SEVENTEEN

Cophylogeny of Figs, Pollinators, Gallers, and Parasitoids

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Cophylogeny provides a framework for the study of historical ecology and community evolution. Plant-insect cophylogeny has been investigated across a range of ecological conditions including herbivory (Farrell and Mitter 1990; Percy et al. 2004), mutualism (Chenuil and McKey 1996; Kawakita et al. 2004), and seed parasitism (Weiblen and Bush 2002; Jackson 2004). Few examples of cophylogeny across three trophic levels are known (Currie et al. 2003), and none have been studies of plants, herbivores, and their parasitoids. This chapter compares patterns of diversification in figs *(Ficus* subgenus *Sycomorus)* and three fig-associated insect lineages: pollinating fig wasps (Hymenoptera: Agaonidae: Agaoninae: *Ceratosolen*), nonpollinating seed gallers (Agaonidae: Sycophaginae: *Platyneura*), and their parasitoids (Agaonidae: Sycoryctinae: *Apocrypta*). Molecular phylogenies of each participant in this tritrophic interaction can illuminate histories of ancient association ranging from codivergence to host switching. We distinguish cospeciation, the simultaneous speciation of a host and an associate (Page 2003), from coevolution, strictly defined as reciprocal evolutionary change between lineages acting as agents of selection upon each other (Stearns and Hoekstra 2000). Tests of cospeciation and alternative hypotheses, including host switching, can evaluate whether evolutionary change in host use was associated with speciation (Coyne and Orr 2004).

Nonpollinating fig wasps have not received as much attention as the pollinating wasps, and so little is known about their evolutionary history (Ulenberg 1985; Bronstein 1991; Lopez-Vaamonde et al. 2001; Cook and Rasplus 2003). Weiblen and Bush (2002) argued from phylogeny that nonpollinating seed gallers are less closely cospeciated with figs than pollinators sharing the same hosts. It is not known if the same is true for fig wasp parasitoids.

Robust phylogeny estimates inform our inference of past evolutionary processes from patterns of diversity seen in the present. Topological congruence between the phylogenies of host organisms and their associated lineages is the first line of evidence for cospeciation. On the other hand, phylogenetic incongruence may indicate other historical patterns of association, including host switching. When host and associate topologies and divergence times are more closely congruent than expected by chance (Page 1996), ancient cospeciation may have occurred. Incongruence between phylogenies requires more detailed explanation, including the possibility that error is associated with either phylogeny estimate. Ecological explanations for phylogenetic incongruence include extinction, "missing the boat," host switching, and host-independent speciation (Page 2003). "Missing the boat" refers to the case where an associate tracks only one of the lineages following a host-speciation event. For most plant-insect interactions, these other events are more common than cospeciation (Farrell and Mitter 1990). A few highly specialized interactions, including obligate pollination mutualisms (Weiblen and Bush 2002; Kawakita et al. 2004), show evidence of cospeciation.

We examined patterns of historical association across three trophic levels in the fig microcosm, comparing mitochondrial DNA phylogenies for pollinators, gallers, and parasitoids to multigene phylogenies of the host figs. Given that nonpollinators play no direct role in fig reproductive isolation, we tested the prediction that gallers and parasitoids show less evidence of cospeciation with their hosts than do pollinators influencing patterns of host gene flow. Some background on fig pollination is needed to understand this prediction in more detail.

Background

Fig Pollination

More than 800 fig species (*Ficus*, Moraceae) occur in the tropics and subtropics worldwide (Berg 1989). Fig species exhibit a wide array of growth forms including shrubs, trees,

climbers, and hemi-epiphytic stranglers. The genus is characterized by an obligate mutualism with pollinating fig wasps (Chalcidoidea: Agaonidae: Agaoninae) and a specialized inflorescence called a syconium, which comprises an enclosed cavity filled with numerous, highly reduced unisexual flowers (Bronstein 1992). At the apex of the syconium is a bract-lined opening, or ostiole, that is the point of entry for pollinating fig wasps. Pollinators depend on figs for the rearing of their offspring, and figs depend on wasps for pollination. Pollinators are very small, approximately a millimeter in body length, and often bear specialized thoracic pockets for the transport of pollen grains (Weiblen 2002). Most fig species are pollinated by unique fig wasp species (Wiebes 1979), but not all cases involve one-to-one species specificity. Molbo et al. (2003) found cryptic pollinator species on the same host in sympatry, whereas Rasplus (1994) reported instances of peripatric pollinator species on widespread hosts with intervening zones of contact (Michaloud et al. 1996).

Chemical volatiles are the primary cues that attract unique pollinators species to receptive figs (Hossaert-McKey et al. 1994; Grison-Pige et al. 2002). Female wasps crawl through the bract-lined ostiole to reach the fig cavity where egg laying takes place. Oviposition involves piercing the style of a pistillate flower and depositing a single egg inside the fig ovule to form a gall (Galil and Eisikowitch 1971). Most Agaoninae actively pollinate fig flowers during oviposition by removing pollen from their thoracic pockets and spreading it across the synstigma, a surface formed by numerous, closely packed pistillate flowers (Jousselin and Kjellberg 2001). Wasp larvae consume fig seeds, a single larva feeding on the contents (typically the endosperm) of a single galled flower (Weiblen 2002). Sexual dimorphism reflects the specialized roles of male and female fig wasps. Females are mated in their galls by males who are typically wingless, blind, and less pigmented than the winged females. The emergence of females from their galls into the fig cavity coincides with anthesis, when males chew an opening in the syconium and females collect pollen from staminate flowers as they exit. The search for receptive syconia, followed by pollination and oviposition, marks the completion of the pollinator life cycle (Jousselin et al. 2001b).

Stability of this mutualism depends on the relative allocation of floral resources to pollen, seeds, and pollinators. Given that pollinators also eat seeds, there is potential evolutionary conflict between seed production and seed consumption (Cook and Rasplus 2003). Fig lineages have resolved this conflict independently by different means (Kameyama et al. 1999). This chapter focuses on the subgenus *Sycomorus*, which is in large part functionally dioecious, segregating the production of seeds and pollinators in two types of figs called "gall figs" and "seed figs" on separate plants (Weiblen et al. 2001). Gall figs contain staminate and short-styled pistillate flowers, whereas seed figs contain only long-styled pistillate flowers (Weiblen and Bush 2002; Weiblen 2004). Because differences in flower morphology affect oviposition success, the former produce pollinators and pollen while the latter produce only seed. There are obvious negative fitness consequences for pollinators of seed figs given that these "tomb blossoms" have ostiole bracts that prevent pollinators from exiting. Nonetheless, seed figs regularly deceive pollinators into visiting despite the absence of any reproductive reward (Grafen and Godfray 1991).

Ficus Subgenus *Sycomorus*

Ficus subgenus *Sycomorus* has attracted the attention of evolutionary biologists interested in explaining the stability of obligate pollination mutualism (Kameyama et al. 1999; Harrison and Yamamura 2003). The group is monophyletic and includes approximately 140 species occurring across Africa, Asia, and the Indo-Pacific (Weiblen 2000). All species of *Ficus* subgenus *Sycomorus* are pollinated by the monophyletic genus *Ceratosolen* (Weiblen 2001). A diverse assemblage of nonpollinating wasps that utilize fig resources is also associated with *Sycomorus* (Kerdelhue et al. 2000). The absence of pollen pockets and the presence of extremely long ovipositors with which to pierce the fig wall distinguish nonpollinators from *Ceratosolen*. However, a few nonpollinator species in Africa (Kerdelhue and Rasplus 1996) and Asia (Jousselin et al. 2001a) with short ovipositors are known to crawl into the fig and oviposit internally.

Nonpollinating Fig Wasps

Nonpollinators occupy two trophic levels in the fig microcosm, as gallers and parasitoids. *Sycomorus* figs are attacked by gallers of the genus *Platyneura* (Sycophaginae). The name *Platyneura* takes priority over *Apocryptophagus* (J. Y. Rasplus, 1997, personal communication) and includes all *Sycomorus* gallers known from Indo-Pacific, the center of *Sycomorus* fig diversity. *Platyneura* are essentially seed parasites. Oviposition in a fig ovule induces an abnormal growth such that *Platyneura* galls are much larger than those of *Ceratosolen*. Each galler occupies a single flower (Galil et al. 1970) and reduces fig fecundity by exactly one seed, while negatively affecting pollinators through competition for floral resources (Weiblen et al. 2001).

Nonpollinators occupying the third trophic level in the fig microcosm are parasitoids of other fig wasps. Three genera of parasitoids (Agaonidae: Sycoryctinae) associated with *Sycomorus* figs are *Sycoscapter*, *Philotypesis,* and *Apocrypta*. They are distinguished by ovipositors associated with three different means of piercing the fig wall. *Sycoscapter* and *Philotypesis* attack *Ceratosolen*, whereas *Apocrypta* are specifically associated with *Platyneura* gallers (Ulenberg 1985). The focus of this chapter is the three-way interaction between *Sycomorus*, *Platyneura*, and *Apocrypta*. *Apocrypta* females pierce the fig wall and deposit solitary eggs in *Platyneura* galls, where these parasitoids kill, consume, or outcompete

galler larvae (Weiblen et al. 2001). *Apocrypta* is not polyembryonic, and, as with the rest of the fig wasp community, a single adult is the product of each gall. Little is known about the systematics of *Apocrypta* apart from that provided by Ulenberg (1985), and no molecular data have been published until now.

Phylogenetic Knowledge

Previous studies on the phylogenetic relationships of *Sycomorus* figs (Weiblen 2000) and pollinators (Weiblen 2001) suggest a history of cospeciation and extreme host specificity. These findings are in broad agreement with predictions from pollinator life history. Cospeciation is expected when associates are vertically transmitted (Herre et al. 1999), and the transportation of fig pollen from cradle to grave by pollinating wasps *(Ceratosolen)* closely approximates this mode of transmission because pollinators tend to select the same host species as their parents. Pollinators rarely make mistakes when choosing host figs, and, in the event that a pollinator enters the wrong host, there is a chance of pollen incompatibility or the failure to lay eggs due to a morphological mismatch between ovipositor and fig flowers (Weiblen and Bush 2002). Only some of these conditions apply to externally ovipositing nonpollinators; the prediction that nonpollinator speciation should be less closely tied to *Sycomorus* speciation is supported by evidence from molecular phylogeny (Weiblen and Bush 2002). Nonpollinator host use may have more to do with the size of the fig and the thickness of the fig wall in relation to their ovipositor length (Weiblen 2004). Phylogenetic conservatism or convergent evolution (Jousselin et al. 2003) in fig size could affect patterns of speciation and host use in nonpollinators and lead to departures from simple expectations of cospeciation.

Overview

This chapter describes a sympatric assemblage of *Sycomorus* figs and the associated fig wasps from a lowland rain forest in Papua New Guinea. New Guinea is the global center of *Sycomorus* fig diversity, with over 60 species on the island. We focus on a set of sympatric and closely related host species because the co-occurrence of close relatives provides opportunities for host switching and departures from one-to-one species specificity. Phylogenies of sympatric *Sycomorus* figs and associated wasps based on multiple gene regions were used to explain the host specificity of nonpollinators and to test hypotheses of cospeciation between figs, pollinators, gallers, and parasitoids. In addition to topological comparisons of host and associate phylogenies, tests of temporal congruence were performed. Different predictions about the timing of divergence are drawn from the contrasting life histories of fig pollinators and nonpollinators. Complete reproductive interdependence of the mutualists, and the observation that extreme pollinator specificity enforces premating reproductive isolation between sympatric fig species, leads to the prediction that divergence of fig and pollinator clades should be temporally congruent. On the other hand, these conditions do not apply to nonpollinators or their hosts, and there is less reason to expect temporal congruence among interacting lineages when the mode of transmission is horizontal (Herre et al. 1999).

Sampling and DNA Sequencing

The study area was located in moderately disturbed lowland rain forest around the Ohu Conservation Area (145°41(E, 5°14″ S, ca. 50–200 m) in the Madang district of Papua New Guinea. The climate at the study area was perhumid (a wet climate with humidity index values above 100) with average annual rainfall of 3558 mm and annual average temperature of 26.5°C (McAlpine et al. 1983). Wasps were reared from 19 sympatric fig species by parataxonomists at the New Guinea Binatang Research Center between 1995 and 2005 (Table 17.1). These fig species are functionally dioecious (Weiblen et al. 2001) as described above. Wasps were reared from gall figs by enclosing each individual ripe fig in a container covered by nylon mesh held with a rubber band. Following the emergence of wasps from their galls and the fig, the entire community from an individual fig was preserved in 70% ethanol. Communities were sorted to genus and morphospecies using a dissecting microscope and digitally photographed.

Table 17.1 lists the 19 *Sycomorus* species and the associated pollinators, gallers, and parasitoids that were examined. Whereas most of the *Ceratosolen* species are known to science, the nonpollinator species have yet to be described. We recognized nonpollinator morphospecies on the basis of body size, ovipositor length, host use, and mitochondrial DNA sequence divergence (S.I.S., unpublished data). *Ceratosolen* pollinators in the study area are extremely specialized and are involved in one-to-one relationships with particular host species (Weiblen et al. 2001). It has been assumed that nonpollinators are similarly specialized (Ulenberg 1985; Weiblen and Bush 2002), but the dissertation research of Silvieus (unpublished data) suggests otherwise.

A molecular phylogeny for 19 sympatric *Sycomorus* host species was inferred from three gene regions: the internal transcribed spacer region of nuclear ribosomal DNA (ITS), glyceraldehyde 3-phosphate dehydrogenase (G3PDH), and granule-bound starch synthase (GBSS or waxy). Amplification of ITS (Weiblen 2000), G3PDH (Strand et al. 1997), and waxy followed published protocols (Mason-Gamer et al. 1998; Evans et al. 2000). Two *Ficus* outgroups were included for rooting purposes: *F. virens*, representing subgenus *Urostigma*, and *F. wassa*, representing subgenus *Sycidium* (Berg and Corner 2005). DNA was extracted from dried leaves using the DNeasy plant mini kit (Qiagen, Valencia, CA). ITS and G3PDH were sequenced directly from polymerase chain reaction (PCR) products, whereas waxy was cloned using the TOPO-TA PCR cloning kit (Invitrogen,

TABLE 17.1

Sympatric *Ficus* Subgenus *Sycomorus* Species and Associated *Ceratosolen* Pollinators, *Platyneura* Gallers, and *Apocrypta* Parasitoids from a New Guinea Lowland Rainforest

Ficus Species	*Ficus Section*	*Trees Sampled*	*Figs Sampled*	*Ceratosolen*	*Platyneura*	*Apocrypta*
adelpha	*Sycocarpus*	9	21	ex *F. adelpha*	*ex* F. adelpha[a]	ex *F. adelpha, bernaysii* & cf. *ternatana*
adenosperma	*Adenosperma*	5	5	*adenospermae*[b]	ex *F. adenosperma* sp. A ex *F. adenosperma* sp. B	
arfakensis	*Sycocarpus*	5	14	*solitarius*	ex *F. arfakensis* & cf. *ternatana*	ex *F. arfakensis*
bernaysii	*Sycocarpus*	21	51	*hooglandi*	ex *F. bernaysii* sp. A ex *F. bernaysii* sp. B	ex *F. adelpha, bernaysii* & cf. *ternatana*
botryocarpa	*Sycocarpus*	21	39	*corneri*	ex *F. botryocarpa*	
congesta	*Sycocarpus*	18	36	*notus*	ex *F. congesta* ex *F. congesta* & *hispidioides*	ex *F. congesta* & *hispidioides*
dammaropsis	*Adenosperma*[c]	18	16	*abnormis*		
hispidioides	*Sycocarpus*	19	27	*dentifer*	ex *F. hispidioides*[a] ex *F. congesta* & *hispidioides*	ex *F. congesta* & *hispidioides*
mollior	*Adenosperma*	5	16	*medlarianus*	ex *F. mollior* sp. A ex *F. mollior* sp. B	
morobensis	*Sycocarpus*	3	5	ex *F. morobensis*		

nodosa	*Sycomorus*	19	23	*nexilis*	ex *F. nodosa* sp. A ex *F. nodosa* sp. B	ex *F. nodosa*
pachyrrhachis	*Sycocarpus*	7	10	ex *F. pachyrrhachis*	ex *F. pachyrrhachis*	
pungens	*Sycocarpus*[d]	23	35	*nanus*		
robusta	*Sycomorus*	3	6	*grandii*[e,g]	ex *F. robusta*[a,e]	
semivestita	*Sycomorus*[f]	1	1	*grandii*[e,g]	ex *F. semivestita*[e]	
septica	*Sycocarpus*	18	32	*bisulcatus*		
subcuneata	*Adenosperma*	3	3	ex *F. subcuneata*[f]	ex *F. subcuneata*	
cf. *ternatana*[h]	*Sycocarpus*	2	2	ex *F.* cf. *ternatana*	ex *F. arfakensis* & cf. *ternatana*[a,e]	ex *F. adelpha, bernaysii* & cf. *ternatana*
variegata	*Sycomorus*	17	27	*appendiculatus*	ex *F. variegata* sp. A ex *F. variegata* sp. B	ex *F. variegata*

NOTE: Total numbers of trees and figs examined for nonpollinators are listed. Nonpollinator morphospecies are named according to the host fig species with which they are associated. All hosts are listed for nonpollinator morphospecies attacking multiple host species.

[a]Cytochrome B sequence unavailable.

[b]400 bp at 3′ end of cytochrome oxidase I unavailable.

[c]Molecular and morphological phylogenetic inferences support the placement of *F. dammaropsis* in section *Adenosperma*. Section *Dammaropsis* sensu Berg and Corner (2005) is embedded within section *Adenosperma* and should not be recognized.

[d]Weiblen (2000) placed *F. pungens* in section *Sycocarpus* based on morphological and molecular phylogenetic evidence. Section *Bosscheria* sensu Berg and Corner (2005) is embedded within section *Sycocarpus* and should not be recognized.

[e]400 bp at 5′ end of cytochrome oxidase I unavailable.

[f]Contrary to Weiblen (2000, 2001), *F. semivestita* is a member of section *Sycomorus* sensu Berg and Corner (2005). Weiblen (2000) included misidentified specimens of *F. subcuneata* in molecular and morphological analysis, erroneously suggesting the transfer of *F. semivestita* to section *Adenosperma*. The pollinator of *F. subcuneata* was misidentified on the basis of the host as *Ceratosolen grandii* in Weiblen (2001) and Weiblen and Bush (2002).

[g]According to morphology and COI sequences *Ceratosolen grandii* was reared from both *F. semivestita* and *F. robusta*.

[h]*Ficus* cf. *ternatana* is an undescribed New Guinea species evidently related to *F. ternatana* of the Moluccas.

Carlsbad, CA). Ten clones were screened for inserts, and plasmids were isolated from four of these using the Qiagen plasmid prep kit. Sequencing followed standard protocols for Big Dye v.3 (Applied Biosystems, Foster City, CA) with an ABI 377 automated DNA sequencer (PE Biosystems, Foster City, CA). Nucleotide sequences for each region were aligned using Clustal X (Thompson et al. 1997), followed by manual editing. Multiple copies of waxy are known in the Rosales (Evans et al. 2000), and therefore it was necessary to ensure that phylogeny reconstruction was performed with orthologous copies. A preliminary survey detected two copies in Moraceae, GBSS1 and GBSS2, which were easily distinguished on the basis of size and intron alignment (W.L.C., unpublished data). Analyses were based solely on GBSS1 because GBSS2 was encountered less commonly in *Sycomorus* figs.

Mitochondrial genes cytochrome oxidase I (COI) and cytochrome B (cytB) were sequenced from pollinating and nonpollinating wasps associated with sympatric *Sycomorus* species. Mitochondrial genes in fig wasps have very high rates of molecular evolution, so they are variable enough to resolve and support relationships among closely related species but are still conservative enough to align without ambiguity (Lunt et al. 1996; Lopez-Vaamonde et al. 2001; Machado et al. 2001; Weiblen 2001; Molbo et al. 2003). Although most morphospecies (hereafter species) were reared from multiple trees and multiple figs per tree (Table 17.1), a single exemplar from each species was included in phylogenetic analysis to reduce computation time. Genomic DNA representing each morphospecies was isolated by pooling up to 10 individuals reared from the same fig in extractions with the DNeasy tissue kit (Qiagen). Amplification and direct sequencing of COI and cytB followed standard protocols for fig wasps (Kerdelhue et al. 1999; Weiblen 2001). Taxon sampling of fig wasps was restricted to the local assemblage of *Sycomorus* associates plus a non-fig-associated chalcid, *Anaphes nitens* (Mymaridae), as an outgroup (C. Lopez-Vaamonde, unpublished data). Sequencing included ~800 bp of COI and ~600 bp of cytB, except in cases noted in Table 17.1 where PCR products were not obtained. Sequences from *Apocrypta*, *Ceratosolen*, and *Platyneura* were analyzed simultaneously, as the genera are reciprocally monophyletic and their relative phylogenetic positions were not the object of this community study.

Phylogenetic Analysis

Phylogenetic analysis was performed with PAUP*4.0b (Swofford 2001) under parsimony and maximum likelihood (ML) criteria. Our experience teaches that the application of multiple optimality criteria in phylogenetic analysis can identify strong inferences that are robust to different assumptions about underlying processes of molecular evolution (Datwyler and Weiblen 2004). Under parsimony, heuristic searches with 1000 random addition sequence replicates explored the possibility of multiple islands of most parsimonious (MP) trees (Maddison 1991). The relative strength of clade support was assessed using nonparametric bootstrap resampling (Felsenstein 1985a) with 1000 replicates and 100 random addition sequence replicates per bootstrap replicate. Although ML is computationally more demanding than parsimony, it assumes explicit models of nucleotide substitution that can be compared statistically. To expedite ML searches, MP topologies were used as starting trees to optimize substitution models, parameter estimates, and search for optimal ML topologies. Substitution models were selected using the Aikake information criterion as implemented in Modeltest (Posada and Crandall 1998; Posada and Buckley 2004), and the best-fitting models with the fewest additional parameters were used in heuristic searches to identify topologies that maximized the likelihood of the data.

Gene regions were analyzed separately and statistical tests of phylogenetic congruence were conducted before combining data sets in simultaneous analyses. MP topologies resulting from searches of separate data sets were compared under ML using the best-fitting substitution model and model parameters for all genes combined (Shimodaira and Hasegawa 1999). If topologies resulting from separate analyses of regions were not significantly different, the results of combined analyses were accepted as the best available estimate of phylogeny.

Regions from *Sycomorus* figs and fig wasps were tested for rate constancy using likelihood ratio tests of models with and without the assumption of a molecular clock (Felsenstein 1985b). When rate constancy was rejected, we applied a nonparametric rate smoothing method to ML branch lengths (Sanderson 1997) as implemented in the program TreeEdit (A. Rambant and M. Charleston, University of Oxford, Oxford, UK) to obtain ultrametric trees for the purpose of molecular dating. As there are no fossils attributed to *Sycomorus* or *Sycomorus*-associated wasps, we used a geological time constraint to estimate divergence times. *Ficus* sections *Papuasyce*, *Dammaropsis*, and *Adenosperma* (Weiblen 2000; Berg and Corner 2005) compose a clade that is endemic to New Guinea and the Solomon Islands. Since neither the island of New Guinea nor its antecedents existed more than ~40 million years from the present (Axelrod and Raven 1982), this age was applied as a maximum constraint for this *Sycomorus* clade and its associated *Ceratosolen* pollinators. The dated fig wasp phylogeny was split into the three component genera for separate reconciliation analyses with the dated host-fig phylogeny.

Reconciliation Analysis

Hypotheses of topological and temporal congruence among the lineages as implied by cospeciation were tested by comparison of dated molecular phylogenies for *Sycomorus* figs, *Ceratosolen* pollinators, *Playneura* gallers, and *Apocrypta* parasitoids. Host and associate phylogenies were reconciled under event-based parsimony and tested for cocladogenesis using TreeMap software (Page 1995). Phylogenetic reconciliation infers the minimal number of evolutionary events

needed to fit an associate phylogeny to a host phylogeny. Cocladogenesis is inferred if phylogenies of hosts and associates are topologically and temporally congruent. Deviations from perfect congruence suggest other evolutionary events including host switching (Percy et al. 2004). It is assumed that associates are restricted to a single host at any point in time, and, if there is a host switch, speciation is assumed with one daughter shifting to the new host (Ronquist 1998). Randomization tested whether reconciled host and associate tree topologies show significantly higher levels of cospeciation than expected by chance. Both host and associate phylogenies, randomized 1000 times using the proportional-to-distinguishable model in TreeMap, generated a null distribution of maximum cospeciation. Failure to reject the null hypothesis suggests that other evolutionary events besides cospeciation are needed to account for host associations in the present day (Hafner and Page 1995). Dates of divergence for *Sycomorus* clades and their inferred ancestral associates were then compared to assess the temporal plausibility of cospeciation. Significant correlation of the age of congruent, ancestral host and associate lineages provides evidence that speciation might have been synchronous. On the other hand, if divergence time estimates are wildly asynchronous, cospeciation is unlikely, and alternative scenarios such as host switching, speciation in the associate but not the host, speciation in the host but not the associate, may be invoked (Percy et al. 2004).

Phylogenies of Figs and Wasps

Sequencing of 19 *Sycomorus* species and two outgroups for three gene regions produced a total of 3040 aligned nucleotide positions. GBSS1 (waxy) provided the most phylogenetic information, with 179 parsimony-informative characters out of 1677 aligned positions (11%). G3PDH and ITS provided 61 and 114 parsimony informative characters (10% and 15%) out of 611 and 752 aligned positions, respectively. Separate heuristic searches of each gene region yielded 216, 10, and 495 MP trees for waxy, ITS, and G3PDH, respectively. Shimodaira-Hasegawa tests indicated that trees resulting from searches of individual gene regions were not significantly incongruent when compared in a likelihood framework incorporating information from all three genes. Likelihood and parsimony analyses were then conducted on the combined data sets. The best-fitting model of nucleotide substitution according to the Aikake information criterion was general time reversible (GTR) with additional parameters for heterogeneity in substitution rates across sites (Γ) and the proportion of invariant sites (I). Parsimony yielded 48 MP trees, and the ML topology was identical to one of these. Model parameters and branch lengths were estimated for the ML tree under GTR + I + Γ. Nonparametric bootstrapping under parsimony supported only half of the clades in the ML tree. Even three congruent gene regions providing 354 parsimony-informative characters failed to provide robust estimates of relationships among recently diverged, New Guinea endemic species in *Ficus* sections *Adenosperma* and *Sycocarpus* (Fig. 17.1). Deeper divergences among sections and subsections, however, were generally well supported according to bootstrap values >70%.

Sequencing of 19 *Ceratosolen*, 20 *Platyneura*, 5 *Apocrypta,* and an outgroup species for two mitochondrial genes provided a total of 1168 aligned nucleotide positions. Cytochrome oxidase I (COI) comprised the larger data set with 436 parsimony informative characters (55%) out of 787 aligned positions, compared to cytochrome B (cytB) with 212 parsimony informative characters (56%) out of 381 positions in total. Heuristic searches of COI alone yielded a single tree, while cytB alone yielded 60 MP trees. Shimodaira-Hasegawa tests indicated that all 60 cytB topologies were significantly less likely ($P < 0.001$) than the COI tree when compared in a likelihood framework incorporating information from both genes. We attribute significant incongruence between nonrecombining mitochondrial genes to homoplasy and the lower number of informative characters from cytB. Heuristic searches of concatenated cytB and COI assuming the best-fitting model of nucleotide substitution (GTR + Γ + I) yielded a topology that was significantly more likely ($P < 0.001$) than either data set alone. Heuristic searches of the two genes in combination under parsimony yielded six trees including a topology identical to the ML tree. We present the ML tree based on the combined data set as the best phylogeny estimate for sympatric *Sycomorus* fig wasps from our study area. The tree was broken along the backbone to yield the individual topologies for pollinators, gallers, and parasitoids shown in Figs. 17.1, 17.2, and 17.3. Nonparametric bootstrap values supported relationships among species groups associated with *Sycomorus* sections and subsections, but clade support within species groups was generally lacking. For example, well-supported *Ceratosolen* clades included pollinators associated with *Ficus* section *Adenosperma*, section *Sycomorus*, and section *Sycocarpus*.

Host Specificity of Nonpollinating Fig Wasps

Gallers and parasitoids exhibited different patterns of association with *Sycomorus* than pollinators (Figs. 17.1, 17.2, and 17.3). *Platyneura* species were as numerous as *Ceratosolen* but did not conform to the pattern of one-to-one specificity that characterizes the mutualists. Departures from one-to-one species specificity included four parasite-free *Sycomorus* species, seven *Sycomorus* species attacked by multiple *Platyneura* gallers, and two *Platyneura* attacking multiple hosts. *Sycomorus* species were commonly attacked by two *Platyneura* that differed in ovipositor length and oviposition timing (Kerdelhue and Rasplus 1996). Species with short ovipositors lay eggs prior to pollination when figs are small in diameter, whereas species with long ovipositors lay eggs after pollination when figs are larger (Weiblen and Bush 2002). Two cases in which sister species of *Platyneura* parasitized the same host are discussed later as examples of adaptive divergence. One *Apocrypta* parasitoid

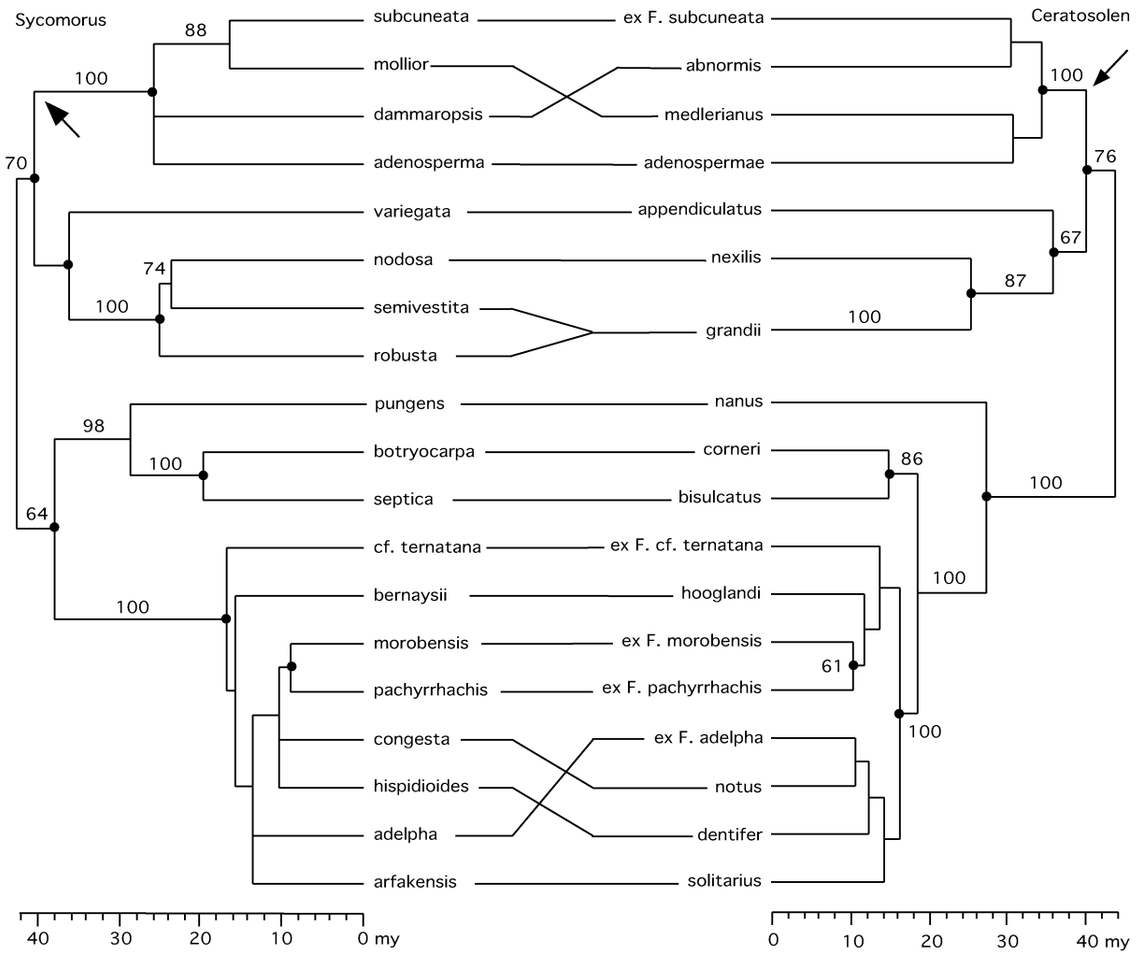

FIGURE 17.1. Cophylogeny of 19 sympatric *Ficus* subgenus *Sycomorus* and their species-specific *Ceratosolen* pollinators. Nonparametric bootstrap values >50% are shown above the nodes. Nonparametric rate smoothing of maximum likelihood topologies with general time reversible plus invariant sites plus heterogeneity in substitution rates across sites (GTR + I + Γ) branch lengths yielded ultrametric trees. Maximum ages for the New Guinea endemic clade (40 million years), represented by *F. adenosperma*, *F. dammaropsis*, *F. mollior*, and *F. subcuneata*, and their respective pollinators (indicated with arrows) were used to calibrate molecular phylogenies with respect to divergence times. The fig wasp community phylogeny was split into three component genera with *Platyneura* and *Apocrypta* shown in Figs. 17.2 and 17.3. Divergence times of congruent fig and pollinator clades marked by dots are compared in Fig. 17.4.

species exhibited broader host ranges than any pollinator or galler. Some parasitoids attacked closely related *Platyneura* on multiple fig species, and other species attacked multiple *Platyneura* on a single *Sycomorus* host. In the most extreme case, a single *Apocrypta* species attacked *Platyneura* on three closely related fig species from section *Sycocarpus*.

Double Dating of Figs and Fig Wasps

Figs and Pollinating Fig Wasps

Previous reconciliation analysis indicated that the extent of codivergence inferred for *Sycomorus* figs and their *Ceratosolen* pollinators is greater than expected by chance (Weiblen and Bush 2002). The same was true for the sympatric, interacting species pairs from New Guinea shown in Fig. 17.1. Heuristic searches maximizing codivergence and minimizing other evolutionary events yielded a reconciled tree with 13 out of 18 possible cospeciation events, four duplications, one host switch, and 16 lineage sorting events. Duplications refer to cases in which a pollinator lineage underwent speciation when the host-fig lineage did not, and lineage sorting refers to the case in which pollinators "missed the boat" and failed to colonize one of the fig lineages resulting from a host-speciation event. The maximum number of inferred cospeciation events was significantly greater than the null expectation based on pairs of randomized trees ($P < 0.001$). Rejecting this rather weak null hypothesis is not terribly informative, however, as nonrandom patterns of historical association could result from processes other than cospeciation. The calibration of molecular phylogenies with respect to divergence times based on

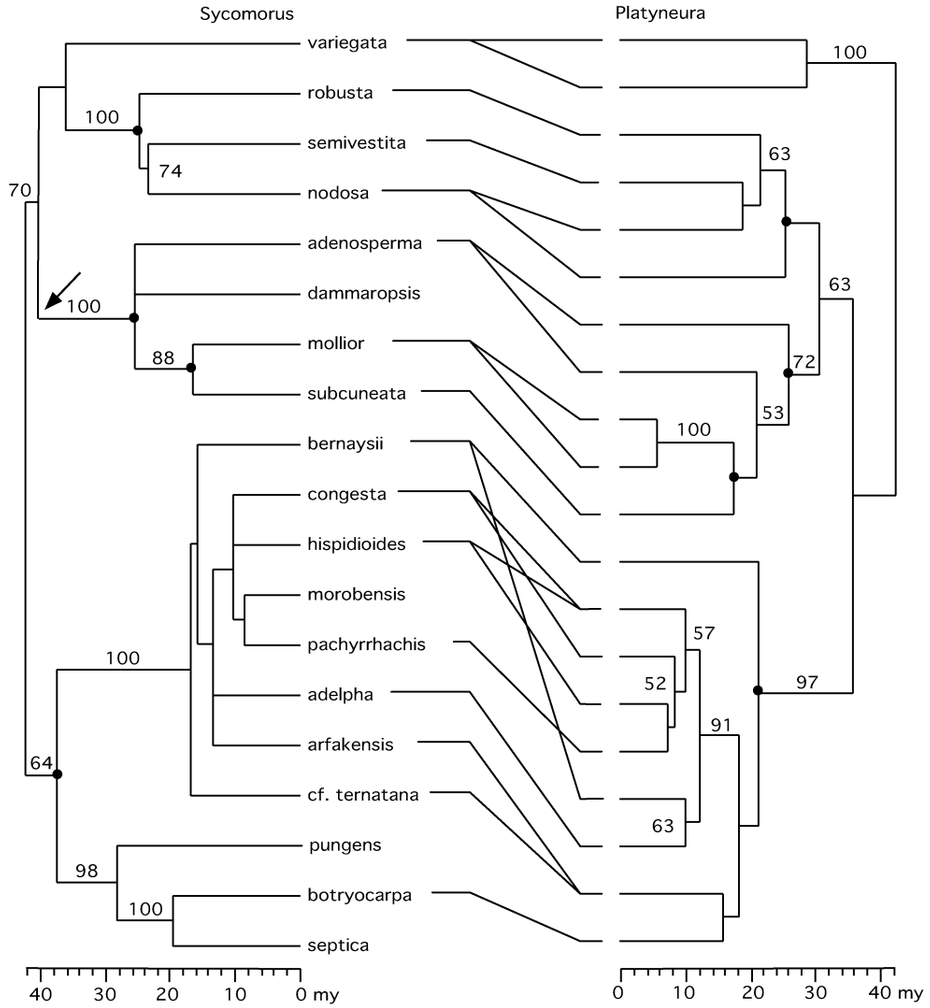

FIGURE 17.2. Cophylogeny of sympatric *Ficus* subgenus *Sycomorus* species and *Platyneura* nonpollinating gallers. The fig wasp community phylogeny was calibrated with respect to time using the 40 million year maximum age constraint for New Guinea endemic *Ceratosolen* as described in Fig 17.1. Divergence times of congruent fig and parasite clades marked by dots are compared in Fig. 17.4.

geological evidence enabled the prediction of temporal congruence of cospeciating fig and pollinator clades to be tested directly. It is important to recognize that 40 million years for the origin of New Guinea represents a maximum age constraint for the origin of fig and fig wasps lineages endemic to the island. In point of fact, the precise age for the origin of these clades could be much more recent, and therefore we concentrate on relative differences in maximum age estimates for interacting lineages.

Upon reconciliation of the fig and pollinator phylogenies shown in Fig. 17.1, the maximum ages of cospeciating lineages (Fig. 17.4A) were not significantly correlated when compared to a null distribution of randomized branch lengths ($P = 0.267$). We attribute the lack of correlation to errors associated with phylogeny estimation and errors in branch length estimation. Reconciliation analysis assumes that phylogeny estimates are without error, and the lack of bootstrap support for relationships within many species groups (Fig. 17.1) suggests that some clades could be spurious. Errors are compounded in reconciliation analysis when a spurious pollinator clade is fitted to a spurious host clade by invoking duplications, host switches, and lineage sorting events. We adopted a strategy to avoid misleading inferences in the face of phylogenetic uncertainty by comparing only the ages of strictly congruent host and associate clades (Rønsted et al. 2005). This strategy minimizes the comparison of spurious clades because host and associate DNA sequences are independent and phylogenetic congruence is unlikely to arise in this case by chance. Congruent clades of *Sycomorus* and *Ceratosolen* pollinators tended to be robust. Six instances of strict congruence were supported by >50% bootstrap, while only a single instance of strongly supported conflict was detected, involving the conflicting positions of *F. pungens* and *C. nanus*. Interestingly, *C. grandii* was

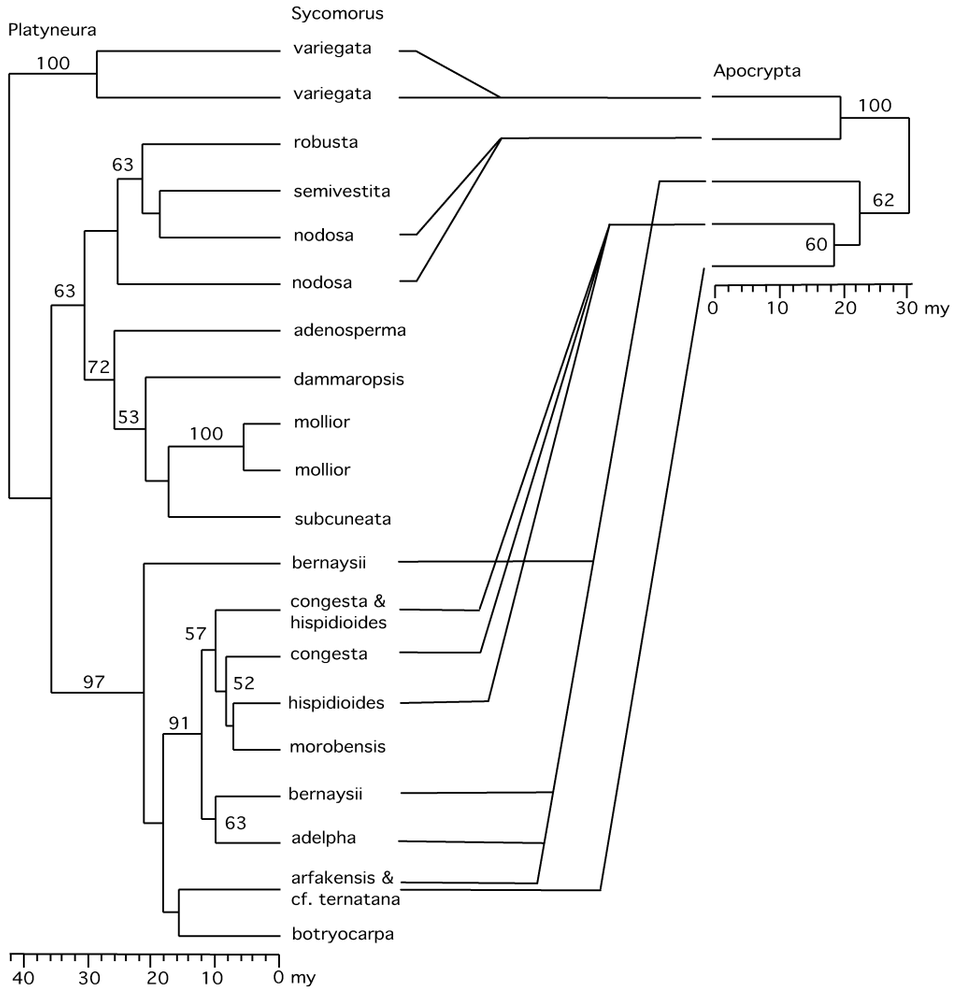

FIGURE 17.3. Cophylogeny of sympatric *Platyneura* nonpollinating galler species and *Apocrypta* nonpollinating parasitoid species. *Ficus* subgenus *Sycomorus* species hosting these wasps are listed between the phylogenies. Nonparametric bootstrap values are shown above the nodes. The fig wasp community phylogeny was calibrated with respect to time using the 40 million year maximum age constraint for New Guinea endemic *Ceratosolen* as described in Fig 17.1.

reared and sequenced on five occasions from *F. robusta* and once from *F. semivestita*, suggesting that this pollinator frequents two hosts and raising the possibility of interspecific hybridization (Machado et al. 2005). Strongly supported conflicts invite further exploration as potential cases of host switching or other evolutionary scenarios besides cospeciation, but they do not bear directly on the overall test of temporal congruence presented here.

Maximum ages of the eight congruent fig and pollinator clades marked in Fig. 17.1 were highly correlated ($R^2 = 0.82$) (Fig. 17.4B), much more so than those of the codiverging clades inferred by reconciliation analysis ($R^2 = 0.56$) (Fig. 17.4A). In contrast to the results of reconciliation analysis, the age correlation of congruent clades was significantly different from null expectations based on randomized branch lengths ($P < 0.005$). Furthermore, the slope of the relationship (0.92) was not significantly different from the slope expected under the cospeciation hypothesis that congruent fig and pollinator clades are of equal age. These results provide compelling evidence for the codivergence of mutualists in the fig microcosm.

Figs and Nonpollinating Gallers

Platyneura gallers show an entirely different pattern of historical association with *Sycomorus* figs than *Ceratosolen* (Fig. 17.2). Weiblen and Bush (2002) reported the extent of cospeciation inferred for figs and gallers to be not significantly different from chance expectations, but the broader sample of taxa included here indicated nonrandom patterns of association between *Platyneura* and *Sycomorus* ($P = 0.011$). Reconciled trees included 9 out of 18 possible cospeciation events, 10 duplications, and 28 sorting events. These findings taken alone suggest that the detection of cophylogeny is sensitive to taxon sampling and that the modes of

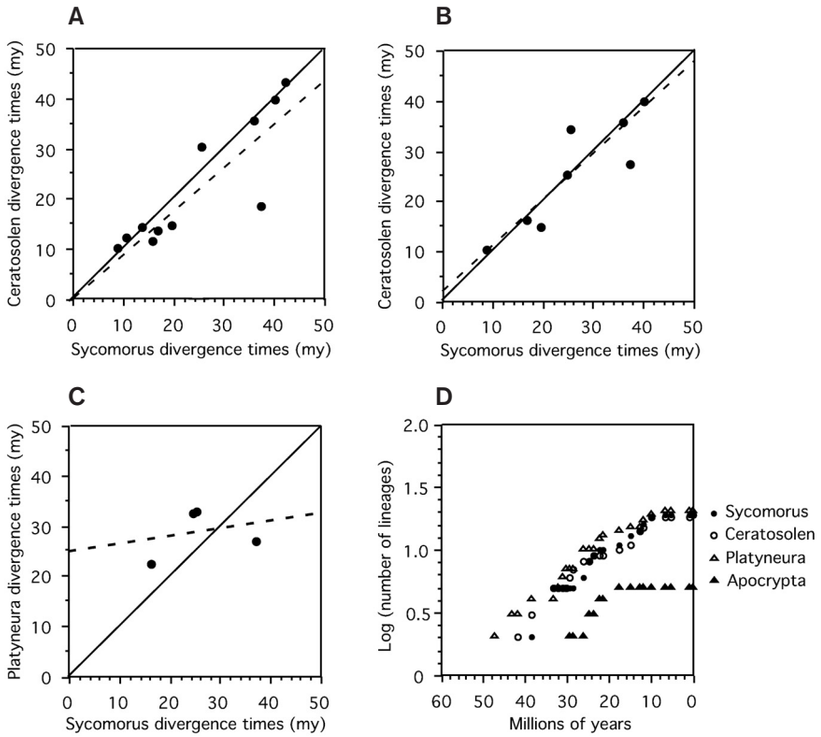

FIGURE 17.4. Temporal congruence of diversifying *Sycomorus* and *Ceratosolen* lineages suggests a history of cospeciation in the fig-pollinator mutualism. Solid lines indicate the slope expected when host and associate lineages are of equal age. Dashed lines indicate the observed regression of host and associate ages. A. The correlation of fig and pollinator cospeciation times ($R^2 = 0.56$) inferred from reconciliation analysis was not significant when compared to a null distribution of randomized branch lengths ($P = 0.267$). B. Maximum ages of congruent fig and pollinator clades shown in Fig. 17.1 were highly correlated ($R^2 = 0.82$). Divergence times of mutualists were significantly correlated compared to chance expectations ($P < 0.005$) based on randomized branch lengths. The slope of the inferred relationship (0.92) was not significantly different from the expectation that codiverged fig and pollinator lineages are of identical age. C. Maximum ages of congruent fig and galler clades shown in Fig. 17.2 were poorly correlated ($R^2 = 0.26$). The correlation of host and parasite divergence times was not significantly different from chance expectations ($P = 0.741$). In addition, the slope of the relationship (0.50) did not support the expectation that fig and parasite clades are of equal age. D. Logarithm of numbers of lineages through time. Figs, pollinators, and gallers originated about the same time and accumulated species at similar rates, while parasitoids appeared later and accumulated species at a slower rate.

speciation in fig pollinators and gallers might not be as different as Weiblen and Bush (2002) suggested. However, a temporal test indicated that the maximum ages of congruent fig and galler clades are poorly correlated ($R^2 = 0.26$) (Fig. 17.4C) and not significantly different from randomly associated branch lengths ($P = 0.741$). In further contrast to the pattern for mutualists, the slope of the relationship for figs and gallers (0.15) strongly deviated from the expectation of codivergence (1.00). The lack of temporal congruence indicates that nonrandom patterns of historical association are not due to cospeciation but some other mode of speciation for fig gallers. That both pollinators and gallers show nonrandom patterns of historical association with hosts but very different temporal patterns of diversification points to the weakness of merely testing for topological congruence in studies of cophylogeny.

Nonpollinating Gallers and Parasitoids

The pattern of historical association for *Apocrypta* parasitoids with their *Platyneura* hosts (Fig. 17.3) was not significantly different from chance expectations ($P = 0.054$). Only two cospeciations, two duplications, and 12 sorting events were inferred from reconciled trees. Although the number of cospeciation events was insufficient for a direct test of temporal congruence, maximum age estimates based on sequence divergence indicate that parasitoids are millions of years younger than their nonpollinating hosts. Topological and temporal evidence

together reject cospeciation as a mechanism for *Apocrypta* diversification. Log-lineage-through-time plots (Fig. 17.4D) suggest that *Apocrypta* colonized New Guinea *Sycomorus* figs more recently and have accumulated species at a slower rate than either *Ceratosolen* or *Platyneura*. That *Apocrypta* are less host specific than pollinators or gallers might explain why parasitoids are less numerous in species (Fig. 17.3).

Modes of Speciation in Fig Pollinators, Gallers, and Parasitoids

Pollinating Wasps

Although the tempo of speciation in *Sycomorus* pollinators and gallers appears quite similar (Fig. 17.4D), modes of speciation appear to be entirely different. Equal ages of congruent fig and pollinator lineages support an overall pattern of codivergence for obligate mutualists (Rønsted et al. 2005). The pattern is consistent with predictions based on intertwined life histories. Pollinators transport host gametes from cradle to grave, and the persistence of a pollinator lineage depends on successful fertilization and colonization of fig flowers in a fashion that approximates the vertical mode of transmission from host parent to offspring exhibited by endosymbionts or the parasitic lice of birds and mammals (Page 1996; Herre et al. 1999; Clayton et al. 2003). The extreme host specificity of *Ceratosolen* can influence *Sycomorus* speciation because pollinator behavior is a central fig reproductive isolating mechanism. Pollinator behavioral responses to volatiles released when fig flowers are receptive determine the extent of gene flow within and among fig populations. If pollinators choose to colonize hosts that are most chemically similar to their natal figs, it is conceivable that quite subtle differences in volatile chemistry (Grison-Pige et al. 2002), perhaps even influenced by nongenetic factors such as soil type or other environmental influences, could result in the complete reproductive isolation of fig subpopulations. Pollinator-mediated disruption of gene flow would allow fig subpopulations to diverge genetically through the accumulation of mutations followed by either drift or selection, the latter raising the possibility of adaptive divergence (Dieckmann and Doebeli 1999).

Why should pollinators exhibit such extreme host specificity as to promote host speciation? It is conceivable that populations have precious little genetic variation in behavioral responses to fig volatiles as a result of intense selection imposed by host figs. Morphological adaptations of figs and the associated wasps are so extreme (Herre 1989; Van Noort and Compton 1996; Weiblen 2004) that the colonization of unrelated figs could result in mismatches of interacting traits including the shape of the wasp head in relation to the fig opening and the wasp ovipositor in relation to the fig flowers. Mismatched pollinators are also at a competitive disadvantage with respect to locally adapted, resident pollinators when it comes to utilizing fig resources (Weiblen and Bush 2002). These considerations lead us to conclude that cospeciation between fig mutualists has occurred more commonly than the evolution of host shifts and expanded host range (Michaloud et al. 1996; Molbo et al. 2003).

Nonpollinating Gallers

Sycomorus gallers have accumulated similar numbers of species in the same time frame as *Ceratosolen,* but modes of speciation appear to be quite different. Historical inferences rejected the cospeciation hypothesis for parasite diversification, and present-day associations support at least two alternative hypotheses. One-to-one specificity does not hold for gallers, as many *Sycomorus* species were attacked by multiple *Platyneura* species (Fig. 17.2). In the cases of *F. nodosa*, *F. adenosperma*, *F. bernaysii*, *F. congesta,* and *F. hispidioides*, nonsister *Platyneura* species were reared from the same host fig, evidence that multiple parasite lineages colonized the same fig species independently. Morphologically and genetically distinct parasite species coexist in the same host by utilizing fig resources at different stages of growth. Gallers with short ovipositors attack the fig early in development when the fig wall is thin, while gallers with long ovipositors attack later in development when the wall is thicker. Speciation mediated by a host shift (Bush 1975; Coyne and Orr 2004) could be more common in fig gallers than pollinators because gallers do not depend directly on fertilization of fig flowers for development and they play no direct role in fig reproductive isolation. They do not interact closely with the fig opening, ovipositing externally by piercing the fig wall and inducing abnormal cellular growth in fig ovaries as a food resource for the offspring. The thickness of the fig wall and the timing of oviposition with respect to fig development appear to be traits that could facilitate a host shift. Sister group comparisons showed that when galler speciation occurred by means of host switching, there is a tendency for *Platyneura* to shift to *Sycomorus* figs with similar wall thickness and to retain similar ovipositor lengths and oviposition habits (Weiblen and Bush 2002). Along these lines, two instances in which a single *Platyneura* species attacked multiple hosts could represent cases of incipient speciation mediated by the colonization of morphologically similar host figs.

The most intriguing cases of galler speciation involved sister *Platyneura* attacking the same host in sympatry, as observed in *F. mollior* and *F. variegata*. Sister *Platyneura* on the same host differ in ovipositor length and oviposition timing and show ~10% mitochondrial DNA divergence (Weiblen and Bush 2002). These cases possibly represent instances of sympatric speciation driven by a phenological shift in oviposition timing as predicted by models of adaptive divergence (Gibbons 1979; Ramadevan and Deakin 1990; Johnson et al. 1996; Dieckmann and Doebeli 1999; Kondrashov and Kondrashov 1999). Although perhaps among the more striking empirical examples of adaptive divergence (Coyne and Orr 2004), it is necessary to recog-

nize that phenological shifts in oviposition habit and the evolution of reproductive isolation could also have occurred in allopatry. In any event, the temporal incongruence of *Platyneura* and *Sycomorus* is consistent with molecular dating evidence from other plant-insect interactions (Percy et al. 2004), indicating that herbivores and their hosts have not cospeciated.

Parasitoids

Sycomorus parasitoids are younger, fewer in species, and less host specific than pollinators and parasites (Fig. 17.3). Abrahamson and Blair (Chapter 14) point out that gallers engineer niches for their use and parasitoids exploit these niches, fueling sequential radiations wherein herbivore speciation drives the speciation of natural enemies. *Apocrypta* reared from different *Ficus* species have very similar mitochondrial DNA sequences (<2% divergence), indicating that fig parasitoids have broader host range than previously thought (Ulenberg 1985; Lopez-Vaamonde et al. 2001). Temporal evidence is consistent with a relatively recent invasion of the *Sycomorus* fig wasp community followed by stepwise colonization of existing host lineages. But why is *Apocrypta* less host specific than other fig wasp lineages? A possible explanation is population size. *Platyneura* gallers are orders of magnitude less abundant than pollinators, and *Apocrypta* parasitoids are similarly rare when compared to *Platyneura*. Many of the figs we sampled did not contain *Platyneura* and few contained *Apocrypta*. Levels of parasitism are possibly limited because parasitoid oviposition search time is wasted on figs lacking *Platyneura* hosts (Weiblen et al. 2001). Species-specific parasitoids may face a higher risk of extinction than less-specialized parasitoids that maintain larger population sizes by utilizing a broader resource base. We have yet to encounter multiple *Apocrypta* species on a single host, and it is thought that a single parasitoid species can attack multiple *Platyneura* species in the same fig. It will be possible in the future to examine the relationship between population size and host range by developing a species concept and molecular phylogeny for the *Sycoscapter* parasitoids of *Ceratosolen*. Since pollinators are orders of magnitude more abundant than gallers, they are the easier host for parasitoids to locate, and we predict that parasitoids of pollinators are more abundant than those of gallers. Host-range differences between parasitoids of pollinators and gallers associated with population size could support the hypothesis that extreme specialization is a dead end (Kelley and Farrell 1998).

Detailed historical inferences in this system will require more robust phylogeny estimates for the interacting lineages, as support for relationships in many species groups was weak even with the inclusion of multiple genes. Nuclear DNA sequence data for fig wasps would be a significant addition. Other possibilities for future study include detailed investigations of particular evolutionary scenarios including host switching, using approaches more sophisticated than maximum cospeciation analysis (Charleston 1998).

Conclusions

The fig microcosm provides a uniquely controlled and bounded environment in which to compare the impact of life-history variation on modes of diversification. Phylogenies for sympatric *Sycomorus* figs and associated fig wasps from New Guinea supported predictions about the extent of codivergence drawn from the contrasting life histories of fig pollinators, gallers, and parasitoids. Complete reproductive interdependence of the mutualists and extreme pollinator specificity enforces premating reproductive isolation between sympatric fig species such that many fig and pollinator clades are of equal age. These conditions do not apply to nonpollinators that appear to have diversified more commonly by processes other than cospeciation, including host switching. Externally ovipositing nonpollinators may colonize new hosts more readily than pollinators because they do not have to enter the fig cavity to lay eggs. Not only must host-shifting pollinators be morphologically compatible with the fig opening and flowers, but there are the additional challenges of pollen compatibility and competition with locally adapted resident pollinators. These considerations combined with the fundamental role that pollinators play in fig reproductive isolation account for the striking differences in modes of speciation for insect lineages that use the same plant resources in very different ways.

Acknowledgments

We thank Brus Isua and the staff of the New Guinea Binatang Research Center for field collecting, Warren Abrahamson, Catherine Blair, and an anonymous reviewer for helpful comments, and the National Research Institute, Forest Research Institute, and Department of Environment of Conservation of Papua New Guinea for permission to conduct the work. This material is based upon work supported by the National Science Foundation under grant DEB 0128833.

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