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Multi-locus phylogenetic analysis of neotropical figs does not support co-speciation with the pollinators: The importance of systematic scale in fig/wasp cophylogenetic studies

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Abstract

For 18 species of Panamanian *Ficus*, representing both basal (*Pharmacosycea*; 4 spp.) and derived (*Urostigma, Americana*; 14 spp.) sections, we sampled multiple individuals per species and analyzed sequence data from multiple (3) genetic markers (*tpi, g3pdh, ITS*). In contrast to previous phylogenetic studies of figs, this sampling design allowed us to evaluate the degree to which different alleles within loci, and different loci within individual species suggest consistent phylogenetic relationships among both distantly and closely related figs. We found multiple instances within both *tpi* and *g3pdh* genes in which different haplotypes were not monophyletic by species. Haplotype and reconciliation analyses suggested that genetic exchange among closely related figs is necessary to fully explain the patterns. In contrast, analyses of multiple loci from multiple individuals in the wasps are monophyletic by species, producing a well resolved species phylogeny. Although combining fig genetic data sets produced a resolved and robust fig topology, no fig phylogenies based on either combined or individual gene trees showed any significant correspondence with the wasp tree. Recent studies have shown that many of the fig species considered here have multiple pollinators and several share genetically indistinguishable pollinators. Together with these genetic results, it appears that a strict co-speciation model does not adequately describe the general evolutionary dynamics of the fig/wasp mutualism, particularly for sympatric, closely related species (within section). Importantly, these results emphasize the need to consider multiple genes, multiple individuals, and systematic scale in order to conduct and interpret robust co-phylogenetic studies of figs and their pollinating wasps.

Keywords: *Ficus*, mutualism, coevolution, cospeciation, phylogeny, cophylogeny, hybridization, paralogy

1. Introduction

Figs (*Ficus spp.* Moraceae) and their pollinating wasps (Agaonidae) constitute perhaps the most tightly integrated pollination mutualism known (Corner, 1952, 1985; Ramirez, 1974; Janzen, 1979; Herre, 1989, 1999; Weiblen, 2004; Cook et al., 2004). The fig-wasp mutualism is both ancient and diverse, originating roughly 90 million years ago (Machado et al., 2001; Rønsted et al., 2005) with over 750 extant species of figs currently recognized (Wiebes, 1979; Berg, 1989). As with other obligate pollination mutualisms found among some flowering plants and their insect pollinators (Pellmyr, 2003; Bronstein, 2002; Herre et al., 1999; Herre, 1999; Kato et al., 2003), it is the insect's recognition and choice of hosts that determines the patterns of host gene flow. Interestingly, although there are relatively few cases of obligate pollination mutualism, those few are often marked by high to extreme speciation and diversification in both partners, raising the question of how host specificity and control of gene flow affects one or both partners.

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This in turn raises a series of interesting questions concerning the processes underlying speciation, coadaptation, and co-evolution in these systems.

Both morphological and recent molecular studies (Ramirez, 1970; Wiebes, 1979; Corner, 1985; Berg, 1989; Berg and Wiebes, 1992; Herre et al., 1996; Machado et al., 2001; Weiblen, 2001, 2004; Jousselin et al., 2003; Jackson, 2004; Cook et al., 2004; Rønsted et al., 2005) broadly support the proposition of co-cladogenesis and coadaptation at a coarse systematic scale (i.e., between recognized genera of pollinating wasps and their respective sections of figs). Although clear incongruencies between fig and wasp phylogenies have been detected (Machado et al., 2001, 2005; Jousselin et al., 2003; Weiblen, 2000, 2001, 2004), results from those studies have been used to suggest that finer scale cospeciation of individual fig and wasp species also should be widespread.

However, there are many reports of what are thought to be single fig species pollinated by two or more species of wasps (based on morphology) (Berg and Wiebes, 1992; Wiebes, 1994; Michaloud et al., 1996; Rasplus, 1996; Kerdelhue et al., 1997; Weiblen, 2002; Cook and Rasplus, 2003). Also, hybrid figs are known to exist both naturally and to have been produced artificially (Ramirez, 1994; Parrish et al., 2003; E.A. Herre, K.C. Jander, pers. obs). In addition, there are several examples of "fig complexes" where the host morphologies are very plastic across a geographic range (e.g., the *F. citrifolia* and *F. americana* species groups) (Berg, 1989).

Finally, recent genetic work based on extensive sampling of wasps associated with several Neotropical host fig taxa has revealed the presence of previously undetected cryptic pollinator species (Molbo et al., 2003; Haine et al., 2006). In stark contrast to the prevailing perception, most host figs studied in detail have turned out to be pollinated by more than one wasp species (Molbo et al., 2003; Lopez-Vaamonde et al., 2002; Haine et al., 2006; Su et al., 2008 in this issue; Rong Chien, Yan-Qiong, James Cook, and Jean-Yves Rasplus, pers. com., 2006 Xingshuabanna Fig Conference).

In some cases, the cryptic species pairs associated with the same host are closest relatives, a finding that is consistent with long-term co-existence on a single host. In other cases, however, the cryptic species pairs associated with the same host are not sister species, indicating a host switch. Further, in some cases, wasps that are genetically indistinguishable regularly pollinate what appear to be different host fig species. These findings undermine the long-held one to one fig-pollinator interaction and add previously unsuspected levels of complexity to the mutualism. For instance, such observed host switching and pollinator sharing should lead to hybridization and genetic introgression between host fig species. Further, these findings provide a potential mechanism for explaining the observed phylogenetic discordance at higher taxonomic levels that persist between existing fig and pollinator phylogenies (Jackson, 2004; Jousselin et al., 2003; Machado et al., 2005).

Existing phylogenetic studies have not been adequately designed to distinguish between strict-sense cospeciation between *individual pairs* of species-specific wasps and figs (at a fine systematic scale), and a much less specific form of codivergence between *groups* of related wasps with *groups* of related figs (see Machado et al., 2005). This difference is crucial with respect to understanding the actual mechanisms underlying coadaptation and coevolution. If strict sense species-specificity is the rule, then coadaptation is expected to take place in a series of isolated lineages. If not, then the coadaptational dynamics will be quite different. A general weakness of most phylogenetic studies addressing evolution in figs and wasps is that they have concentrated on a relatively small number of species that represent very ancient, distantly related taxonomic subdivisions within the genus *Ficus* or the associated genera of pollinator wasps.

Moreover, these studies tend to sample one or a very few genes from only one "representative" individual per host and pollinator species. Such sampling differentially emphasizes ancient processes and will inevitably tend to bias interpretation towards cospeciation. A rigorous understanding of the process of speciation (and the degree to which strict-sense cospeciation is actually the dominant pattern in recent or ongoing co-evolutionary processes) requires extensive sampling of multiple loci for multiple individuals within many sets of closely related fig species and their pollinators (Machado et al., 2005).

Here, we present genetic data from multiple individuals using multiple genes in two distantly related groups of sympatric, relatively closely related figs and their associated wasps. We analyze the data using different phylogenetic methods. Our data show that, within each group, the wasp phylogeny does not correspond to that of the figs. Further, consistent with recent studies demonstrating a greater complexity in wasp-fig associations than has been appreciated, our data suggest that the prevalence of strict sense cospeciation has been exaggerated in this mutualism.

2. Materials and Methods

Priming, amplification and sequencing reactions

Leaf material was collected from several individuals of 18 Neotropical fig species found along the Panama Canal, around Barro Colorado Island. Four species represent the most basal section of figs (*Pharmacosycea* sect. *Pharmacosycea*), and 14 represent a more derived section (*Urostigma* sect. *Americana*). The low-copy nuclear glycerol-3-phosphate dehydrogenase gene (*g3pdh*) and triosephosphate isomerase gene (*tpi*) were amplified and

sequenced in up to nine individuals of each species. To provide a comparison between the two distantly related Neotropical sections, sequences from a palaeotropical species, *F. benjamina,* were also included. Sequence data was also generated for the internal transcribed spacer (*ITS*) for single individuals of each Neotropical species. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen). The primers used for PCR and sequencing have been described elsewhere (Strand et al., 1997). PCR products were directly sequenced in both directions using standard protocols. If the sequence from an individual was polymorphic, the PCR product was cloned using the pGEM-T Easy Vector System (Promega), and 8 to 10 colonies were PCR amplified and sequenced. A subset of the sequences has been previously published (Machado et al., 2005), and new sequences are available in GenBank under accession numbers EU081757–EU081772.

Sequence alignment and likelihood mapping

Sequences were aligned by eye after preliminary alignment in ClustalX (Thompson et al., 1997), resulting in three individual gene data sets: *g3pdh* (723 bp), *tpi* (530 bp) and *ITS* (271 bp). An alignment of 25 cytochrome oxidase I (COI) (816 bp) sequences from fig pollinating wasp associated with 14 different species of Neotropical figs was constructed using sequences published elsewhere (Machado et al., 2001; Molbo et al., 2003; Machado et al., 2005). Phylogenetic signal in each of the individual gene and combined data sets was evaluated using likelihood mapping based on quartet analyses (Strimmer and von Haeseler, 1997) as implemented in TreePuzzle (Schmidt et al., 2002).

Phylogenetic analysis of individual gene data sets

Phylogenetic analyses were conducted in PAUP* v4.0b (Swofford, 1998). Preliminary NJ trees were reconstructed using maximum likelihood genetic distances. These preliminary trees identified monophyletic groups of conspecific haplotypes that were nearly identical (1–3 bp different). To improve resolution and create simplified phylogenies, these 'phylotypes' (i.e., monophyletic groups of haplotypes from a single species) were reduced to single representatives, which resulted in edited *g3pdh* and *tpi* data sets of 19 and 26 sequences respectively. The phylotypes are listed in Table 1.

Phylogenies for the edited *g3pdh* and *tpi* fig's phylotypes and for the pollinator's COI were estimated using both maximum likelihood (ML) and maximum parsimony (MP). ML searches used a GTR model (Yang 1994), with corrections for both the proportion of invariant sites (I) and rate heterogeneity (G), which were estimated from the data. For MP searches, each phylogeny was reconstructed using the general program settings: tree-bisection reconnection (TBR) swapping algorithm, initial tree obtained by 'simple' stepwise addition, gaps were treated as fifth characters, ACCTRAN character state optimization was employed and multiple states considered uncertain. Confidence intervals for ML and MP estimates were obtained using 100 and 500 non-parametric bootstrap replicates respectively (Felsenstein, 1985).

To further characterize these phylogenies, Mr Bayes (Huelsenbeck and Ronquist, 2001) was used to apply Bayesian inference (BI) with the following program settings: estimation with a full GTR+I+G model, four MCMC chains, with 10,000,000 generations and a sample frequency of 1,000 generations. *Pharmacosycea* (*F. insipida*) sequences were designated outgroups for each analysis. All other settings were default. Tracer v1.0.1 (http://evolve.zoo.ox.ac.uk/software) was used to assess the stationarity of parameters and appoint an appropriate burn-in for each search (typically 10,000 generations). Log-likelihood ratio tests (Huelsenbeck et al., 1996) were used to assess the statistical reliability of accepted topologies against alternatives in which monophyly was enforced on all conspecific sequences. Free and constrained trees were estimated for complete *g3pdh* and *tpi* data sets using ML as described above; twice the difference in likelihood was then compared against the chi-squared distribution, where total estimated parameters determine the degrees of freedom.

Haplotype analysis

Lack of bifurcating diversification and violation of normal phylogenetic assumptions can occur when a data set includes markers that do not subscribe to a purely dichotomous phyletic model (e.g. if there is intragenic recombination). In this case, it is appropriate to analyze such data using statistical parsimony (Templeton et al., 1992). The *g3pdh* and *tpi* data sets were analyzed using TCS v1.18 (Clement et al., 2000). This program estimates a distance matrix between all sequences, based on the number of mutational steps. A haplotype network was reconstructed after determining the 'probability of parsimony' (as defined by Templeton et al., 1992) for each pairwise comparison between sequences. Identical sequences were collapsed into single haplotypes. Given their dissimilarity, *Pharmacosycea* and *Americana* sequences were treated separately. To evaluate the clustering of haplotypes by fig species, 100 randomized trees were generated for both *g3pdh* and *tpi* in PAUP*. The number of times that two conspecific sequences were closest relatives was noted in each randomized tree to generate a distribution. This was compared to the observed frequency of conspecific neighbors in the haplotype network. Haplotypes were considered significantly clustered by species if the observed number of conspecific links exceeded the first standard deviation of the links in the randomized data sets.

Table 1. Phylotypes inferred from neighbour-joining phylogenies of all sequences.

Locus	Phylotype	Constituent sequences	Sister taxon	Bootstrap support[a]	Representative sequence[b]
G3pdh	bullenei	BUL, BUL38	colubrinae 2	<50	BUL
	citrifolia	CIT, CIT90, CIT92.1, CIT92.2, CIT2, CIT28	colubrinae 1/costaricana/perforata 2	55	CIT90
	colubrinae 1	COL, COL95.1, COL95.2, COL94	costaricana/perforata 2/ turbinata	82	COL95.2
	colubrinae 2	COL33.1, COL33.2	bullenei	79	–
	costaricana	COS	perforata 2	–	COS
	dugandii	DUG	citrifolia/colubrinae 1/ costaricana/perforata 2	–	DUG
	nymphaefolia	NYM	obtusifolia 2	–	NYM
	obtusifolia 1	OBT41, OBT1, OBT2, OBT8.2, OBT8.3	triangle 1/perforata 1	84	OBT1
	obtusifolia 2	OBT	nymphaefolia	–	–
	obtusifolia 3	OBT56, OBT60	popenoei	98	–
	paraensis	PAR, PAR26.1, PAR26.2, PAR101	citrifolia/colubrinae 1/paraensis	51	PAR26.2
	perforata 1	PER68	triangle 1/obtusifolia 1	–	PER68
	perforata 2	PER65	costaricana	–	–
	perforata 3	PER1, PER2, PER16.1, PER16.2, PER58	pertusa	51	–
	pertusa	PET, PET27, PET7	perforata 3	77	–
	popenoei	POP77.1, POP77.2	obtusifolia 3	100	POP77.1
	triangle 1	TRI, TRI108.1, TRI108.2, TRI2	perforata 1/obtusifolia 1	65	TRI2
	trigonata 1	TRG74.1, TRG74.2, TRG111.1, TRG111.2	triangle 1/obtusifolia 1	59	TRG74.2
	turbinata	TUR	costaricana/perforata 2	–	TUR
Tpi	bullenei	BUL38.1, BUL38.3, BUL63.3, BUL63.5, BUL63.1	popenoei	98	BUL63.5
	citrifolia	CIT, CIT92.1, CIT90.3, CIT92.3, CIT28.1, CIT90.1, CIT2	trigonata 3	97	CIT
	colubrinae 1	COL95.4, COL94.1	costaricana/turbinata/pertusa 2	85	COL95.4
	colubrinae 2	COL95.2	triangle 3/bullenei/nymphaefolia	–	–
	costaricana	COS, COS39.2	turbinata	65	COS39.2
	dugandii	DUG	costaricana/turbinata/pertusa 2/ colubrinae 1	–	DUG
	nymphaefolia 1	NYM64.3	triangle 1/obtusifolia 1	–	–
	nymphaefolia 2	NYM1, NYM2	triangle 3/bullenei	90	NYM2
	obtusifolia 1	OBT57.1, OBT60.1, OBT60.3, OBT41.2, OBT56.2, OBT57.3, OBT8.1, OBT56.1, OBT41.1	triangle 1/trigonata 2	<50	OBT8.1
	obtusifolia 2	OBT, OBT59.3	costaricana/pertusa 2/colubrinae 1/ dugandii	<50	–
	paraensis 1	PAR, PAR15.1, PAR26.1, PAR103.1	perforata 1	100	–
	paraensis 2	PAR15.2	costaricana/dugandii/obtusifolia 2	–	PAR15.2
	perforata 1	PER4.3, PER4.1, PER58.4	paraensis 1	<50	–
	perforata 2	PER58.1, PER16.1, PER68.1, PER103.1	triangle 1/obtusifolia 1	50	PER16.1
	pertusa 1	PET7.2	triangle 1/trigonata 2/obtusifolia 1	–	–
	pertusa 2	PET27.1, PET	costaricana/turbinata	50	–
	pertusa 3	PET7.1	costaricana/dugandii/obtusifolia 2/ paraensis 2	–	–
	popenoei	POP1, POP2, POP77.1, POP77.3, POP30.5, POP30.2	bullenei	54	POP77.3
	triangle 1	TRI108.2, TRI109.4, TRI109.1	trigonata 2	<50	TRI109.4
	triangle 2	TRI, TRI109.3	triangle 1/obtusifolia 1/citrifolia/ trigonata 3	<50	–
	triangle 3	TRI106.3, TRI106.2	trigonata 4	<50	–
	trigonata 1	TRG74.1	perforata 1/paraensis1	–	–
	trigonata 2	TRG111.1, TRG111.2	triangle 1	<50	TRG111.1
	trigonata 3	TRG	citrifolia	–	–
	trigonata 4	TRG74.2, TRG111.4, TRG111.3	triangle 3	100	–
	turbinata	TUR	costaricana	–	TUR

[a]Support values are given where a phylotype comprises >1 sequence and are derived from 100 non-parametric bootstrap replicates of the original NJ phylogeny (see methods).

[b]A representative sequence is named where a phylotype was subsequently selected for the combined data set; a dash – indicates that the phylotype was not considered to be consistent with other loci and was excluded from the combined data set.

Phylogenetic analysis of combined data sets

In order to maximize phylogenetic signal and to obtain a 'best guess' of the *Americana* phylogeny, *g3pdh* and *tpi* sequences for one individual for each *Americana* species were added to ITS sequences to produce a combined data set of 1524 bp (with the exception of *F. pertusa* and *F. bullenei* for which no ITS was available).

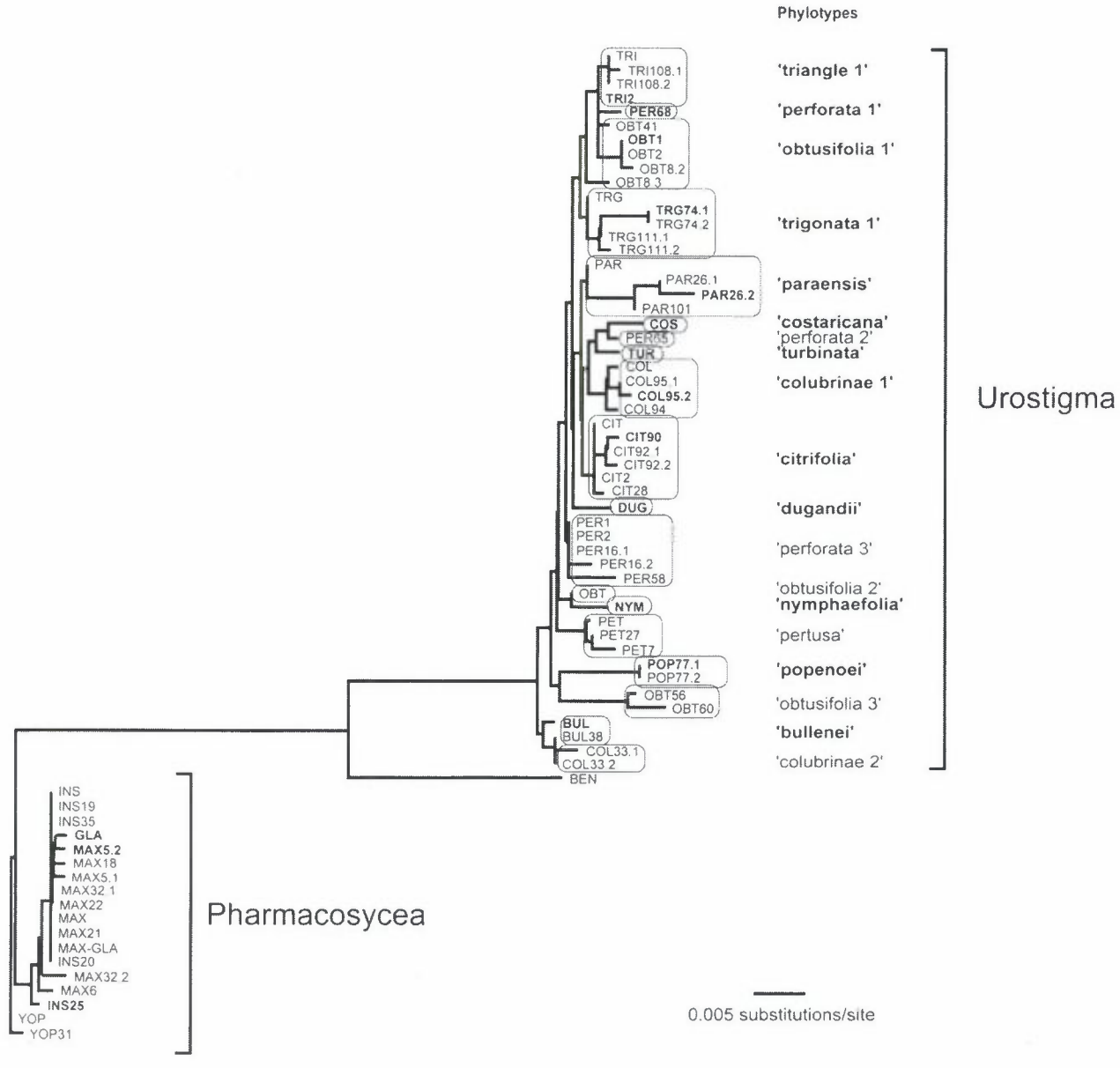

Figure 1. Neighbour-joining heuristic tree for all *g3pdh* sequences. Genetic distances were estimated with a full ML model. Monophyletic groups of conspecific sequences are squared and designated 'phylotypes' at right. Sequences are identified by a three-letter species code followed by individual and clone numbers (e.g., 33.2) where available. Phylotypes and individual sequences shown in bold were subsequently selected for the combined genes data set. Sample labeled "BEN" corresponds to the sequence from *F. bengalensis*.

In each edited tree (see above) there were a number of phylotypes (i.e., monophyletic groups of haplotypes from a single species); many species possessed unrelated phylotypes that present alternative histories. By definition, the species tree should represent a single history that averages over the histories of individual genes and lineages. Therefore, to create a combined data set *g3pdh* and *tpi* phylogenies were pruned to maximize their agreement; single sequences from each species that presented a common history were then selected from each tree (see Table 1). There are too few species (4) for conducting a meaningful cophylogenetic analysis of *Pharmacosycea*.

Putative orthologous sequences (those that share a recent common ancestor and were separated by a speciation event) were identified on the basis of relative position. For example, there are two *F. nymphaefolia tpi* phylotypes: 'nymphaefolia 2' is identified as orthologous to 'triangle 3' and 'trigonata 4', while 'nymphaefolia 1' is orthologous to 'triangle 1' and 'trigonata 2'. Of the two, 'nymphaefolia 2' is considered to present a common history with the 'nymphaefolia' *g3pdh* phylotype in Fig. 2 based on neighboring branches that they share. Taking this process further, *g3pdh* phylotypes 'triangle 1', 'perforata 1', 'obtusifolia 1' and 'trigonata 1' (Fig. 1) were identified as

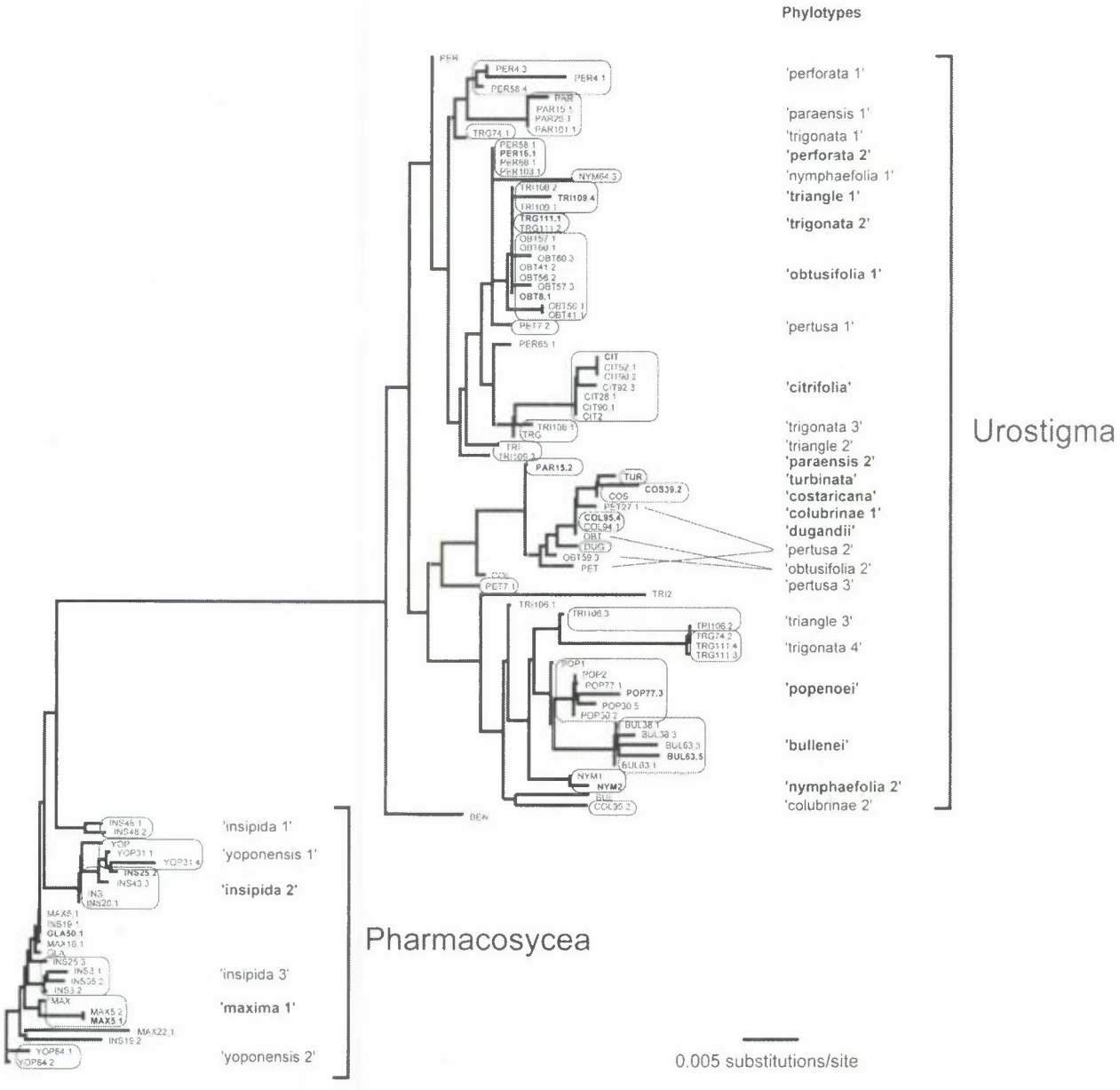

Figure 2. Neighbour-joining heuristic tree for all *tpi* sequences. Genetic distances were estimated with a GTR model. Sample labeled "BEN" corresponds to the sequence from *F. bengalensis*.

putative orthologs and concatenated with those *tpi* phylotypes ('triangle 1', 'perforata 2', 'obtusifolia 1' and 'trigonata 2'; Fig. 2) that present a common history. On this basis, 'perforata 2', 'obtusifolia 2' and 'obtusifolia 3' in Figure 1, as well as 'triangle 2', 'trigonata 3' and 'obtusifolia 2' in Fig. 2, are putative paralogs (i.e. sequences separated by a gene duplication event) and were excluded. Similarly, 'turbinata', 'costaricana', 'colubrinae 1', 'dugandii' and 'paraensis' were considered putative *g3pdh* orthologs and were concatenated with *tpi* phylotypes 'turbinata', 'costaricana', 'colubrinae 1', 'dugandii' and 'paraensis 2' on the basis of consistent relationships within and between gene trees. Finally, phylotypes for *F. bullenei, F. popenoei* and *F. nymphaefolia* were united based on their basal position in both gene trees; this made 'nymphaefolia 1' in Fig. 2 a putative paralog.

The combined data set was analyzed with likelihood mapping (see above) and phylogenetic estimation was carried out as before using ML, MP and BI. In order to check the legitimacy of combining the three separate data

partitions, the edited ML trees for *g3pdh* and *tpi* were compared using two-tailed Shimodaira-Hasegawa (SH) (Shimodaira and Hasegawa, 1999) tests in PAUP*. ML trees estimated using the *g3pdh* and *tpi* partitions of the combined data set were similarly compared with one another, and each with the ITS partition. 1000 replicates with full optimization were used for each test. P values presented in the results section represent the common level of statistical significance for all possible phylogeny comparisons.

Reconciliation analyses

Having obtained a combined genes topology for the *Urostigma Americana* phylogeny, reconciliation analyses (Page and Charleston, 1997, 1998) were used to compare this with individual gene phylogenies and with the COI fig-wasp phylogeny. GeneTree v1.3.0 (Page, 1998; http://taxonomy.zoology.gla.ac.uk) was used to reconcile the *g3pdh* and *tpi* gene phylogenies with the combined genes tree, hereafter termed the 'species' tree. The program calculates the minimum number of gene duplications required to account for the distribution of gene copies. Concomitant to this is the number of gene losses also required. These two processes – gene duplication and loss – were used to explain unrelated phylotypes derived from single species. It therefore provided a minimum estimate for gene paralogy in the absence of cross-pollination and given a correct tree topology.

TreeMap v2.0.2 (Charleston and Page, 2002; http://taxonomy.zoology.gla.ac.uk) was used to assess the correspondence between the fig species tree and the pollinating wasp phylogeny. The program employs a four-event model of cophylogeny, comprising '*codivergence*' (synchronous cladogenesis or phylogenetic tracking), '*duplication*' (unilateral duplication of an associate), '*loss*' (the unilateral extinction or disappearance of an associate) and '*switching*' (the unilateral transfer of an associate to another host) (Page, 1990, 1994; Charleston, 1998).

This model is used to explain incongruence in the most parsimonious way. The program searches through all possible combinations to locate all solutions that are potentially optimal under some set of event costs (Charleston, 1998) and, in so doing, determines the level of correspondence (in the form of the number of codivergence events). Randomization tests were carried out in TreeMap to evaluate the statistical significance of observed correspondence; the observed maximum number of codivergence events was considered significant if this value was recovered in no more than 5 of 100 randomized cophylogenies.

3. Results

Phylogenetic signal

The multiple alignments for *g3pdh* and *tpi* contained 70 and 102 distinct sequences respectively. These data sets comprised 19 species of *Ficus* (4 *Pharmacosycea*, 14 *Urostigma Americana* and *F. benjamina*) and between 1 and 13 sequences per species. Alignment was straightforward as variation was low for both genes. Maximum likelihood genetic distances ranged between 0.003 and 0.084 substitutions/site for *g3pdh* and between 0.002 and 0.066 for *tpi*. Most variation derived from the *Pharmacosycea-Americana* split; the average genetic distance between these groups was 0.062 and 0.05 for *g3pdh* and *tpi* respectively, while within *Americana* it was 0.013 (*g3pdh*) and 0.02 (*tpi*) and within *Pharmacosycea* it was 0.004 (*g3pdh*) and 0.009 (*tpi*). There were 85 (12% of total characters) and 65 (12%) parsimony-informative characters for *g3pdh* and *tpi*, respectively. However, as indicated by the genetic distances, 39 and 23 related to the *Pharmacosycea-Americana* split. Relative to the COI data for wasps, which is known to possess effective phylogenetic signal (Molbo et al., 2003; Machado et al., 2005), likelihood mapping indicated that there is low phylogenetic signal in the fig sequences as shown by a large proportion of unresolved quartets. Of the three fig loci, *tpi* produced the lowest number of unresolved quartets (7.4%), although these are still notable compared with COI, where only 1.2% of quartets are unresolved.

Gene topologies

Figs. 1 and 2 show neighbour-joining phylogenies for all sequences. The splits between *Ficus* sections are robust, as are clusters of identical or near-identical conspecific sequences or "phylotypes" (e.g. 'colubrinae 1' phylotype in Fig. 1, and 'citrifolia' phylotype in Fig. 2). Uncertainty relating to the relationships between sequence clusters accounts for the relatively low robustness in each of these trees. Indeed, it was obvious that many species retained multiple, unrelated phylotypes. For example, five *F. obtusifolia* sequences cluster together in Fig. 1 close to sequences from *F. triangle* and *F. perforata*, forming a single phylotype. Additionally, there are *F. obtusifolia* sequences related to *F. nymphaefolia* ('obtusifolia 2') and to *F. popenoei* ('obtusifolia 3'). However, where monophyly was enforced for conspecific sequences, estimates were significantly worse than the best, unconstrained trees for both *g3pdh* ($2\Delta\ln L = 223.84$, $p<0.001$) and *tpi* ($2\Delta\ln L = 462.74$, $p<0.001$). This shows that, while likelihood mapping indicates that signal is generally poor, there is genuine phylogenetic information at some nodes. In short, where there are differences between sequence clusters, these are real and significant and they

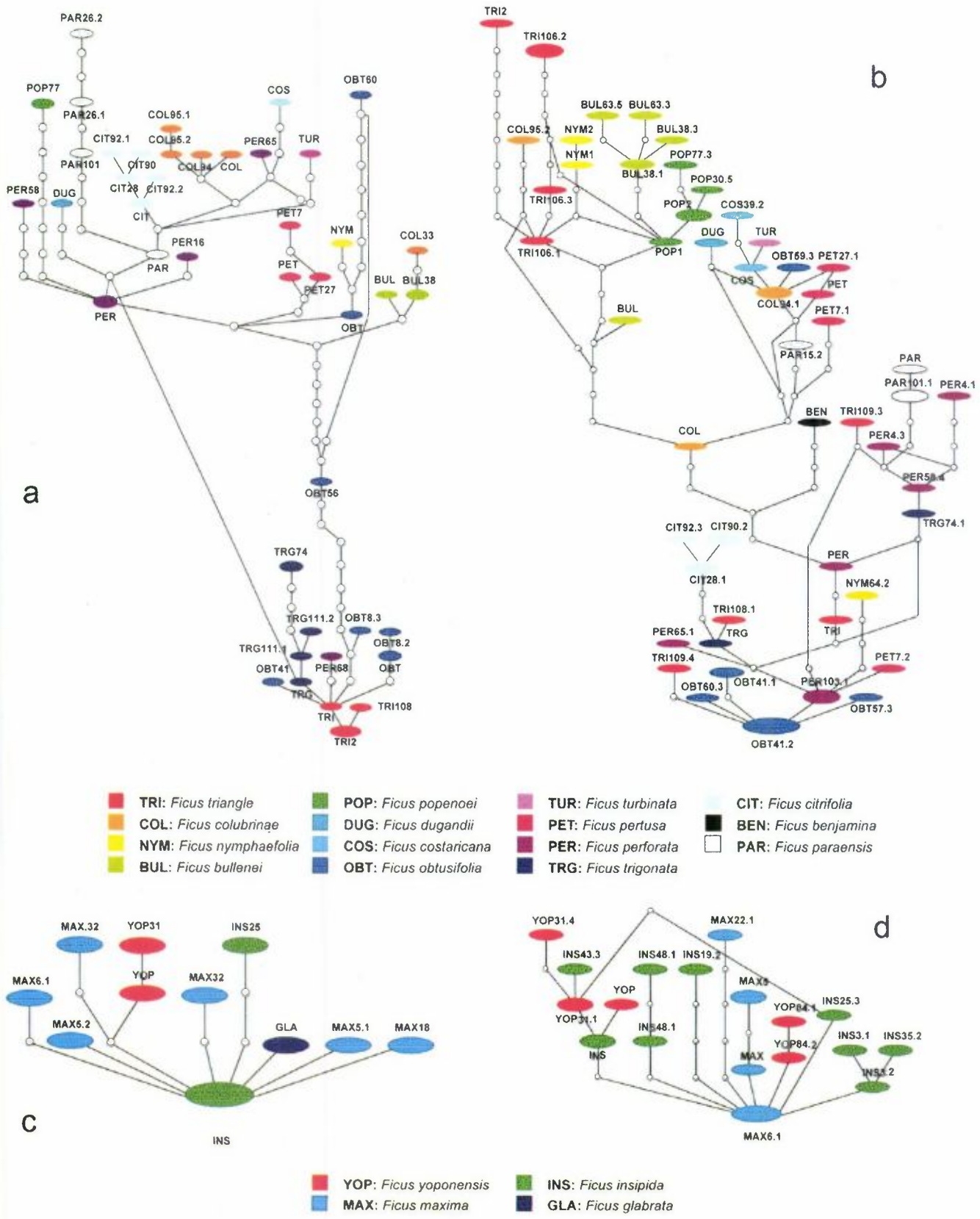

Figure 3. Haplotype networks for a) *g3pdh* (*Americana*), b) *tpi* (*Americana*), c) *g3pdh* (*Pharmacosycea*) and d) *tpi* (*Pharmacosycea*). Identical sequences were grouped together and are reflected by the size of the ellipse. Lines indicate single mutational steps between haplotypes; open circles indicate intermediate mutational states not seen in the alignment.

either suggest the admixture of sequences from different fig species or the presence of paralogous gene copies.

The first step in simplifying phylogenetic variation before conducting additional analyses was to create phylotypes from monophyletic groups of near-identical sequences (see Table 1). For example, in both Figs. 1 and 2, four *F. paraensis* sequences (including individuals PAR, PAR26 and PAR101) cluster together and are separated by a few substitutions, and constitute phylotype 'paraensis' in both trees. However, despite reducing the data to phylotypes, resolution and robustness were not improved in the ML and MP trees. Bayesian analysis of the edited *g3pdh* and *tpi* data sets often generated nodal posterior probabilities between 60–90; however, the probabilities of each sampled tree showed that all topologies were equally bad ($p<0.001$). Accordingly, no best tree was identified. This uncertainty notwithstanding, some gross similarities were evident from the full and edited trees; *F. triangle, F trigonata, F. obtusifolia* and *F. perforata* group closely in both Figs. 1 and 2. In Fig. 2, *F. nymphaefolia* (phylotype 1) also clusters with this clade but has no putative homolog in Fig. 1 (where *F. nymphaefolia* would correspond to phylotype 2 in Fig. 2). *F. costaricana, F. turbinata* and *F. colubrinae* are closely related in both trees, although, again, *F. colubrinae* is represented by other phylotypes elsewhere. In addition, *F. paraensis* and *F. citrifolia* have an affinity, as do *F. bullenei* and *F. popenoei.* Beyond these results, the phylogenies were unable to establish relationships between these species groups and the status of other phylotypes remained ambiguous.

The second step in simplifying the data for the creation of a combined data set was the reduction of each data partition to a set of putative homologs. As stated previously, phylotypes 'triangle 1', 'perforata 1', 'obtusifolia 1' and 'trigonata 1' in Fig. 1 (*g3pdh*) appear similarly placed to 'triangle 1', 'perforata 2', 'obtusifolia 1' and 'trigonata 2' in Fig. 2 (*tpi*). From their placement, these are assumed to be homologs of their respective loci.

Haplotype analyses

If the sequences used to construct *g3pdh* and *tpi* phylogenies have not evolved in a tree-like manner, typical phylogenetic analyses may not be appropriate to elucidate their relationships. The small amount of variation in these data, which was insufficient to produce resolved phylogenies but still provided some phylogenetic structure, comprises a series of mutations that may be shared among species or that are unique to each species. Haplotype networks constructed using these mutations are shown in Fig. 3 and roughly correspond to the phylogenies. Haplotypes group according to species; the number of connections between haplotypes of the same species was significant, that is, far outside the standard deviation of connections seen in 100 randomized networks. *G3pdh* had 27 conspecific connections, while randomized networks of this data set produced 2.3 on average (±1.62 SD). *Tpi* had 24 conspecific connections and produced an average of 2.0 when randomized (±1.43 SD).

Combined fig phylogeny

The combination of *g3pdh* and *tpi* sequences with additional sequence data for ITS produced better resolution and robustness in the phylogeny. ML, MP and BI all largely agreed on a topology comprising two clades. The first included *F. triangle, F. trigonata, F. obtusifolia, F. perforata* and *F. citrifolia*. The second included *F. costaricana, F. dugandii, F. turbinata, F. colubrinae* and *F. paraensis* as one clade and *F. nymphaefolia, F. popenoei* and *F. bullenei* as another. ML and MP topologies are shown in Fig. 4. The improvement made by combining the data is seen in likelihood mapping (not shown), where the number of unresolved quartets fell to 4.0%. Also, whereas Bayesian inference of *g3pdh* and *tpi* trees failed to produce a single most favorable topology (all trees equally bad), the search for the combined data set identified a topology with highest probability. Optimal ML and BI topologies were identical. However, this result was achieved after the removal of putative paralogs from the *g3pdh* and *tpi* datasets, as described above. The assignation of homology was based largely on placement of sequences within the edited trees, and in the absence of a complete knowledge of how many paralogs exist for each locus, and how many are represented here. Furthermore, there is the issue of data compatibility. Combining the data resulted in improved phylogenetic signal and robustness; likelihood mapping suggested that there was little conflict within the data set, as only 1.7% of quartets produced conflicting topologies. However, the individual gene trees were significantly different when compared using the SH test, both for the edited trees (*g3pdh* vs. *tpi*: $p<0.001$) and for the combined data set (all comparisons among *g3pdh*, *tpi* and ITS, $p<0.02$). Therefore, the combined data set must be considered preliminary, although its topology is consistent with both individual gene phylogenies.

Reconciliation analyses

The comparison of edited phylogenies for *g3pdh* and *tpi* with the combined tree generated estimates for the level of paralogy. The clustering of conspecific phylotypes in unrelated clades in Figs. 1–3 suggested that distinct gene copies, predating the origin of morphospecies, were present. By reconciling the gene and species trees, using duplication and loss events, GeneTree arrived at 12 and 11 duplication events for *g3pdh* and *tpi*, respectively. This large number of duplications suggests that not only do species possess poorly related gene copies, but also the relationships between related copies do not reflect the species phylogeny.

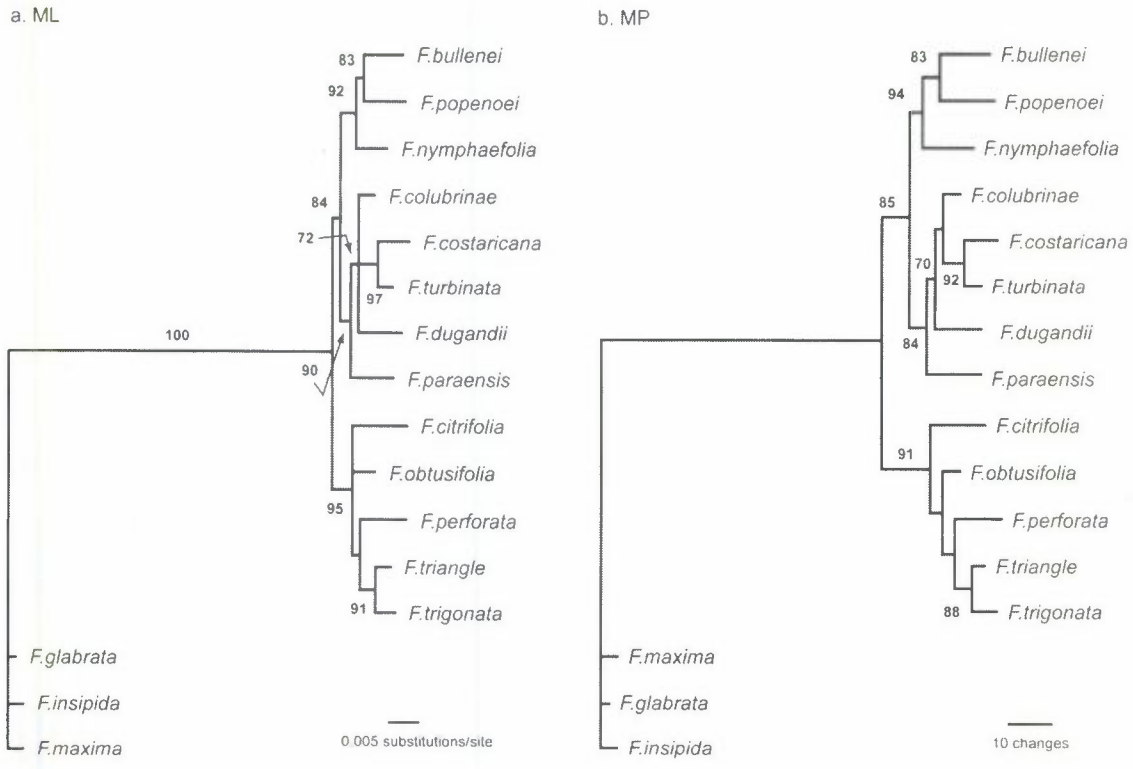

Figure 4. Combined data set phylogenies for Neotropical *Ficus* using a) ML and b) MP. Figures shown in bold are non-parametric bootstrap values.

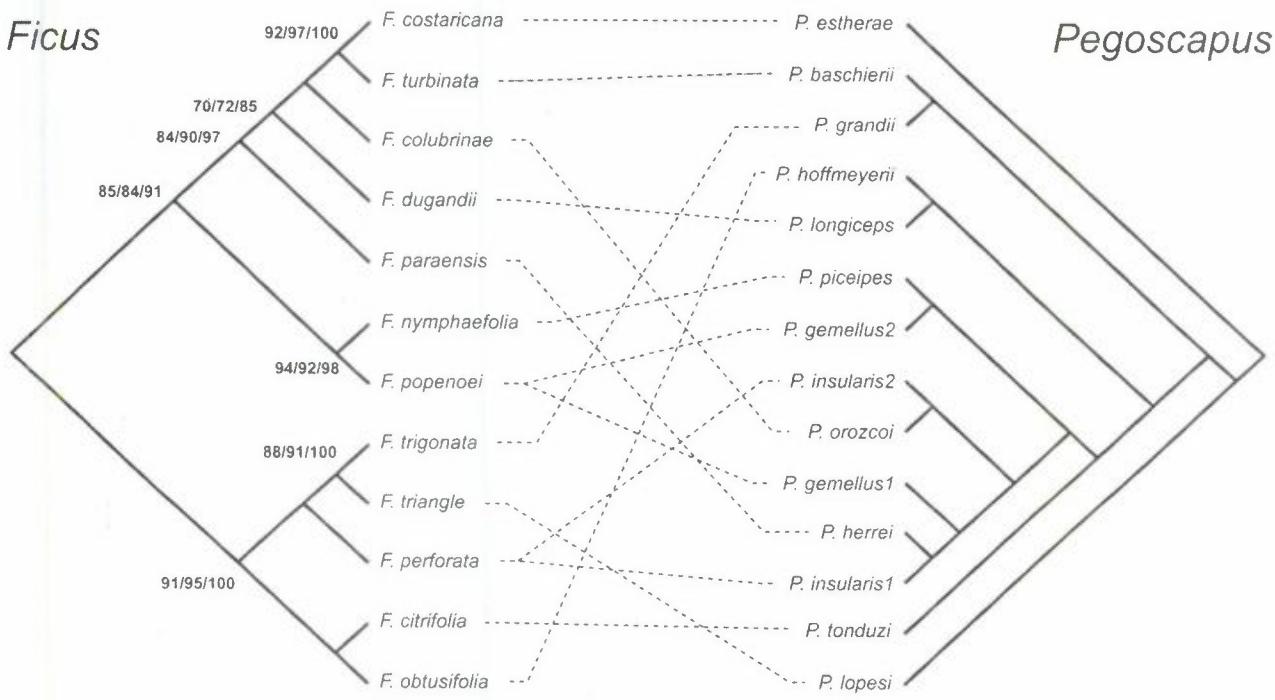

Figure 5. Tanglegram showing correspondence between *Ficus* (*Americana*) species phylogeny (right) and their *Pegoscapus* spp. pollinators (left). Associated mutualists are linked by a dashed line. Figures in bold denote bootstrap proportions in the format ML/MP/BI.

Such a number demands a high duplication rate and either equally frequent loss of copies or poor sampling of available genetic diversity.

Reconciliation of the combined fig phylogeny and wasp phylogeny failed to identify any significant codivergence. Fig. 5 shows the extent of mismatch between the two phylogenies. Reconciliation of wasp and fig phylogenies (of any method) produced a minimum of 3 codivergence events and a maximum of 5. Such was the incongruence between fig and wasp phylogenies that exhaustive searches for reconciled trees could not be completed. However, it is known that 7–8 codivergence events would be required for significance using TreeMap, given the number of taxa included (Jackson, 2004b). If switching events are excluded, reconciliation of fig and wasp phylogenies required very large numbers of duplications and losses; 40 and 45 losses were required for MP and ML topologies respectively. Reconciliation of gene tree topologies simplified to contain only putative orthologs identified in Figs. 1 and 2 did not produce a different result; *g3pdh* and *tpi* topologies only generated 2–6 codivergence events in both cases. Hence, in a similar fashion to the reconciliation of gene and species phylogenies, without horizontal movement of associates, very frequent duplication and extinction are necessary to explain incongruence.

4. Discussion

This study has utilized multiple loci from multiple individuals per species to evaluate the distribution of alleles within and among fig species representing two distinct, sympatric sections of figs (*Pharmacosycea Pharmacosycea* and *Urostigma Americana*). In addition to determining the degree to which different alleles and different loci show a coherent and congruent sorting by species, this sampling allows us to draw inferences about processes that influence co-evolutionary processes in sympatric figs and their wasps at both coarse and fine systematic scales (among and within fig section, respectively). We find three main results. First, the two nuclear genes analyzed in detail, as well as all other genes reported (Herre et al., 1996; Machado et al., 2001; Jousselin et al., 2003; Weiblen, 2001, 2004; Rønsted et al., 2005), clearly reflect deep genetic divergence between the *Pharmacosycea* and *Americana* sections, but relatively little within them (see Herre et al., 1996; Machado et al., 2001, 2005). Second, both genes possess multiple haplotypes that are not monophyletic by fig species. Third, despite clear, consistent phylogenetic resolution of the associated pollinator wasp species based on multiple genes sampled in multiple individual wasps, neither individual nor combined data sets in the figs support co-divergence between the fig hosts and their associated wasp pollinators. None of these results is easily reconciled with the proposition of species specificity and strict-sense co-speciation in the fig-wasp mutualism at this fine scale of sympatric species within a section. Indeed, these results stand in stark contrast to what would be expected if these fig species were classical "good" biological species and represented distinct gene pools.

Low divergence and the population structure of Ficus *species*

The figs studied here and their associated wasps are morphologically distinct according to section. The two distantly related fig/wasp lineages (*Pharmacosycea/Tetrapus* and *Americana/Pegoscapus*) are distinguished by a range of morphological and behavioral characters that ensure host specificity (between section) and make hybridization between them extremely unlikely (e.g., passive versus active pollination, and inverted versus interdigitated bracts forming the ostioles, respectively). Both mitochondrial and nuclear markers consistently show that pollinator wasp species are deeply and consistently well resolved phylogenetically, both within and among the genera that are associated with different fig sections (Herre et al., 1996; Machado et al., 2001; Molbo et al., 2003; Machado et al., 2005; and unpublished data). Calibrations of the deep genetic divergence found among the pollinator wasp genera suggest deep temporal splits between the associated fig sections (Machado et al., 2001, 2005). The *Pharmacosycea* species group, the basal group of all *Ficus*, appears to have split 60 to 87 MY ago from the rest of *Ficus*, and the *Urostigma* (*Americana*) section appears to have split from its closest relatives (apparently the African section, Galoglychia) around 40 to 55 MY ago (Machado et al., 2001; Rønsted et al., 2005; Rønsted and Savolainen, 2007). Further, assuming strict one-to-one fig-wasp specificity and co-speciation, the data from the wasps would suggest that some extant *Pharmacosycea* fig species diverged from each other about 40 MY ago, and that some extant *Urostigma* (*Americana*) fig species diverged at least 21 (±6.5) MY ago (Machado et al., 2001; Rønsted et al., 2005; Peñalver et al., 2007).

However, these expectations are not consistent with published reports based on analyses of several loci indicating that only slender genetic distances separate the fig species within these sections (Herre et al., 1996; Machado et al., 2005). Further, the present analyses of *G3pdh, tpi,* and *ITS* are consistent in showing surprisingly little divergence between fig species within sections. Based on the absolute and relative genetic differences among the wasps, we would expect to observe much greater differentiation among the figs, particularly if strict sense co-speciation was the primary mode of diversification. What accounts for these differences in genetic differentiation observed between the wasps and figs? We are presently unable to assign the *relative* importance of a slow substitution rate, unsorted ancestral polymorphisms (either

of which are expected given a relatively recent origin of the selected fig species), the consequences of gene paralogy, or homogenizing processes among fig populations (e.g., hybridization followed by genetic introgression). However, several inferences can be made.

There are few other *g3pdh* or *tpi* data sets with which the present divergence levels can be compared. Levels of *g3pdh* divergence are indeed an order of magnitude higher among various Poaceae and Solanaceae available in public databases. Conversely, Malcomber (2002) used *tpi* in a phylogeny of *Gaertnera* spp. (Rubiaceae: Rubicoideae) and found small genetic distances (average = 0.01±0.003 s/site) among 20 species; this was explained by a rapid and recent radiation. However, these genes are not thought to evolve especially slowly (Strand et al., 1997). If the antiquity of the pollinator lineages suggests equally ancient origins for the host trees (as is strongly supported by the abundant demonstration of long-term coevolution between groups of figs and wasps (Machado et al., 2001, 2005; Jousselin et al., 2003; Weiblen, 2001, 2004; Rønsted et al., 2005), this would also rule out a recent radiation of these figs. The most likely scenario is that these sections represent ancient lineages whose extant species have, surprisingly, not diverged as other considerations suggest they ought to.

The default explanation for these patterns could include both gene paralogy and lack of sorting of ancestral polymorphisms. But given that the precise placement of sequences was not robust, confirmation of the existence of possible paralogy was necessarily tentative. Figs. 1 and 2 suggest two distinct clades of *g3pdh* that represent paralogs that could predate *Americana*. However, although there may be more than one *tpi* copy, there is no evidence for the large number (12) required to explain the incongruence between gene and species trees, purely in terms of gene duplication and loss. Even allowing for misplacements, there was substantial incongruence between species and gene trees, and it is not thought that *g3pdh* and *tpi* occur in the high copy numbers required for this explanation to apply in these fig species (Strand et al., 1997). With respect to unsorted ancestral polymorphisms, Neotropical fig species maintain large population sizes and display high genetic variability (Herre, 1996; Nason et al., 1996; Machado et al., 2005; this study). Previously, these large populations and profuse gene flow (Nason et al., 1998) within them have been thought to build up variability within species, and perhaps slow genetic differentiation (sorting) between them.

However, recent analyses suggest that this is not a likely explanation for the observed lack of differentiation. Machado et al. (2005) used sequence data from 3 loci (including *tpi* and *g3pdh*) and 3 *Urostigma Americana* species to test a simple model of species divergence (isolation model) in which genetic divergence among species pairs occurred without gene flow. This isolation model was rejected for two species pairs using a conservative test that takes into account the possibility that lack of divergence is the result of unsorted shared ancestral polymorphisms, and thus patterns of genetic divergence (or lack of divergence) among those species pairs are the result of introgression. This conclusion was not overly surprising given that the target fig species shared at least one common pollinator species (Molbo et al., 2003; Machado et al., 2005). Introgression between fig species would prevent genetic divergence and also account for the high levels of intraspecific variation. Consistent with this idea, breakdowns of specificity by pollinators have often been documented (Ramirez, 1970; Ware and Compton, 1992; Berg and Wiebes, 1992; Wiebes, 1994; Michaloud et al., 1996; Rasplus, 1996; Lopez-Vaamonde et al., 2002, Molbo et al., 2003). These cases are especially notable given the traditional dogma of strict host specificity and a 'one-to-one rule' between figs and pollinators (Ramirez, 1970; Janzen, 1979; Weiblen, 2002). Even if only 1% of foundresses mistakenly move between different figs, this might result in substantial gene flow if, as has been observed, entry results routinely in successful fertilization (Compton, 1990; Ware and Compton, 1992; Compton, 1993; Ramirez, 1994; Parrish et al., 2003).

Further, although no single haplotype was shared by different species, there are several incidences where the closest relatives of a haplotype are found in related species and this would coincide with the rare, but consistent, "mistakes" made by foundresses. The present analyses are sufficient to support the plausibility of introgression in homogenizing relatively closely related (within sections) species. Moreover, the Machado et al. (2005) study on Neotropical figs described above and a study of Indonesian figs using microsatellites (Parrish et al., 2003) have also demonstrated the occurrence of fig hybridization in nature. We suggest that genetic analysis of fast-evolving markers or divergence population genetics studies of multilocus sequence datasets will be useful to evaluate the relative importance of hybridization in obscuring the phylogenetic signal at this fine scale.

Discordant cophylogeny and the effect of systematic scale

Having realized the dangers of accepting a species phylogeny based on any single history among many, it was nonetheless necessary to estimate one for reconciliation analysis. The combined data set generated a strong phylogenetic signal with apparently little conflict according to likelihood mapping. However, the data partitions produced significantly different topologies and so more data are required to corroborate the preliminary tree shown in Fig. 4. In selecting the congruent parts of the gene trees, the combined data set also included the most robust elements; therefore, three clades comprising, first, *F. costaricana, F. turbinata, F. colubrinae* and *F. dugandii*, second, *F. obtusifolia, F.* near *trigonata ("F. triangle")*,

F. trigonata and *F. perforata*, and third, *F. popenoei, F. nymphaefolia* and *F. bullenei* emerge in both gene and species phylogenies, as well as haplotype analyses. The positions of *F. citrifolia* and *F. paraensis* are less certain and reflect the absence of signal towards the base of the tree. Nonetheless, the lack of correspondence between fig and wasp trees is strongly supported by the fact that none of these species clusters have corresponding pollinator clades. For example, in Fig. 5, closely related wasp species such as *P. gemellus1, P. herrei* and *P. insularis1* correspond to fig species spread throughout the tree. Were basal nodes solely responsible for the incongruence, phylogenetic error might have a deciding role. However, Fig. 5 demonstrates that terminal nodes are especially incongruent and, in fact, codivergence events were only observed towards the base of reconciled trees produced by TreeMap.

The observed cophylogenetic incongruence appears to be inconsistent with the general conclusions of almost all previous studies of fig-wasp cospeciation (Cook and Rasplus, 2003; Machado et al., 2001; Jousselin et al., 2003; Weiblen, 2000, 2001, 2004; Weiblen and Bush, 2002). Certainly, comparative analyses spanning the entire mutualism suggest mutual diversification of relatively closely related groups (usually genera) of wasps with their corresponding groups (usually sections or subsections) of figs. Yet, precisely because these studies generally focus on results based on samples of relatively few representatives of each already greatly diverged group, they are effectively insensitive to processes operating at a contemporary scale, like the recent and on-going cladogenic events that were specifically addressed by this study. Previous analyses of *Pegoscapus* spp. have shown that seemingly host-specific pollinators contain cryptic species (Molbo et al., 2003). Furthermore, where nominal wasp species contain cryptic clades these are not necessarily sister species; at this scale, pollinator diversification is characterized not only by cospeciation but also by unilateral lineage duplications and host switching between related figs (Molbo et al., 2003). Likewise, these data suggest that the ecological processes implied by broad comparisons (i.e., cospeciation and host specificity) do not adequately describe the complexity of contemporary divergence in Neotropical figs.

In the only previous study from a different geographic region attempting to match phylogenies of figs with pollinators within a wasp genus, Weiblen and Bush (2002) analyzed the cophylogeny of *Ceratosolen* pollinators with *Sycomorus* figs. By sampling from among a single genus, one might suppose that any comparisons would involve close relatives.

Yet, *Ceratosolen* is a large clade and its lineages are, in fact, highly diverged and relatively old. The sampling was such that ML genetic distances ranged between 0.107 and 0.569 (for *Ceratosolen* spp., mean 0.373). Thus, despite sampling wasps within a recognized genus, Weiblen and Bush (2002) were nonetheless observing very deep nodes within the agaonid phylogeny. By contrast, the genetic distances among the *Pegoscapus* spp. studied here range between 0.001 and 0.209 (mean 0.109). Even after accounting for a higher substitution rate among *Ceratosolen* over *Pegoscapus*, it is known that the divergence times within *Ceratosolen* are much older (Machado et al., 2001). Therefore, the *Ceratosolen* sampling addresses a fundamentally different temporal scale when compared to the present *Pegoscapus* analysis.

The apparently contradictory patterns observed between studies conducted at broad and fine scales could be resolved if one accepts that gene flow continues between diverging populations (within a section of figs), but diminishes as they become optimized to local conditions and introgression carries fitness penalties. Thus, if speciation is a gradual process of population delineation and may even be discontinuous (i.e. populations can coalesce as well as fragment), one should expect closely related fig and pollinator species to lack strict host specificity, due to imperfect derivation of volatile attractant profiles. This state of incomplete differentiation among figs can explain why pollinators apparently diversify independently of their hosts during, and shortly after, speciation (Molbo et al., 2003), and may even explain how fig species diversity is generated (Baker, 1961; Machado et al., 2005).

Linking population differentiation to observations made at broader scales raises the issue of how reliable such broad scale phylogenies based on limited individual and gene sampling are as useful tools to infer the ongoing evolutionary processes that produce them. The effect of extinction following clade diversification must be to delete aspects of the historical record. This means that the historical record will become incomplete and simplified with age; therefore, addressing the cophylogeny question at different scales will likely give different answers. Put into context, the fig/wasp cophylogenies reported by previous studies record divergence of major lineages long separated by vicariance at the global or continental scale (Herre et al., 1996; Machado et al., 2001; Weiblen, 2000, 2001; Weiblen and Bush, 2002; Jackson, 2004). The impression of codivergence is enforced by comparisons of such distantly related lineages. Co-evolutionary studies at the local scale records a period of imperfect host specificity, events that can not be detected by sampling one gene from a few individuals representing ancient and already highly diverged groups of figs and wasps.

The importance of systematic scale to understanding the complexity of cophylogeny may parallel what was observed in a study of active pollination by *Pleistodontes* pollinators (Cook et al., 2003). Here, several gains and losses of active pollination behavior are observed within a single genus, although phylogenies of the entire agaonid family had only previously suggested one origin (Machado et al., 2001). Thus, the historical record of pollination behavior is greatly

simplified when one addresses the question at a broad systematic scale.

Conclusion

We found no evidence to support the proposition of strict sense cospeciation at a relatively fine systematic scale (species within fig section) between sympatric figs and their pollinator wasps. Specifically, the combination of low divergence and incongruence between gene and 'species' trees is consistent with the mixture of haplotypes from different species (as would result from hybridization and introgression). This would also account for the discordance between all forms of fig phylogeny and the wasp tree, which cannot adequately be explained using lineage duplication and extinction events. Taken together, the demonstration of cryptic pollinator species and the lack of congruence of the phylogenies of Neotropical (and other groups of) figs and fig-wasps show that the diversification of this mutualism is inadequately described by a simple codivergence model that assumes one-to-one species specificity, and strict-sense co-speciation. We suggest that previous approaches to fig/wasp cophylogeny have contributed to an overly simplified interpretation of fig-wasp co-evolution for at least three reasons. First, sampling relatively few species to represent distantly related fig and associated wasp taxa obscure evolutionary processes that occur during and shortly after speciation, and will inherently bias interpretations towards strict-sense co-speciation. Second, it is now clearly established that there are frequent breakdowns in strict one-to-one wasp-host fig species specificity. Several workers have found that multiple pollinator wasps can be associated with what are thought to be single host figs, and that at least sometimes these wasps successfully pollinate more than one host. Third, data collected from different genetic markers and different individuals within a fig species can support alternative histories. This last observation suggests that these breakdowns in specificity lead to hybridization and introgression which play an integral part in fig-wasp co-evolutionary dynamics.

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