegoscapus</i> sp. 1"	21	Cuatro Caminos, Oaxaca	AB308336
	ASD 023	" <i>P. sp. 2</i> "	35	Arroyo Azul, Oaxaca	AB308337
<i>F. pertusa</i>	ASD 032	" <i>P. silvestrii</i> "	31a	Tuxtlas, Veracruz	AB308340
	ASD 032	" <i>P. silvestrii</i> "	31c	Tuxtlas, Veracruz	AB308341
	ASD 009	" <i>P. sp. 3</i> "	41	Tehuantepec, Oaxaca	AB308338
<i>F. americana</i>	ASD 033	" <i>P. sp. 4</i> "	40	Tuxtlas, Veracruz	AB308339
	ASD 048	" <i>P. standleyi</i> "	07	Tamul, San Luis Potosí	AB308342
<i>F. cotinifolia</i>	ASD 010	" <i>P. sp. 5</i> "	30	Tehuantepec, Oaxaca	AB308338
	ASD 041	" <i>P. kraussii</i> "	36	Gómez Farías, Tamaulipas	AB308343
	ASD 052	" <i>P. kraussii</i> "	37	Tamul, San Luis Potosí	AB308344
	ASD 011	" <i>P. kraussii</i> "	45	San Pedro Huamelula, Oaxaca	AB308345
<i>F. glyxicarpa</i>	GIM w/n	" <i>P. sp. 6</i> "	20	Chamela, Jalisco	AB308346
	ASD et al. 055	<i>P. sp. 7</i>	58 (E)	La Cañada Cuernavaca, Morelos	AB308347
<i>F. aurea</i>	ASD 024	" <i>P. jimenezzi</i> "	12	Temazcal, Oaxaca	AB308348
	ASD 029	" <i>P. sp. 8</i> "	13	Tuxtlas, Veracruz	AB308349
	ASD 030	" <i>P. sp. 8</i> "	27	Tuxtlas, Veracruz	AB308350
	ASD 042	<i>P. sp. 9</i>	04	Alta Cima, Tamaulipas	AB308351
<i>F. calyculata</i>	ASD 039	<i>P. bruneri</i>	03	Valle de Tehuacán-Cuicatlán, Oaxaca	AB308352
	ASD 014	<i>P. bruneri</i>	43	San Mateo del Mar, Oaxaca	AB308353
<i>F. trigonata</i>	ASD 012	" <i>P. sp. 10</i> "	11	San Pedro Huamelula, Oaxaca	AB308354
	ASD et al. 060	" <i>P. sp. 10</i> "	56 (C1)	Las Estacas, Morelos	AB308355
	ASD et al. 060	" <i>P. sp. 10</i> "	56 (C2)	Las Estacas, Morelos	AB308356
	GIM w/n	" <i>P. sp. 11</i> "	32	San Carlos, Sonora	AB308357
<i>F. petiolaris</i>	GIM w/n	<i>P. sp. 12</i>	14	Las Palmas, Baja California Sur	AB308358
	GIM w/n	<i>P. sp. 12</i>	16	Las Palmas, Baja California Sur	AB308359

(Wiebes, 1979; Herre, 1996; Machado et al., 2001; Weiblen, 2000). It was long thought that each fig species had only one associated species of fig wasp as its pollinator, and each wasp is only associated with one species of fig, generally called the "one-to-one" rule. Molecular phylogenetic studies generally support the hypothesis of coevolution between recognized genera of pollinating wasps and their respective sections of *Ficus* (Herre et al., 1996; Kerdelhué et al., 1999; Machado et al., 2001; Weiblen, 2001; 2004; Weiblen and Bush, 2002; Jusselin et al., 2003; Rønsted et al., 2005). However, these phylogenetic studies have focused mainly on testing the hypothesis of fig-wasp coevolution at a higher taxonomic level. Although these data suggest cospeciation between figs and pollinating wasps, the degree and extent of the "one-to-one" rule and the specifics of cospeciation between figs and wasps are not well understood. For example, Kerdelhué et al. (1997) reported three different *Ceratosolen* species as pollinators of *Ficus sur* (subgenus *Sycomorus*), and Molbo et al. (2003) suggested the existence of cryptic

species of pollinating wasps in some Panamanian fig species. A critical review of host specificity and its coevolutionary implications in fig/fig-wasp mutualism has been published recently (Machado et al., 2005). It is thus important to conduct detailed population genetic analyses by sampling throughout the distributional range for each fig species to test the degree of species-specificity in the fig-wasp mutualism. In this study, we used mitochondrial COI sequences to analyze the phylogenetic relationships of fig-pollinating wasps collected from the fig of subgenera *Pharmacosycea* and *Urostigma* growing at localities in Mexico.

2. Materials and Methods

Sampling

We collected fig-pollinating wasps from syconia of 36 fig trees distributed in 21 localities in Mexico; 4 fig species

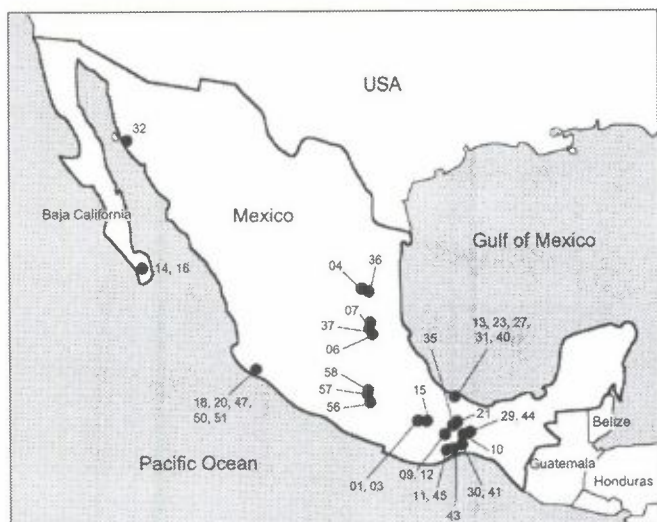
Phylogenetic analysis

Figure 1. Locality map of samples used in this study. The sample numbers correspond to that in Table 1 and phylogenetic tree.

belonged to the subgenus *Pharmacosycea* (Miquel) section *Pharmacosycea* Miquel and 10 fig species to the subgenus *Urostigma* Gasparrini section *Americana* Miquel (Table 1 and Fig. 1). Vouchers are deposited at the herbarium of the Universidad Nacional Autónoma de México (MEXU-UNAM). The fig-pollinating wasps were preserved in 99% ethanol until used for DNA extraction.

DNA extraction, PCR and sequencing

Total DNA was extracted from a single individual wasps using a QIAamp DNA Mini Kit (QIAGEN, Germany). DNA for each specimen was finally dissolved in 200 μ l elution buffer, and 4 μ l of the DNA solution was used as a template for amplification of DNA fragments by polymerase chain reaction (PCR). A fragment of the mitochondrial DNA containing an approximately 1000 bp 5'-region of the cytochrome oxidase subunit I (COI) gene was amplified using a primer pair

(forward:

COI1-1-Ple 5'-TTAATTGGAAATGATCAAATTTATAAT-3';

reverse:

COI-2M 5'-ACATAATGAAAATGTGCTACTACATAATA-3')

designed for this study. The thermal conditions for amplification were as follows: denaturation at 94°C (5 min); followed by 35 cycles of denaturation at 94°C (30 sec), annealing at 50°C (30 sec), extension at 72°C (2 min); and final extension at 72°C (7 min). Direct sequencing was performed on an automated ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Inc., HITACHI). The COI sequence data of Panamanian fig-pollinating wasps were taken from GenBank database.

Sequence alignments were carried out using the multiple-alignment program CLUSTAL W, version 1.81 (Thompson et al., 1994) with default settings (Gap Opening Penalty = 10.0; Gap Extension Penalty = 0.20; Delay Divergent Sequences = 30%; DNA Transitions Weight = 0.50). A NEXUS format file of the complete alignment was used for neighbor-joining (NJ) (Saitou and Nei, 1987), maximum likelihood (ML) (Felsenstein, 1981) and maximum parsimony (MP) analyses with PAUP 4.0b (Swofford, 2001). Construction of the NJ trees was performed using evolutionary distance computed by Kimura's two-parameter method (Kimura, 1980). For ML analysis, heuristic searches were carried out using the HKY85 model of the nucleotide substitutions with tree-bisection-reconnection (TBR) branch-swapping under the default settings. The starting tree was obtained via stepwise addition, and the starting branch lengths were obtained using the Rogers-Swofford approximation method (Rogers and Swofford, 1998). Trees with approximate likelihoods 5% or further from the target score were rejected without additional iteration. A heuristic search was also performed for MP analysis under the default settings. All trees were evaluated using the bootstrap test (Felsenstein, 1985) based on 1000 replicates for the NJ and MP, and 100 replicates for the ML analysis.

3. Results*Properties of the COI gene sequence*

An 868 bp sequence of the COI gene was included in the analyses after excluding the end positions of the sequences due to the fact that they were missing from some samples. A total of 287 (33%) characters were variable (217 at the codon third position, 54 at the first position and 16 at the second position) among the sequences of all the pollinating wasps determined, and 207 (23.8%) characters were variable (176 at the codon third position, 25 at the first position and 6 at the second position) within the genus *Pegoscapus*. The base substitutions, which mostly occurred at silent sites (the third position of codon and a part of the first position), were randomly distributed throughout the sequences. The maximum sequence difference of the COI gene region examined for all the fig pollinating wasps was 0.181 (Kimura's two-parameter distance), which was the difference between the "*Tetrapus americanus*" (01a: one specimen of the pollinators of *Ficus maxima*) and the *Pegoscapus* sp. (04a: the pollinator of *F. calyculata*), while that within the genus *Pegoscapus* was 0.107 between the "*Pegoscapus* sp. 1" (21a: a specimen of the pollinator from *F. microcarpa*) and the "*Pegoscapus* sp. 10" (11a: a specimen of the pollinator from *F. petiolaris*).

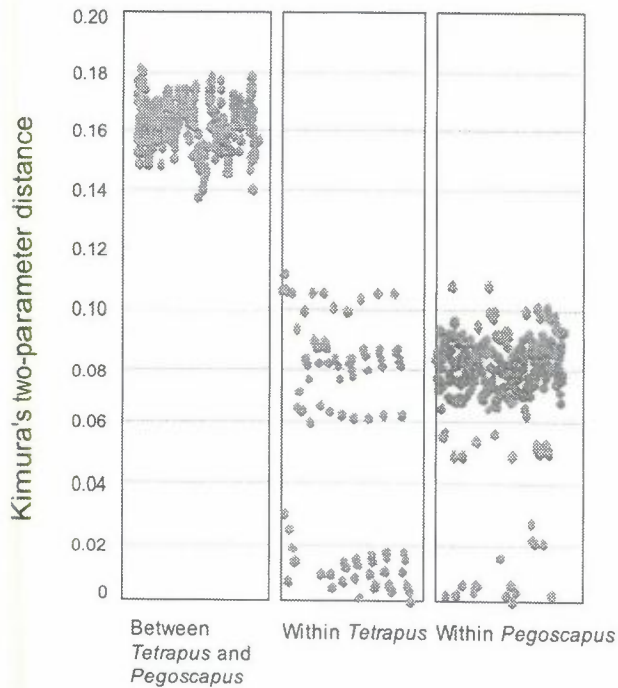


Figure 2. Kimura's two-parameter distance between both genera (*Tetrapus* and *Pegoscapus*) and within the genera.

Also, the sequence differences between the two pollinator genera, *Tetrapus* and *Pegoscapus*, were approximately 0.16 (Fig. 2). The evolutionary distances at three positions (1st, 2nd and 3rd), which were corrected for multiple substitutions with Kimura's two parameter method (Kimura, 1980), exhibited an almost linear correlation with the observed p (base substitution percentage) (Fig. 3), suggesting that the base substitution of the COI gene was not saturated within the range of the sequence differences detected in Mexican fig-pollinating wasps. The COI gene sequences were AT-rich (ranged from 72.22% to 75.79%) and their G + C contents were nearly constant.

Phylogeny of Mexican fig-pollinating wasps

We first used the mitochondrial COI gene sequence to analyze the phylogenetic relationships of fig wasps, including both the pollinating and non-pollinating wasps, collected from all the fig species (Table 1). The phylogenetic tree of the COI gene, which was constructed by the NJ method and rooted with *Apis mellifera*, *Drosophila melanogaster* and *Bombyx mori*, showed that the fig-pollinating wasps were clearly grouped into a single clade with a robust support of 99% bootstrap (BS) value, and all the non-pollinating wasps were their sister groups (data not shown). Then, we used non-pollinating wasps as the outgroup to analyze the phylogenetic relationships of

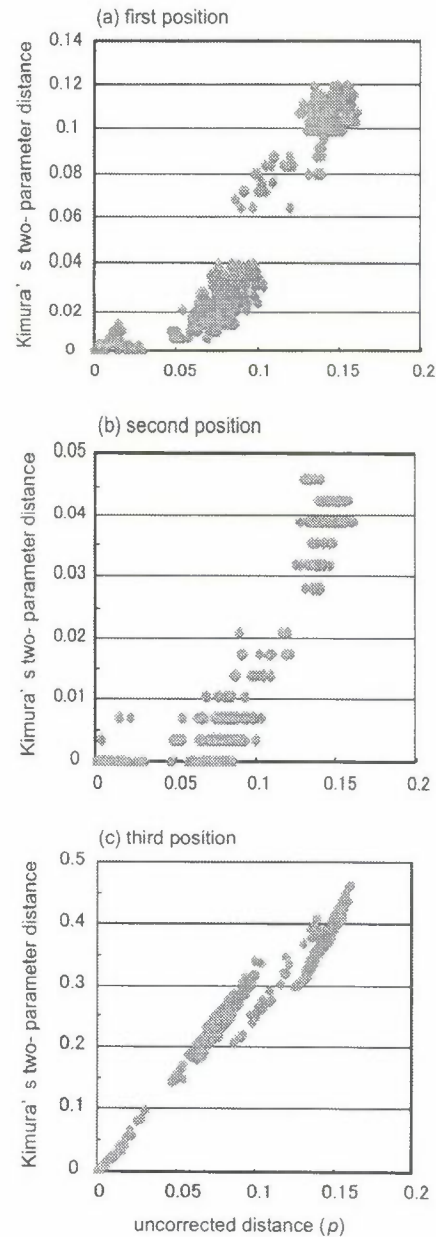


Figure 3. Saturation plots of each codon position calculated through pairwise sequence comparisons.

pollinating wasps. The three methods (NJ, ML and MP) gave essentially the same tree topology and the NJ tree was shown (Fig. 4). Although *T. americanus* (23a) appears to have different grouping between NJ and ML/MP trees, neither one has robust support with the bootstrap value (Fig. 4). The results showed *Tetrapus* (100% BS) as sister to a clade with *Pegoscapus* (87–100% BS) and *Eupristina*. The phylogenetic relationships within genera of pollinators, on the other hand, were largely inconsistent with their host

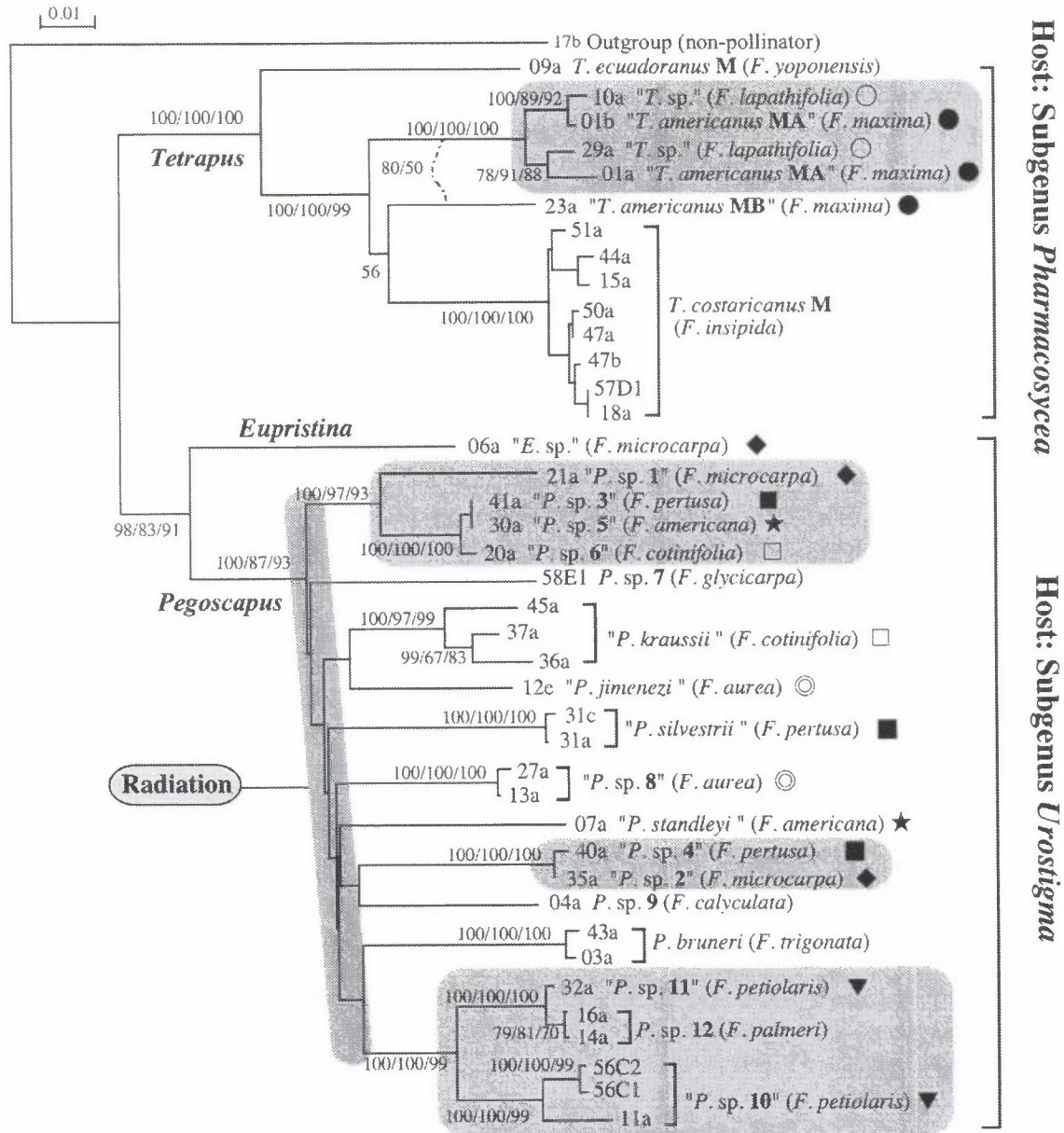


Figure 4. NJ-phylogenetic tree of Mexican fig-pollinating wasps based on the mitochondrial COI gene sequence. The numbers in each branching point indicate the bootstrap percentage of NJ/ML/MP (those less than 50% are not shown). The dotted line shows the grouping of *Tetrapus americanus* (23a) on ML and MP trees. The tree was outgroup-rooted using the COI gene sequences of a non-pollinating wasp. Locality numbers shown in the end of branches correspond to those in Table 1 and Fig. 1. The symbols after the *Ficus* species name indicate the pollinating wasps, which are from same *Ficus* species, but separated into different lineages. The clusters including the pollinating wasps from different *Ficus* species are shaded.

fig species. For example, the pollinating wasps from *F. maxima* in the *Tetrapus* clade were separated into at least two lineages (23a and 01a/01b), and one (01a/01b) of them was related to the pollinators (10a/29a) of *F. lapathifolia* so closely as to be indistinguishable on the tree (Fig. 4). In the *Pegoscapus* clade the pollinating wasps appear to have radiated into 11 lineages within a short time. Most fig species-associated wasps did not cluster into a single clade

per each host species. *Ficus pertusa* associated wasps were clearly divided into three different lineages, and two of these were each clustered with the associated wasps of *F. microcarpa* and *F. americana*/*F. cotinifolia*, and were hardly distinguishable from each other by sequence differences. Two independent lineages of the associated wasps were also found in *F. cotinifolia*, *F. americana*, *F. aurea* and *F. petiolaris*, respectively. In some lineages,

meanwhile, the wasp species from more than two *Ficus* species were closely clustered together with only small sequence differences; one striking case is the wasp collected in *F. petiolaris* in Sonora and the wasp collected in *F. palmeri* in Baja California, both fig species are endemics and geographically separated by the Gulf of California (Fig. 4). It is also interesting to note some lineages of wasp pollinators are differentiated within the same host fig species but in different geographic regions; such is the case of *T. costaricanus* and *F. insipida* (Fig. 4).

Relationship between the fig-pollinating wasps of the Mexico and Panama

In order to discover the biogeographical differences between the fig-pollinating wasps of Mexico and Panama, we constructed a phylogenetic tree (Fig. 5) by adding COI gene sequences (from GenBank; www.ncbi.nlm.nih.gov) of Panamanian fig-pollinating wasps (Molbo et al., 2003; Machado et al., 2005) to our data set. A 358 bp sequence overlapping the two data sets was used in this analysis. The result showed essentially the same topology as when using only Mexican samples; the two pollinator wasp genera, *Tetrapus* and *Pegoscapus* were monophyletic (Fig. 5). Within the *Tetrapus* clade, the pollinator wasps of *F. insipida* from Panama formed an independent lineage, while the Mexican wasps presented another lineage and clearly clustered with other wasp species associated with the *F. maxima*, *F. glabrata* and *F. lapathifolia* (Fig. 5). Several lineages of the pollinating wasp species of *F. maxima* were scattered throughout the *Tetrapus* clade, and only one (*T. americanus* PA) of these was independent, implying that these lineages of the *F. maxima*-associated wasps do not have a common origin. *Ficus yoponensis* associated wasps from Mexico and Panama constituted a single lineage. In the *Pegoscapus* clade, sixteen lineages of Panamanian fig-wasps were recognized in this analysis, but almost all these lineages were phylogenetically independent from Mexican wasps except for one lineage, *P. silvestrii* which showed a close relationship to the pollinator of Mexican *F. pertusa* (Fig. 5). The divergence within the two pollinator species, *T. ecuadoranus* and *P. silvestrii* probably indicates the biogeographical differentiation of the fig wasps between Mexico and Panama.

4. Discussion

Radiation of phylogenies of Pegoscapus wasps

Eleven lineages were recognized in the phylogenetic tree of *Pegoscapus* wasps. However, it is noteworthy that the branching order of these phylogenetic lineages could not be determined with certainty, because branches of only very short lengths with low bootstrap values support these

basal nodes (Fig. 4). Two possible explanations for the ambiguous branching order of these lineages may be considered: the first is that the nucleotide substitutions of the COI gene are saturated; and the second is that these *Pegoscapus* lineages radiated within a short time. The first possibility, saturation of nucleotide substitutions, does not seem to be the case because the actual percentage of substitutions is linearly correlated to the evolutionary distance that is corrected for multiple substitutions by Kimura's method (1980) (Fig. 3). Consequently, a reasonable interpretation for tree topology would be that a radiation of the *Pegoscapus* occurred. Judging from the result shown in Fig. 5, the divergence of Panamanian lineages of *Pegoscapus* would also be included in such a radiation event. The divergence time between two *Pegoscapus* species (*P. hoffmeyerii* and *P. gemellus*) was estimated to be 21 (± 6.5) million years ago (Mya) (Machado et al., 2001) originally based on fossil data of *Pegoscapus* wasps. On the other hand, the divergence of the two *Pegoscapus* species corresponds to the radiation of this genus (Fig. 5). Thus, the occurrence of the radiation of *Pegoscapus* wasps can be presumed to be about 21 Mya. According to this divergence time and the average sequence difference (8.15%) between those lineages, the evolutionary rate of the COI gene sequence of *Pegoscapus* wasps was calculated to be 0.39% per million years (Myr). The calculated rate is close to that estimated from ground beetle COI sequences, about 0.01 *D* unit (Kimura's two parameter distance) of the COI sequence corresponding to about 2.7 Myr (about 0.37% per million years) (Su et al., 2004), although the COI gene seems to evolve fast in some other insects (Brower, 1994; Farrell, 2001; Caccone and Sbordoni, 2001).

More than one lineage is detected in the pollinating wasps from the same Ficus species

The phylogenetic relationships within each genus (*Tetrapus* and *Pegoscapus*) of pollinators are largely disordered. Wasps pollinating the same fig species do not cluster together into a single group in most of the cases; rather, they are separated into more than one different lineage on the COI tree, grouping with pollinators from other fig species, while some pollinators from different fig species are clustered into the same lineage, some of which show identical sequences (Figs. 4 and 5). These results are inconsistent with the hypotheses of the "one-to-one" rule and cospeciation of fig-wasp mutualism, and strongly suggest that more than one associated wasp species per fig species. For example, the pollinating wasps (*T. americanus*) of *F. maxima* of subgenus *Pharmacosyceae* are separated into at least three clearly different lineages: the "*T. americanus* MA" from Mexico; the "*T. americanus* PA" from Panama; and the "*T. americanus* MB/PB" from both regions (Fig. 5).

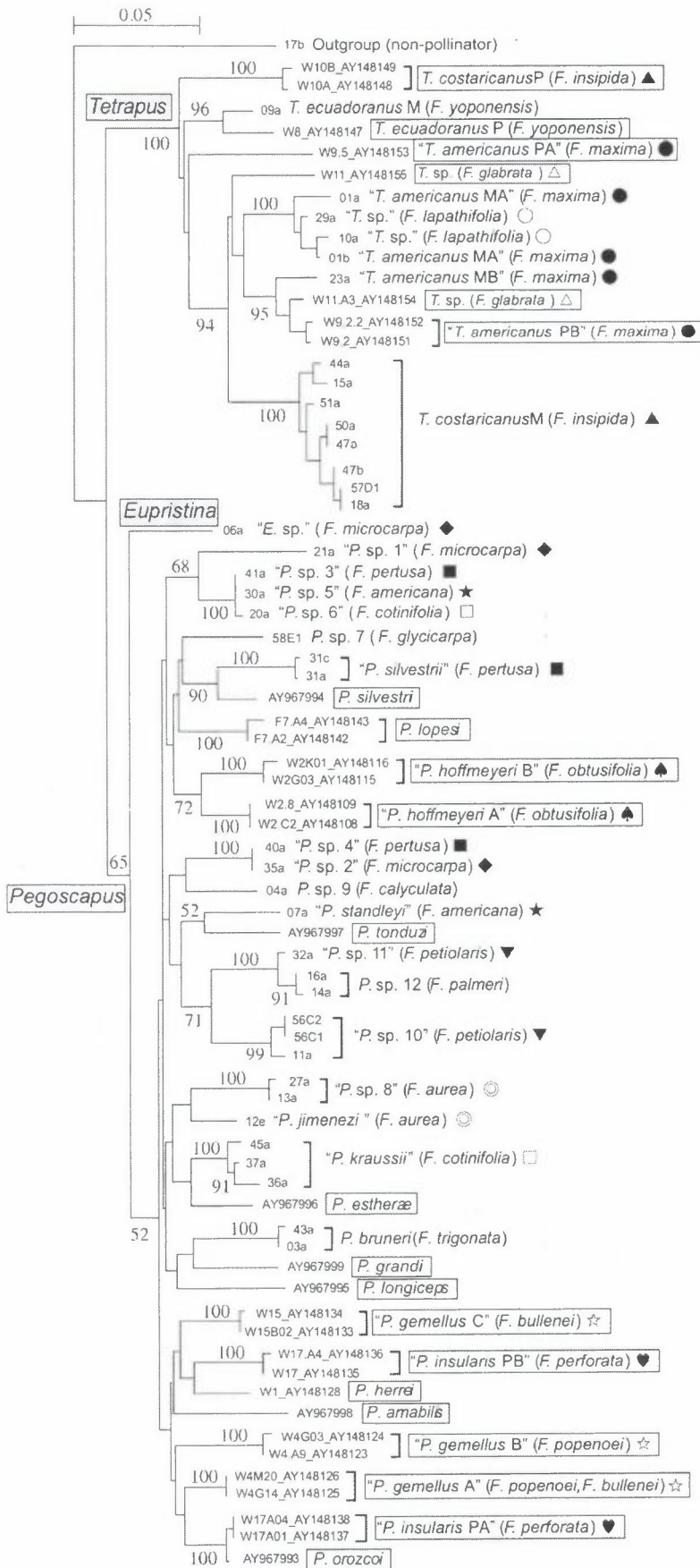


Figure 5. Phylogenetic tree (NJ) of the fig-pollinating wasps from Mexican and Panamanian *Ficus* species based on the mitochondrial CO1 gene sequence. For the descriptions, see Fig. 4. The sequence data of Panamanian samples (boxed) are from GenBank database, and their original sample numbers and sequence accession numbers are shown before sample name.

These results suggest that *F. maxima* may have three associated wasp species including one that was previously reported as a cryptic species of *T. americanus* in Panama (Molbo et al., 2003). Also, the pollinating wasps of *F. insipida* split into two completely independent lineages, one in Mexico and another in Panama, suggesting the existence of two wasp species pollinating *F. insipida* in different geographic regions. Similarly, multiple species of pollinating wasps were also observed in the genus *Pegoscapus*, such as the pollinators of *F. pertusa*, *F. cotinifolia*, *F. americana* and *F. petiolaris*. Combined with the previous study (Molbo et al., 2003), molecular findings strongly suggest that multiple species of pollinator wasps per fig species are likely to exist extensively in the two *Ficus* sections, *Pharmacosycea* and *Americana*.

No molecular evidence is found in this study to support the cospeciation hypothesis of fig-wasp mutualism

Morphological studies and the “one-to-one” species-specificity of the interaction between figs and pollinating wasps have led to the hypothesis of coevolution and cospeciation between them (Ramirez, 1974; Wiebes, 1979; Berg, 1989). Recent molecular phylogenetic analyses have also provided some evidence to support the presence of cladogenesis and coadaptation between recognized genera of pollinating wasps and their respective fig sections (Herre et al., 1996; Machado et al., 2001; Weiblen, 2001; 2004; Jousselein et al., 2003). However, these results do not mean that strict cospeciation between figs and pollinators has taken place at any taxonomic level or within any groups. Machado et al. (2005) suggested that a strict-sense cospeciation of one-to-one species specific figs and wasps should not be the default paradigm for formulating hypotheses to explain the diversification mode for fig and wasp species. Our results have revealed numerous cases of more than one wasp species pollinating one fig species in the two fig sections, *Pharmacosycea* (subgenus *Pharmacosycea*) and *Americana* (subgenus *Urostigma*), and, in addition, such species are not clustered into a single group, but fall into independent lineages (Figs. 4 and 5). In other words, the wasp lineages (or species) pollinating the same fig species do not always have a common origin. This suggests that host-switching has taken place in these species, and may be accompanied by a morphologically convergent (parallel) evolution to adapt to the same host fig species. An interesting example of host shift is that some *Pegoscapus* wasps are likely to pollinate *F. microcarpa* which is an introduced species in the Americas, and normally pollinated by *Eupristina* species. A pseudogene or mitochondrial introgression may be considered as explanations for these results, but a supplementary analysis of nuclear 28S rRNA gene sequence of these wasps showed essentially the same tree topology as the mitochondrial COI gene (data not shown). The coincidence of the results

between nuclear and mitochondrial genes strongly precludes the possibility of a pseudogene or mitochondrial introgression. The findings obtained in this study, therefore, do not provide total support for the cospeciation hypothesis, and indicate that host-switching occur in the figs/fig-wasp mutualism. Of course, there may be many cases where coevolutionary relationships exist in fig-wasp associations, but the occurrence of cospeciation between figs and their pollinators would depend on the strictness of the “one-to-one” relationships of the fig-wasp mutualism, which might differ between fig/wasp groups. Given such a viewpoint, there may be another implication in our results, that is, the “one-to-one” relationships between these monoecious figs and their pollinators, *Pharmacosycea-Tetrapus* and *Americana-Pegoscapus* are rather loose. This contrasts with the extremely strict one-to-one relationships, we found between Japanese dioecious figs and their pollinating wasps (Azuma and Su, data not published).

Fig species usually have wide distribution ranges, and some species are geographically separated by big barriers such as seas and mountains. Results using only one or a few specimens as the representatives of a wasp species to analyze its phylogenetic position may cause researchers to draw misleading conclusions regarding the speciation mode. This is because the possibility exists that more than one species (or phylogenetically independent lineages) of wasps pollinate one host fig species in different distribution ranges as found in the present study. In addition, fig wasps may have undergone morphologically convergent evolution under the pressure of natural selection in their adaptation to fig species. Fig taxonomy is another problem in that apparently identical or closely similar *Ficus* species growing in two different regions may be considered to be the same species and given a single name, when in fact they are distinct. For example, the Mexican *F. insipida* and the Panamanian *F. insipida* may be (phylogenetically) different species. Therefore, extensive phylogenetic studies of figs and fig-pollinating wasps are needed to test the “one-to-one” rule and the cospeciation of fig-wasp mutualism including the whole geographic distribution range of the species.

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