FROM PATTERN TO PROCESS: ECOLOGY AND EVOLUTION OF HOST SPECIFICITY IN THE FIG-POLLINATOR MUTUALISM

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Annika M. Moe

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George D. Weiblen, Advisor

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I dedicate this work to my family on both sides of the Pacific,

and to the forests of New Guinea for teaching me humility and perseverance.

ABSTRACT

One of the greatest challenges in the study of coevolution, indeed, for biology in general, is to understand how evolutionary and ecological processes shape patterns in nature. Ecologists routinely observe patterns of association among organisms, such as parasites infecting hosts or insects pollinating flowers and systematists routinely infer patterns of phylogenetic relationship. Such patterns invite explanation and suggest hypotheses about the evolutionary process, but it is difficult to investigate contemporary processes, including natural selection and, of course, impossible to directly observe historical processes. Observation of patterns in various ecological contexts, inference of phylogenetic patterns, model and simulation of processes, and direct experimentation aim to test specific predictions about the role of ecology in shaping evolutionary trajectories, and evolutionary processes in shaping ecological associations.

The fig-wasp pollinator mutualism provides a unique opportunity to examine fundamental processes of coevolution, namely, reciprocal adaptation where interacting partners are the agents of selection. Because pollinating wasp reproduction is directly linked to host plant reproduction, it is possible to estimate the fitness consequences of interaction for both partners simultaneously. By manipulation of interacting individuals and species, or by examination of natural variation within and among populations, it may be possible to estimate the strength and direction of selection on each mutualistic partner.

This work employs molecular genetic patterns, ecological observations, and direct experimentation to investigate host specificity in *Ceratosolen* (Agaonidae, Hymenoptera)

pollinators of *Ficus* subgenus *Sycomorus* (Moraceae) and potential processes affecting the origin and evolution of species diversity in this system.

The first chapter examines genetic variation in *Ceratosolen* pollinators of widespread *Ficus* across the geographic range of several host species. Deep mitochondrial DNA sequence divergence between host-specific populations distributed across Wallacea suggests host conservatism during ancient range expansion and subsequent isolation by distance. Geographic patterns of sequence divergence and host association are more consistent with a model of allopatric speciation than speciation by host switching. The second chapter investigates pollinator host choice by morphotyping and DNA barcoding of floral visitors in a community of closely related and sympatric fig species. Host specificity was very high, but rare pollinator sharing among sympatric fig species was observed at a rate of 1-2%. Even such rare events could be evolutionarily significant and pose challenges for species delimitation.

The third chapter examines fitness consequences of pollinator sharing by experiment. A new method of manipulating fig pollinators investigated the reproductive consequences of intra- and interspecific pollinator visitation for both mutualistic partners. When pollinators were introduced to a novel host species, hybrid seed set was comparable to results of conspecific crosses. Hybrids germinated, established, and grew at rates comparable to non-hybrids. Pollinator fitness, however, was compromised after oviposition in the novel host. Although heterospecific pollinators induced gall formation, offspring did not develop to maturity in the new host. Microsatellite genotypes of a New Guinea fig community indicated a substantial but not absolute barrier to gene flow among sympatric species. That hybrids constituted fewer than 2% of individuals in populations may be explained by selection against pollinator host switching in this system.

Collectively, these studies suggest that the extreme species-specificity of associations between *Ceratosolen* pollinators and *Sycomorus* figs is maintained by the fitness cost of colonizing new hosts. At the same time, hybridization resulting from rare instances of pollinator sharing in even the most extremely specialized of pollination mutualisms has the potential to influence diversification and coevolution.

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CHAPTER 1

MOLECULAR DIVERGENCE IN ALLOPATRIC *CERATOSOLEN* POLLINATORS (AGAONIDAE) OF GEOGRAPHICALLY WIDESPREAD *FICUS* SPECIES (MORACEAE)

The diversification of tropical phytophagous insects has been attributed to a combination of ecological specialization and geographic isolating mechanisms (Coyne and Orr 2004; Waser and Ollerton 2006; Tilmon 2008; Schemske et al. 2009). Many studies have investigated local host plant associations as a mechanism driving insect speciation (Tilmon 2008). Fewer studies have examined what Mayr and Dobzhansky (Dobzhansky 1937; Mayr 1942) supposed was the predominant mode of speciation involving the geographic isolation and divergence of populations. Studies of pollinating seed predators in the family Agaonidae (fig wasps) have particularly focused on the evolution of host specificity to account for speciation (Herre et al. 2008).

The mutualism between figs (Moraceae: *Ficus*) and their wasp pollinators (Chalcidoidea: Agaonidae) is one of the most species-specific plant/pollinator interactions (Ollerton 2006). Due to reproductive interdependence, this mutualism was once thought to involve a one-to-one pollinator species to host species ratio (Janzen 1979; Weiblen 2002). Under this scenario, reproductive isolation and speciation in one partner could cause speciation in the other partner, resulting in highly congruent phylogenies as predicted by models of co-speciation in vertically transmitted parasites and their hosts (Page 2003). Although molecular phylogeny has revealed congruence between some fig and pollinator lineages (Weiblen and Bush 2002; Jousselin et al. 2008), numerous cases of incongruence provide evidence of processes other than cospeciation (Machado et al. 2005; Jackson et al. 2008; Su et al. 2008; Renoult et al. 2009). Early pollinator species concepts were shaped by the assumption of 1:1 species specificity (Wiebes 1979) whereas more recent studies have identified departures from this pattern of association. Multiple cryptic species are known to pollinate the same *Ficus* species in sympatry (Molbo et al. 2003; Molbo et al. 2004; Peng et al. 2008) and sampling of pollinators across the host species geographic range has identified cryptic, allopatric species (Haine et al. 2006; Su et al. 2008). Pollinator sharing, in which one species of wasp pollinates more than one species of *Ficus* (Machado et al. 2005) provide indirect evidence of hybridization among fig species (Parrish et al. 2003). Incongruence between fig and pollinator phylogenies from the Neotropics (Machado et al. 2005; Marussich and Machado 2007; Su et al. 2008) and Africa (Renoult et al. 2009) has been interpreted as evidence of speciation by host switching, but other processes, such as differential rates of dispersal and allopatric speciation, could also produce such a pattern. Phylogeographic data are needed to shed light on the role geographic isolation in fig wasp speciation.

Molecular surveys of fig pollinators have been of limited geographical extent and records of pollinator associations across the range of widespread host species are few. Haine et al. (2006) found several cryptic species pollinating widespread *F. rubiginosa* in Australia but no evidence of host switching. Su et al. (2008) found a different situation in Mexico, where cryptic wasp species pollinating *F. petiolaris* did not form a monophyletic group, suggesting repeated and independent colonization of the host by different pollinator lineages.

Unequal dispersal rates between figs and wasps could facilitate speciation by host switching if a local pollinator colonized an exotic fig or an exotic pollinator colonized a local fig. Examples of host switching are known from fig trees cultivated outside their native range that were subsequently colonized by local pollinators (Janzen 1979; Compton 1990; Ramirez 1994). African fig pollinators are postulated to have switched hosts in crossing the Mozambique channel to colonize Madagascar (Kerdelhué et al.

1999). Fig wasps with more limited dispersal (Harrison and Rasplus 2006) that pollinate figs distributed across oceanic islands provide opportunities to examine the relative roles of geographic isolation and host specificity in pollinator speciation.

Compared to studies of neotropical and African fig pollination during the past decade (Arnold 1997; Machado et al. 2005; Marussich and Machado 2007; Jackson et al. 2008; Su et al. 2008; Renoult et al. 2009), there have been few comparable studies in Southeast Asia, the center of fig diversity. The island region of Wallacea is a biogeographical transition zone that marks the meeting of two continental shelves, the Sunda, linking Borneo and Java to the Asian mainland, and the Sahul, connecting New Guinea to Australia (Figure 1.1). The two plates came into contact around 50 mya and brought distinct flora and fauna into close proximity across Wallacea (Evans et al. 2003; Schulte et al. 2003; Lourie and Vincent 2004; Beck et al. 2006; Braby and Pierce 2007; Jonsson et al. 2008; Muellner et al. 2008). Given that fig pollination arose after the break-up of Pangea (Zerega et al. 2005), fig and pollinator species spanning the Wallace Line must have achieved their current distribution by dispersal across the Makassar strait or the Philippine Sea and subsequent range expansion. In this regard, Wallacea is fertile ground for detecting host switches in conjunction with long-distance dispersal and/or range expansion.

The wasp genus *Ceratosolen* pollinates several sections of mainly dioecious Australasian *Ficus* (Wiebes 1982). Several *Ceratosolen* species have very broad distributions across Wallacea and have been collected from several sites within their range (Machado et al. 2001; Weiblen 2001; West et al. 2001; Lin et al. 2008). For these reasons, *Ceratosolen* wasps are an ideal focal group for this study.

We take advantage of such widely distributed taxa in Wallacea to investigate three questions. (1) Did geographic isolation of pollinator populations result in allopatric divergence? Specifically, are pollinators of figs with widespread geographic ranges sufficiently diverged to comprise cryptic species? (2) Were pollinator-host associations conserved through the process of allopatric divergence? Specifically, are pollinators of widespread fig species monophyletic? (3) How old are cryptic species of *Ceratosolen* and is the timing of divergence consistent with allopatric speciation?

We use mitochondrial DNA sequences to investigate these questions. Despite recent criticism (Zink and Barrowclough 2008), mitochondrial DNA can illuminate patterns of genetic structure consistent with the geographic isolation of populations and the conservatism of host associations. Conflicting mitochondrial and nuclear gene trees, where the former indicates monophyly and the latter non-monophyly, could arise due to the maternal inheritance of mitochondrial DNA if female gene flow is more restricted than male gene flow (Zink and Barrowclough 2008). However, the opposite is true of fig wasps, where females are the only dispersing sex. Mitochondrial DNA is therefore appropriate for detecting regional genetic differentiation at the scale of thousands of kilometers.

MATERIALS AND METHODS

Taxon Sampling. We sampled *Ceratosolen* pollinators of six *Ficus* species with widespread geographic ranges, four of which span Wallacea (Table 1.1). We also included a pair of sister species, *Ceratosolen pygmaeus* and *Ceratosolen nanus*, whose geographic ranges meet at the Wallace Line, and whose hosts are sister species (Berg and

Corner 2005). This pair enabled comparison of the extent of divergence between sister *Ceratosolen* species occupying non-overlapping regions of Wallacea (Berg and Corner 2005) to divergence within species distributed across Wallacea. Additionally, geographically isolated populations of *Ceratosolen abnormis*, a New Guinea endemic, were sampled in order to calibrate mitochondrial DNA divergence by the timing of known geologic events (see Molecular Dating).

During 1995-2008, ripe figs containing galled flowers were collected from host trees prior to wasp emergence and figs were sealed in containers covered with a fine mesh. As the adult wasps emerged from ripe figs, they were collected and preserved in 70% ethanol. Voucher specimens are deposited at the Bell Museum of Natural History (MIN).

In addition to *Ceratosolen* pollinators of six widespread *Ficus* species (Table 1.2), phylogenetic analyses included cytochrome oxidase I (COI) sequences from 32 *Ceratosolen* species (Table 1.3). The purpose of including all available *Ceratosolen* sequences was to enable tests of monophyly. Such broad sampling is needed to detect cases of host switching in which the pollinators of widespread hosts are not monophyletic. Additionally, outgroup sequences were obtained from GenBank (Table 1.4). For distance, parsimony and Bayesian analyses, the outgroup included two *Kradibia* species, sister group to *Ceratosolen* (Cruaud et al. 2009). In the case of divergence time estimation, the outgroup was expanded to include representative sequences from 14 genera of pollinating fig wasps in order to improve temporal calibration of the phylogeny.

DNA Extraction, PCR, and Sequencing. Sequences of 410-801 bp of cytochrome oxidase I (COI) were obtained directly from individual wasps or from GenBank accessions. DNA was extracted using a Qiagen Dneasy Tissue Kit. We amplified approximately 500 base pairs of mitochondrial COI using primers SW2618 and Pat (Simon et al. 1994; Machado 1998)**.** Amplification was performed on an Eppendorf mastercycler thermocycler with 1 min at 94˚C followed by 36 cycles of 30 s at 94˚C, 1 min at 45˚C, 30 s at 68˚C, followed by a final extension of 5 min at 72˚C. The amplified PCR products were purified using a Qiagen Qiaquick PCR purification kit. Sequencing reactions were performed using a BigDye Terminator v3.1 Cycle Sequencing Ready reaction kit on an Eppendorf mastercycler thermocycler with 1 min at 96˚ C followed by 26 cycles of 10 s at 96˚ C, 5 s at 50˚ C, 4 min at 60˚ C, then analyzed on a ABI Prism 377 DNA Sequencer.

Phylogenetic Analyses. Sequences were edited in Sequencher 4.0 software, aligned by eye and redundant haplotypes were excluded from analysis. Modeltest 3.7 (Posada and Buckley 2004) was used to perform an Aikake Information Criterion test to identify the best-fitting model of evolution. A general time reversible model of evolution with invariant sites and gamma distribution of variable sites (GTR + G + I) was chosen. A neighbor-joining tree was constructed in PAUP 4.0 (Swofford 2001) under GTR + G + I. Uncorrected p-distances were calculated in PAUP 4.0 (Swofford 2001). Although uncorrected p is not the most sophisticated measure genetic distance, it was chosen to facilitate comparison with the DNA barcoding literature (Hebert et al. 2003; Hebert et al. 2004). Under maximum parsimony criterion, a heuristic search was performed with

10,000 sequence addition replicates. Parsimony bootstrap analysis was performed with 1000 replicates with 10 addition sequence replicates per bootstrap replicate. A Bayesian estimate of phylogeny with branch lengths and posterior probabilities was obtained with Mr. Bayes 3.1.2 (Huelsenbeck and Ronquist 2001) by sampling 4000 trees from two simultaneous runs of four chains over $2x10^6$ generations of MCMC analysis and a GTR + G + I model of evolution. The final standard deviation of split frequencies was 0.019, indicating the two runs had converged onto a stationary distribution.

Resulting phylogenies were rooted with *Kradibia gestroi* and *Kradibia tentacularis*, the latter formerly *Liporrhopalum* (Cruaud et al. 2009). For purposes of Bayesian molecular dating (see below), trees were rooted with *Tetrapus,* sister to all other genera of pollinating fig wasps (Cruaud et al. 2009; Lopez-Vaamonde et al. 2009).

Molecular Dating. A molecular clock hypothesis for *Ceratosolen* was rejected on the basis of a chi-square log likelihood ratio test between trees with and without the clock enforced (δ = 619.8 $p<0.001$). We used Bayesian methods and an exponential relaxed molecular clock model of evolution in BEAST (Drummond and Rambaut 2007) to construct an ultrametric tree. Fourteen genera of Agaonidae were included to more accurately date the age of focal *Ceratosolen* species. Monophyly of pollinator clades strongly supported by multiple mitochondrial and nuclear loci were enforced as topological constraints (Cruaud et al. 2009; Lopez-Vaamonde et al. 2009).

The tree was calibrated by assigning strong prior distributions to three nodes within *Ceratosolen* and to one clade of non-pollinating fig wasps. A clade endemic to New Guinea was given normal prior distribution with a mean of 40 my and a standard deviation of 0.5 my, based on the age of the island of New Guinea (Hill and Gleadow 1989; Haig and Medd 1996). The New Guinea endemic clade includes *C. abnormis* and other pollinators of *Ficus* sections *Adenosperma, Dammaropsis* and *Papuasyce* (Weiblen 2000; Berg 2005; Berg and Corner 2005). Under vicariance, the extent of molecular divergence between highland and lowland populations of *C. abnormis* should date from the time when the interior highlands were isolated from the lowlands by the orogenesis of the central New Guinea cordillera 4.7-5.8 mya (Rawlings and Donnellan 2003). Divergence of the highland endemic species, *C. sp* ex *F. microdictya*, from its lowland sister species, *C. armipes*, should also derive from the same geological event. Therefore, these two nodes were given a normal prior distribution with a mean of 4.75 mya and a standard deviation of 0.5 my.

Pegoscapus fossil specimens from Dominican amber are the oldest known specimens of the genus (Lopez-Vaamonde et al. 2009). Dating of Dominican amber has been estimated by different sources to be anywhere between 15 and 45 my old (Lambert et al. 1985; IturraldeVinent and MacPhee 1996). The *Pegoscapus* crown group was therefore given a normal distribution prior with a mean of 30 my and a standard deviation of 5 my. The BEAST analysis was performed over two runs of 10,000,000 generations. The GTR+G+ I parameters from modeltest were used as priors on the model of evolution, the tree prior was assigned a Yule process and 18,000 trees sampled from the two runs were combined to build an ultrametric tree.

RESULTS

One hundred *Ceratosolen* sequences yielded an 801 bp alignment including 89 unique haplotypes and 541 variable sites, 427 of which were parsimony-informative.

Twenty-five sequences were missing ~400 bp from the 5' end of COI but these were included in the analysis based on simulations demonstrating that data sets as small as 200 characters and missing up to 50% of the data performed equally as well as complete data sets (Wiens 2006).

Compared to the 2% divergence threshold for Hymenoptera species recognition in the DNA barcoding literature (Hebert et al. 2003), genetic distances between sister species of *Ceratosolen* were large. For example, named sister species *C. pygmaeus* and *C. nanus* were 11.9-17.8% divergent. Comparable divergence within named taxa provides evidence for the existence of unnamed species, which we provisionally call cryptic species. Large genetic distances (up to 18.8%) within named species were associated with geographic isolation. For example, lowland and highland samples of *C. abnormis* populations were 15.6-17.1% divergent and represent vicariance associated with the uplift of the New Guinea central cordillera. Four species showed strong regional differentiation across Wallacea, suggesting either isolation by distance or vicariance. Malaysian *C. fusciceps* was 7.8-8.5% divergent from Australian samples. *Ceratosolen appendiculatus* was 15.7-18.8% divergent between a New Guinean clade and an Indo-Malayan clade. *Ceratosolen corneri* showed 9.3-11.4% divergence between New Guinea samples and Philippine samples. Three cryptic species within *C. solmsi* showed deep divergence among India, China and Southeast Asia (15.8-17.8%). *Ceratosolen bisulcatus* from New Guinea was 7.7-11.9% divergent from Asian morphotypes and a black Taiwanese morphotype was 7.8-14.4% divergent from the remaining Asian samples.

Pollinators of each focal *Ficus* species were monophyletic across all analyses except *C. bisulcatus* (Figs. 1.2 & 1.3). The relationship among monophyletic *C. corneri* and three *C. bisulcatus* lineages, corresponding to black Australian, black Asian and yellow Asian morphotypes, was unresolved (Figure 1.3). Whereas genetic distance indicated similarity among all *C. bisulcatus* samples, parsimony bootstrapping failed to support monophyly of the species. Bayesian analysis grouped the New Guinean clade *C. bisulcatus* with *C. corneri*, but with low posterior probability (0.78).

Molecular dating identified several independent dispersal events across the Wallace Line in a time interval between 8.4 mya and 18.8 mya (Figure 1.4). The split between Wallacean sister species, *C. nanus* (New Guinea) and *C. pygmaeus* (Philippines) occurred at least 14.9 mya. Cryptic species diverged at a minimum of 8.4 mya in *C. corneri*, 14.1 mya in *C. bisulcatus*, 16.0 mya in *C. fusciceps*, and 18.8 mya in *C. appendiculatus*. The root node of the tree, and origin of agaonid pollinators, was dated to 57.9 mya, within the 51-78 mya interval postulated for the host plants by Ronsted et al. (2005).

DISCUSSION

Although the role of ecological mechanisms driving speciation in fig pollinators has received much attention, relatively few studies have examined species divergence with respect to geography. Our results suggest that allopatric speciation could potentially explain much *Ceratosolen* diversity in Wallacea. Mitochondrial haplotype diversity is consistent with recent studies identifying cryptic species and challenging the old paradigm of 1:1 species-specificity in fig pollination but our findings differ from previous work in suggesting that divergence of geographically isolated populations rather than host switching may provide the explanation.

Allopatric Divergence. Phylogeographic patterns in *Ceratosolen* across Wallacea support the hypothesis of allopatric speciation in several respects. First, individuals sampled from New Guinea populations of *C. abnormis,* isolated by an alpine barrier, were reciprocally monophyletic. Second, there was strong support for the sister relationship of *C. nanus* and *C. pygmaeus* whose ranges are separated by the Wallace Line (Figure 1.2). In addition, three species with distributions straddling the Wallace Line were monophyletic (*C. appendiculatus, C. corneri,* and *C. fusciceps*) and molecular divergence within each of these taxa was geographically structured. In other words, there was support for the reciprocal monophyly of samples taken from either side of the Wallace Line. Would these patterns of reciprocal monophyly hold with more extensive sampling? If not, more recent dispersal events would be suggested. Either way, the most parsimonious explanation of deeply diverged lineages existing on different sides of the Wallace Line is divergence associated with ancient dispersal events from one continental plate to another. Deep divergence was also identified in the monophyletic *C. solmsi* across South Asia, but relationships between Indian, Chinese, and Southeast Asian lineages were unresolved. More extensive sampling could shed light on whether vicariance or isolation by distance can account for this divergence.

Ceratosolen bisculatus was the only taxon in which cytochrome oxidase was equivocal with respect to monophyly. There was strong support for New Guinea and Tawainese endemic clades, as well as a broadly distributed clade ranging from Java to southern Taiwan, but relationships between these three and *C. corneri* were unclear. The discovery of two sympatric species pollinating *F. septica* in southern Taiwan may be an example of sympatric speciation, but is not necessarily inconsistent with allopatric speciation because geographic structure in the broadly distributed clade may be interpreted as a range expansion from Java to the Philippines and Taiwan.

Molecular divergence time estimation provides further insight on *Ceratosolen* phylogeography. Minimum age estimates suggest that widespread, named taxa are of Miocene origin (Figure 1.4) and the geographic localization of mitochondrial DNA haplotypes is evidence that dispersal across major oceanic or alpine barriers at some point accompanied divergence of geographically isolated populations. Vicariance biogeography can explain divergence between highland and lowland *C. abnormis* but it cannot account for divergence within taxa that straddle the Wallace Line because fig pollination arose after the break-up of Pangea (Zerega et al. 2005) and the two plates comprising Wallacea did not reach proximity until the Eocene. *Ceratosolen* lineages spanning the Wallace Line must therefore have achieved their current distribution by dispersal across the Makassar Strait or the Philippine Sea during the past 50 mya (Figure 1.1). Minimum age estimates for these dispersal events (8-18 mya) fall within a period when range expansion might have been facilitated by lower sea levels due to Miocene cooling and the growth of the Antarctic ice cap (Evans et al. 2003; Schulte et al. 2003; Lourie and Vincent 2004; Beck et al. 2006; Braby and Pierce 2007; Jonsson et al. 2008; Muellner et al. 2008). Given the complete dependence of fig pollinators on host trees for survival, it is noteworthy that ancient dispersal in *C. appendiculatus*, *C. corneri*, and *C. fuscipes* appears not to have been associated with the colonization of novel host species during range expansion. On the contrary, host associations appear conserved in the focal *Ceratosolen* species.

Host Conservatism. The classical assumption of extreme host conservatism that underpinned fig wasp taxonomy (Wiebes 1979) has received much recent scrutiny (Machado et al. 2005; Jackson et al. 2008; Su et al. 2008; Renoult et al. 2009) and cases of incongruence between fig and pollinator phylogeny have been attributed to host switching. However, speciation by host switching was not evident from phylogenetic analysis of *Ceratosolen* in Wallacea (Figs. 2-4). Although dispersal across thousands of kilometers, and potentially beyond the range of natal host species, would provide opportune conditions for switching to a novel host (Janzen 1979), *Ceratosolen* host associations appear to have been maintained in each case of ancient dispersal across Wallacea (Figure 1.4). *Ceratosolen bisulcatus* could provide evidence of a host switch, in the event that one of three *C. bisulcatus* clades turned out to be more closely related to *C. corneri*, but the relationship was ambiguous according to cytochrome oxidase sequences alone. Additional gene sampling is needed to better resolve *Ceratosolen* phylogeny and additional taxon sampling can evaluate speciation hypotheses more broadly. At the same time, the extreme mitochondrial DNA divergence in monophyletic *Ceratosolen* pollinators of widespread hosts suggests that at least some examples of allopatric speciation and host conservatism in Wallacea are unlikely to be overturned.

Cryptic species. As in previous palaeotropical studies of more limited geographic scope (Haine et al. 2006; Lin et al. 2008), we identified ancient, divergent lineages pollinating geographically widespread fig species. This suggests the need for a comprehensive re-assessment of *Ceratosolen* species limits, where morphological characters distinguishing divergent lineages may yet be found. In the case of *C. solmsi* and *C. bisulcatus*, named subspecies (Wiebes 1982) might be elevated in rank but new names will also be needed to recognize, for example, divergent lineages of Tawainese *C. bisulcatus* differing in coloration (Lin et al. 2008). For the purpose of present discussion, we refer to genetically divergent lineages as "cryptic species". Mitochondrial DNA divergence among 93% of studied Hymenopteran congeneric species pairs is 8-16% and averaged 11.5 ($\pm$3.8%) (Hebert et al. 2003). However, species delimitation by this criterion alone remains controversial (DeSalle et al. 2005; Meyer and Paulay 2005; Brower 2006)**.** Given the extreme variation among higher taxa in the degree of divergence between sister species, critics of DNA barcoding have advocated the use of divergence estimates from known sister species in the group of interest as thresholds for cryptic species recognition. In the case of *Ceratosolen*, divergence between sister species was similar to hymenopteran data with 7.6-20.4% divergence and an average of 11.3 ($\pm$4%). Comparable divergence among regional populations of six named species provided strong evidence of cryptic species in every case. In the event of future taxonomic revision, named subspecies of *C. bisculatus* and *C. solmsi* could be elevated to species, and regional populations of *C. appendiculatus, C. corneri,* and *C. fusciceps* across Wallacea are likely to yield a number of new species. In the case of the *C. abnormis*, New Guinea highland and lowland populations could also be recognized as different species.

Evolutionary Consequences. Evidence of allopatric divergence in *Ceratosolen* pollinators of geographically widespread fig species has implications for fig wasp dispersal and the breeding structure of host populations. Population genetic studies of neotropical strangler figs inferred long-distance pollen movement and fig populations covering more than 600 km^2 (Nason et al. 1996, 1998). Fig wasp trapping studies in Asia (Compton et al. 2000; Harrison and Rasplus 2006) detected pronounced differences in dispersal ability of pollinators according to the breeding system and population density of the host. Dioecious figs typically have much higher population densities and flower more often than monoecious figs (Harrison and Shanahan 2005). Consistent with evidence from the neotropics, pollinators of monoecious strangler figs were trapped at great distances from the nearest trees whereas dioecious fig pollinators, including *Ceratosolen,* were very rarely encountered above the forest canopy. Given that fig pollinators are attracted to the nearest available tree, local populations of dioecious figs could effectively limit *Ceratosolen* dispersal distances (Harrison and Rasplus 2006). Geographically localized mitochondrial DNA haplotypes, such as the distinct haplotypes of *C. nanus* in mainland New Guinea and nearby New Britain separated by less than 500 km, or highland and lowland *C. abnormis* separated by less than 200 km, suggest that dispersal distances in *Ceratosolen* are more restricted than in pollinators of monoecious figs (Nason et al. 1996, 1998). Consequently, gene flow among dioecious fig populations may also be limited.

Studies of geographically widespread tropical tree species have identified population genetic structure consistent with isolation by distance (Dick and Heuertz 2008). If pollinators of widespread dioecious fig species have undergone allopatric speciation, then the same mechanism of geographic isolation could result in host speciation. Evaluation of the cryptic species hypothesis is more challenging for figs than for their pollinators given the limited sequence divergence observed among closely related fig species, even for rapidly evolving gene regions (Ronsted et al. 2007; Silvieus 2007; Ronsted et al. 2008b). On the other hand, geographic variation in fig morphology is well-known and subspecies are recognized in the taxonomic literature. *Ficus botryocarpa*, for example, is divided into ssp. *botryocarpa* in the Philippines and ssp. *subalbidoramea* in New Guinea (Berg and Corner 2005) that happen to coincide with the divergent lineages of *C. corneri*. *Ficus hispida* and *F. racemosa* include many regional synonyms with no currently recognized subspecies but each are considered “highly variable” (Berg and Corner 2005). *Ficus septica* varieties include a widespread v. *septica* and a Philippine endemic *v. salicifolia* (Corner 1965). Might sympatric *C. bisulcatus* in Taiwan have resulted from local pollinator specialization on *v. salicifolia* in the Philippines followed by subsequent dispersal and colonization of v. *septica* in southern Taiwan? Hyper-variable plant molecular markers such as microsatellites are needed to examine the extent of co-variation in fig and pollinator population genetic structure.

CONCLUSIONS

Simple models of allopatric speciation deserve consideration alongside scenarios of ecological specialization in accounting for the diversity of tropical phytophagous insects as revealed by 'DNA barcodes'. Examination of *Ceratosolen* pollinators associated with six widespread *Ficus* species suggests a common pattern of genetic structure corresponding to geographic distance and consistent with allopatric divergence. Pollinator associations appear to be conserved across large host species ranges. Deep divergence within named pollinator species suggest that deviations from 1:1 species-specificity may not necessarily arise from host switching. Although this conclusion differs from that reached for neotropical pollinators of monoecious *Ficus* species (Molbo et al. 2003; Machado et al. 2005; Su et al. 2008), the divergence of *Ceratosolen* mitochondrial DNA haplotypes appears so ancient that our findings are unlikely to be overturned by nuclear DNA sequences, given their longer coalescent times. Comparative studies would be helpful in determining the mechanisms underlying these differences, which might be due to variation among regional host plant lineages in breeding systems and population density.

TABLE 1.1. Focal *Ceratosolen* species and localities sampled. Numbers in parentheses indicate number of samples from each locality.

Pollinator	Host	Host section	Samples	Haplotypes	Localities
C. corneri Wiebes	*F. botryocarpa* Miq.	*Sycocarpus*	10	9	New Guinea (8), New Britain (1), Philippines (1)
C. bisulcatus Mayr	*F. septica* Burm.f.	*Sycocarpus*	14	14	New Guinea (5), Indonesia (2), Taiwan (6), Philippines (1)
C. solmsi Mayr	*F. hispida* Blanco	*Sycocarpus*	5	4	Cambodia (1), Malaysia (1), India (2), China (1)
C. appendiculatus Mayr	*F. variegata* Blume	*Sycomorus*	10	5	New Guinea (6), Singapore (2), Indonesia (1), Australia (1)
C. fusciceps Mayr	*F. racemosa* L.	*Sycomorus*	4	3	Malaysia (1), Australia (3)
C. abnormis Wiebes	*F. dammaropsis* Diels	*Adenosperma*	12	10	New Guinea lowland (9), New Guinea highland (3)
C. nanus Wiebes	*F. pungens* Reinw. ex Blume	*Bosscheria*	11	11	New Guinea (11)
C. pygmaeus Grandi	*F. minnahassae* (Teijsm. & de Vriese) Miq.	*Bosscheria*	1	1	Philippines (1)

TABLE 1.2. GenBank accession numbers and locality information for focal *Ceratosolen* species. Where collection numbers were unavailable, the source publication is listed in parentheses.

Haplotype	Pollinator species	Locality	Collection no. or citation	GenBank accession
BOT1	*C. corneri*	New Guinea, Ohu	B135	[AF200386]
BOT2	*C. corneri*	New Guinea, Ohu	B150.5	[GU434044]
BOT3	*C. corneri*	New Guinea, Ohu	B135.5	[GU434045]
BOT4	*C. corneri*	New Guinea, Ohu	B47	[GU434046]
BOT5	*C. corneri*	New Guinea, Baitabag	G065	[GU434047]
BOT6	*C. corneri*	New Guinea, Ohu	B240.1A	[GU434048]
BOT7	*C. corneri*	New Guinea, Ohu	B239.2A	[GU434049]
BOT8	*C. corneri*	E. New Britain, Mt. Kavangi	GW428	[GU434050]
BOT9	*C. corneri*	Philippines, Luzon	GW2116.2	[GU434051]
DAM1	*C. abnormis*	New Guinea lowlands, Ohu	B110	[GU434084]
DAM2	*C. abnormis*	New Guinea lowlands, Ohu	B169.4	[GU434085]
DAM3	*C. abnormis*	New Guinea lowlands, Ohu	B186.3	[GU434086]
DAM4	*C. abnormis*	New Guinea lowlands, Ohu	B186.4	[GU434087]
DAM5	*C. abnormis*	New Guinea lowlands, Ohu	B62	[GU434088]
DAM6	*C. abnormis*	New Guinea lowlands, Ohu	B52	[GU434089]
DAM7	*C. abnormis*	New Guinea lowlands, Baitabag	G054	[GU434090]
DAM8	*C. abnormis*	New Guinea lowlands, Baitabag	G107	[GU434091]
DAM9	*C. abnormis*	New Guinea lowlands, Baitabag	G082	[GU434092]
DAM10	*C. abnormis*	New Guinea highlands, Mu	GW2142-1A	[GU434083]
HIS1	*C. solmsi solmsi*	Cambodia, Angkor Wat	GW2720	[GU434064]
HIS2	*C. solmsi marchali*	India, Mumbai	GW2783-1A	[GU434065]
HIS3	*C. solmsi marchali*	China, Xishuangbanna Garden	(Jiang et al. 2006)	[AY842421]
HIS4	*C. solmsi solmsi*	Malaysia	(West et al. 2001)	[AF302054]

TABLE 1.2 CONTINUED

Haplotype	Pollinator species	Locality	Collection no. or citation	GenBank accession
MIN1	*C. pygmaeus*	Philippines, Luzon	GW2104	[GU434076]
PUN1	*C. nanus*	New Guinea, Baitabag	G077	[AF200382]
PUN2	*C. nanus*	New Guinea, Ohu	B175.5	[GU434066]
PUN3	*C. nanus*	New Guinea, Baitabag	G094	[GU434067]
PUN4	*C. nanus*	New Guinea, Niksek	GW1119	[GU434068]
PUN5	*C. nanus*	New Guinea, Ohu	B62	[GU434069]
PUN6	*C. nanus*	New Guinea, Baitabag	G120.0	[GU434070]
PUN7	*C. nanus*	New Guinea, Baitabag	GW1746.2	[GU434071]
PUN8	*C. nanus*	New Guinea, Ohu	B190.2	[GU434072]
PUN9	*C. nanus*	New Guinea, Baitabag	GW2055.1	[GU434073]
PUN10	*C. nanus*	New Guinea, Ohu	B232.1A	[GU434074]
PUN11	*C. nanus*	E. New Britain, Malpas	GW467	[GU434075]
RAC1	*C. fusciceps*	Australia, Darwin	GW1075	[GU434081]
RAC2	*C. fusciceps*	Malaysia, Penang	GW2713	[GU434082]
RAC3	*C. fusciceps*	Australia, Atherton Tablelands	GW2724B	[AF200379]
SEP1	*C. bisulcatus bisulcatus*	New Guinea, Ohu	B170	[GU434052]
SEP2	*C. bisulcatus bisulcatus*	New Guinea, Niksek	GW1122	[GU434053]
SEP3	*C. bisulcatus bisulcatus*	New Guinea, Baitabag	GW2024.1	[GU434054]
SEP4	*C. bisulcatus bisulcatus*	New Guinea, Ohu	B214.1A	[GU434055]
SEP5	*C. bisulcatus bisulcatus*	New Guinea, Ohu	B214.3A	[GU434056]
SEP6	*C. bisulactus jucundus*	Indonesia, Sebesi	FS17-2A	[GU434057]
SEP7	*C. bisulactus jucundus*	Indonesia, Sebesi	FS17-2B	[GU434058]
SEP8	*C. bisulactus jucundus*	S. Taiwan	FS336-2	[GU434062]
SEP9	*C. bisulactus jucundus*	S. Taiwan, Lanyu Island	FS584-3	[GU434063]
SEP10	*C. bisulactus jucundus*	Taiwan	(Lin et al. 2008)	[EF440181]

TABLE 1.2 CONTINUED

Haplotype	Pollinator species	Locality	Collection no. or citation	GenBank accession
SEP11	*C. bisulactus jucundus*	Philippines	(Machado et al. 2001)	[AY014986]
SEP12	*C. bisulactus jucundus*	S. Taiwan	FS62-8	[GU434059]
SEP13	*C. bisulactus jucundus*	C. Taiwan	FS46-11	[GU434060]
SEP14	*C. bisulactus jucundus*	N. Taiwan	FS11-11	[GU434061]
VAR1	*C. appendiculatus*	New Guinea, Ohu	B198.3	[AF200374]
VAR2	*C. appendiculatus*	Australia, Cape Tribulation	GW2746	[GU434077]
VAR3	*C. appendiculatus*	Borneo, Kalimantan Barat	GW892	[GU434078]
VAR4	*C. appendiculatus*	Singapore, Botanical Garden	GW1888.2	[GU434079]
VAR5	*C. appendiculatus*	Singapore, Botanical Garden	GW1081	[GU434080]

TABLE 1.3. GenBank accession numbers and locality information for non-focal *Ceratosolen* species. Where collection numbers were unavailable, the source publication is listed in parentheses.

Pollinator species	Locality	Collection number	GenBank accession
C. adenospermae	New Guinea, Ohu	B316.4A	[DQ679075.1]
C. arabicus		(Machado et al. 2001)	[AY014988.1]
C. armipes	New Guinea, Salemben	GW622	[AF200391]
C. capensis		(Machado et al. 2001)	[AY014994.1]
C. constrictus		(West et al. 2001)	[AF302055]
C. dentifer	New Guinea, Ohu	B149.5	[DQ679123]
C. emarginatus		(Jiang et al. 2006)	[AY842419]
C. galili		(West et al. 2001)	[AF302056]
C. grandii	New Guinea, Ohu	B308.3A	[DQ679170]
C. gravelyi		(Jiang et al. 2006)	[AY842420]
C. hooglandi	New Guinea, Ohu	B55.1	[DQ679089]
C. medlarianus	New Guinea, Ohu	B305.4A	[DQ679133]
C. nexilis	New Guinea, Ohu	B181.5	[DQ679138]
C. notus	New Guinea, Ohu	B8.1	[DQ679108]
C. pilipes		(Machado et al. 2001)	[AY014984]
C. solitarius	New Guinea, Ohu	B279.9A	[DQ679088.1]
C. sp. ex *F. adelpha*	New Guinea, Ohu	B78.1	[DQ679076]
C. sp. ex *F. arbuscula*	New Guinea, Crater Mt.	JE1.1	[GU434093]
C. sp. ex *F. aurantiacafolia*	New Guinea, Baitabag	GW122.03	[GU434098]
C. sp. ex *F. microdictya*	New Guinea, Kaironk	GW954.3	[GU434099]
C. sp. ex *F. morobensis*	New Guinea, Ohu	B163.3	[DQ679135]

TABLE 1.3 CONTINUED

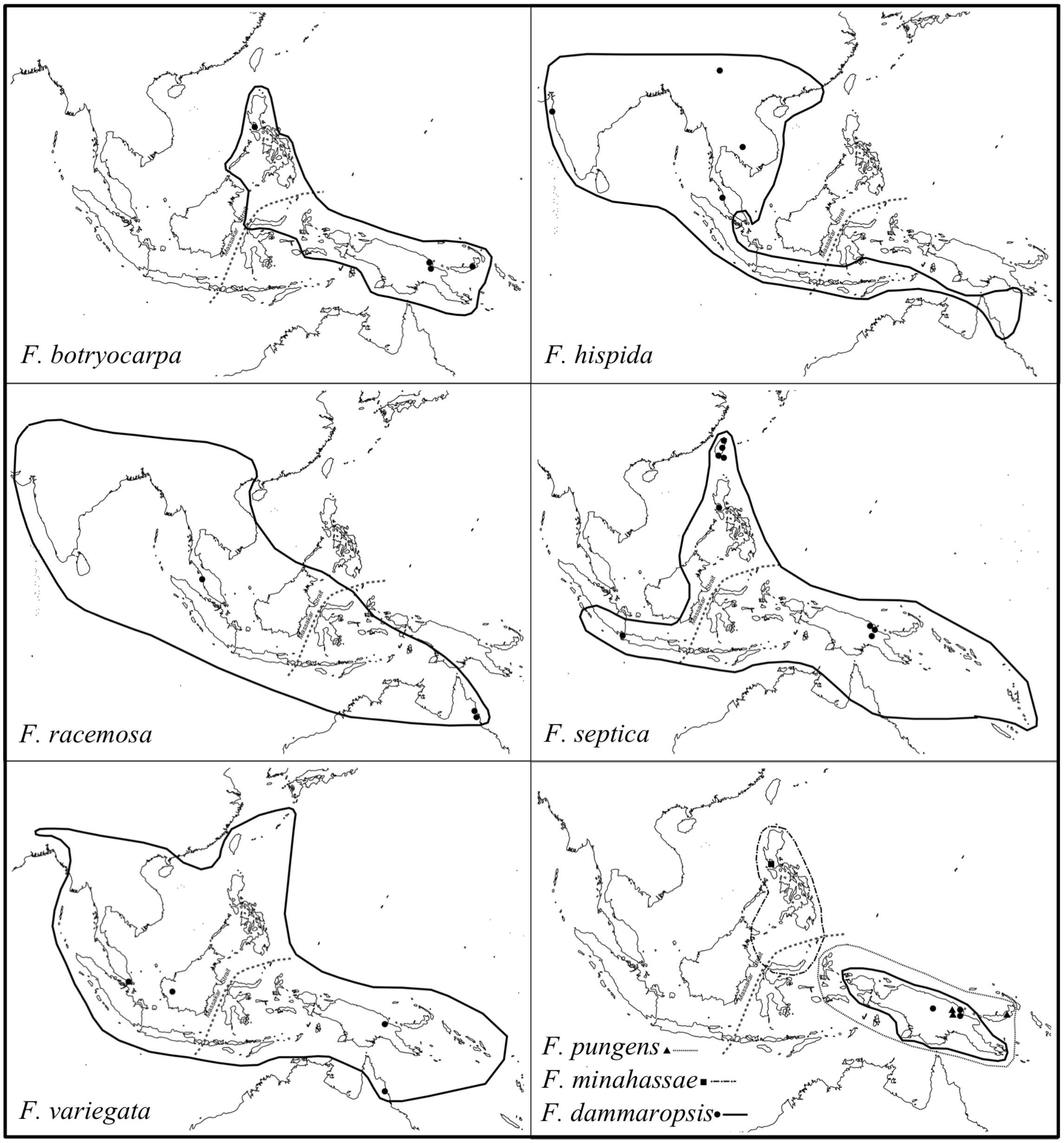
Pollinator species	Locality	Collection number	GenBank accession
C. sp. ex *F. ochrochlora*	New Guinea, Crater Mt.	GW735	[GU434095]
C. sp. ex *F. pachyrrhachis*	New Guinea, Ohu	B318.1A	[DQ679150]
C. sp. ex *F. rubrijuvenis*	New Guinea, Ohu	B81.1A	[GU434094]
C. sp. ex *F. satterthwaitei*	Philippines, Luzon	GW2102.1A	[GU434096]
C. sp. ex *F. saurauioides*	New Guinea, Baitabag	GW2006B.1	[GU434097]
C. sp. ex *F. subcuneata*	New Guinea, Ohu	GW1687.A	[DQ679176]
C. vechti	Malaysia, Endau-Rompim	GW1086	[AF200389]
C. vestustus		(Machado et al. 2001)	[AY014985]
C. wui		(Lin et al. 2008)	[EF440119]

TABLE 1.4. GenBank accession numbers for outgroup sequences

Pollinator species	GenBank accession
Alfonsiella longiscapa	[AY642454.1]
Courtella armata	[AY014978]
Dolichoris boschmai	[AY642459]
Elisabethiella baijnathi	[AY014975]
Eupristina verticillata	[AF302053]
Kradibia gestroi	[AY014983]
Kradibia_tentacularis	[AY014993]
Nigeriella excavata	[AJ971655]
Pegoscapus gemellus	[AY148134.1]
Platyscapa soraria	[AY014982.1]
Pleistodontes froggatti	[AY014980]
Tetrapus sp.	[AY148155.1]
Valisia intermedia	[AY642456.1]
Watersoniella sp.	[AY642462]
Wiebesia pumilae	[AY014995]

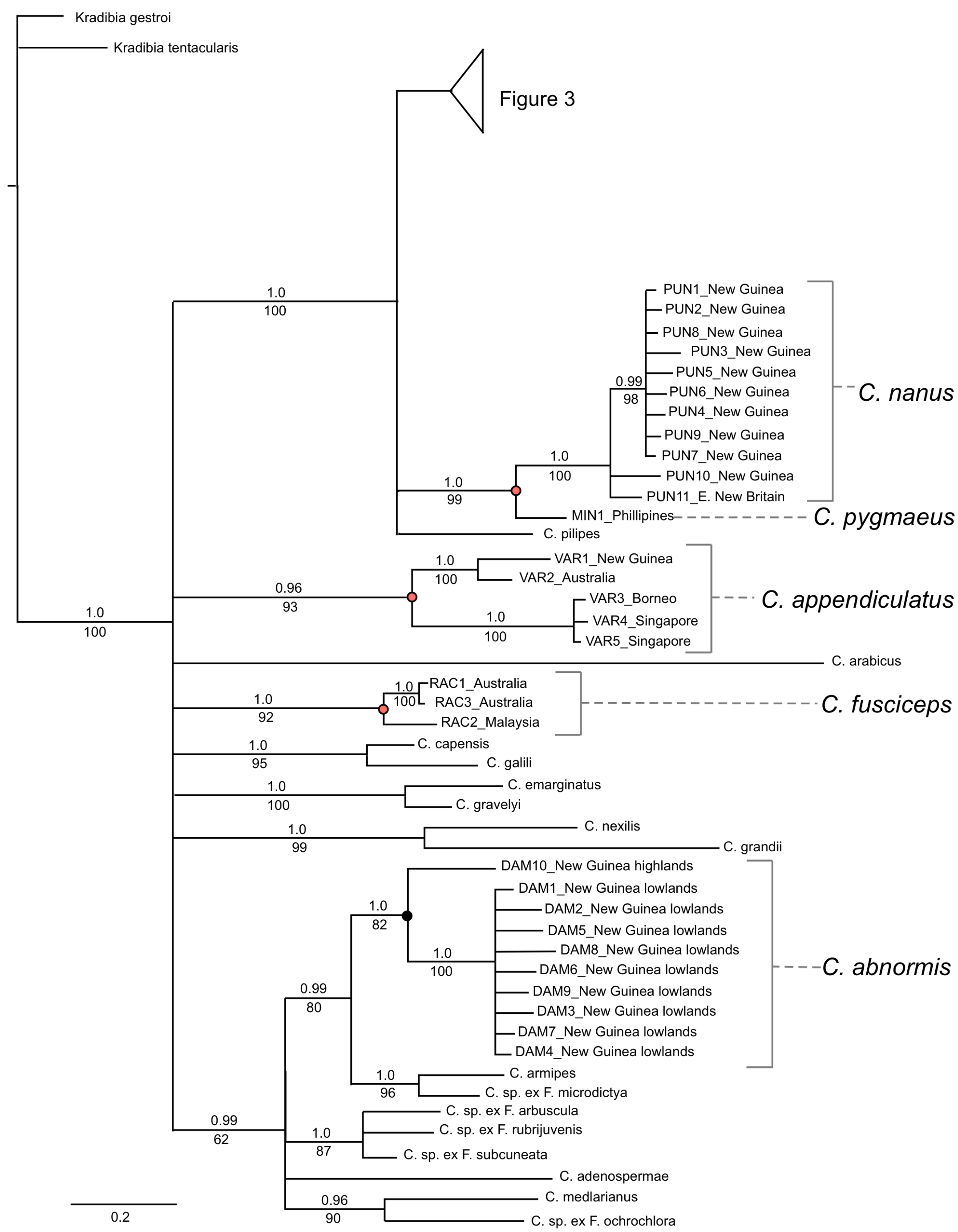
FIGURE 1.1. Maps of Wallacea illustrating geographic distributions of focal *Ficus* species and sampling localities. The Wallace Line (dashed) is a major biogeographical transition zone that marks the contact of the Sunda shelf (Borneo, Java, and mainland Asia) to the Sahul shelf including New Guinea and Australia. Geographic ranges (solid lines) and sampling localities (circles) are shown for five widespread taxa (*F. botryocarpa*, *F. hispida*, *F. septica*, *F. racemosa*, and *F. variegata*). The map on the lower right map illustrates distributions of three locally endemic taxa sampled for comparison with widespread species: *F. dammaropsis* (solid line & circles), *F. minahassae* (dashed line & square), and *F. pungens* (dotted line & triangles).

FIGURE 1.1.


F. botryocarpa
F. hispida
F. racemosa
F. septica
F. variegata
F. pungens
F. minahassae
F. dammaropsis

FIGURES 1.2 & 1.3. *Ceratosolen* mitochondrial DNA phylogeny according to Bayesian analysis. Taxon labels for six widespread species include the first three letters of the *Ficus* host, followed by haplotype number and the locality. Bayesian posterior probabilities are listed above the branches with parsimony bootstrap values beneath. Poorly supported nodes (<0.95 posterior probability) are not shown. Branch lengths are proportional to genetic distance. Shaded circles mark dispersal events associated with the Wallace Line. Solid circles mark notable divergence not associated with the Wallace Line.

FIGURE 1.2.



Kradibia gestroi
Kradibia tentacularis
Figure 3
PUN1_New Guinea
PUN2_New Guinea
PUN8_New Guinea
PUN3_New Guinea
PUN5_New Guinea
PUN6_New Guinea
PUN4_New Guinea
PUN9_New Guinea
PUN7_New Guinea
PUN10_New Guinea
PUN11_E. New Britain
C. nanus
MIN1_Phillipines
C. pygmaeus
C. pilipes
VAR1_New Guinea
VAR2_Australia
VAR3_Borneo
VAR4_Singapore
VAR5_Singapore
C. appendiculatus
C. arabicus
RAC1_Australia
RAC3_Australia
RAC2_Malaysia
C. fusciceps
C. capensis
C. galili
C. emarginatus
C. gravelyi
C. nexilis
C. grandii
DAM10_New Guinea highlands
DAM1_New Guinea lowlands
DAM2_New Guinea lowlands
DAM5_New Guinea lowlands
DAM8_New Guinea lowlands
DAM6_New Guinea lowlands
DAM9_New Guinea lowlands
DAM3_New Guinea lowlands
DAM7_New Guinea lowlands
DAM4_New Guinea lowlands
C. abnormis
C. armipes
C. sp. ex F. microdictya
C. sp. ex F. arbuscula
C. sp. ex F. rubrijuvenis
C. sp. ex F. subcuneata
C. adenospermae
C. medlarianus
C. sp. ex F. ochrochlora
0.2

FIGURE 1.3.

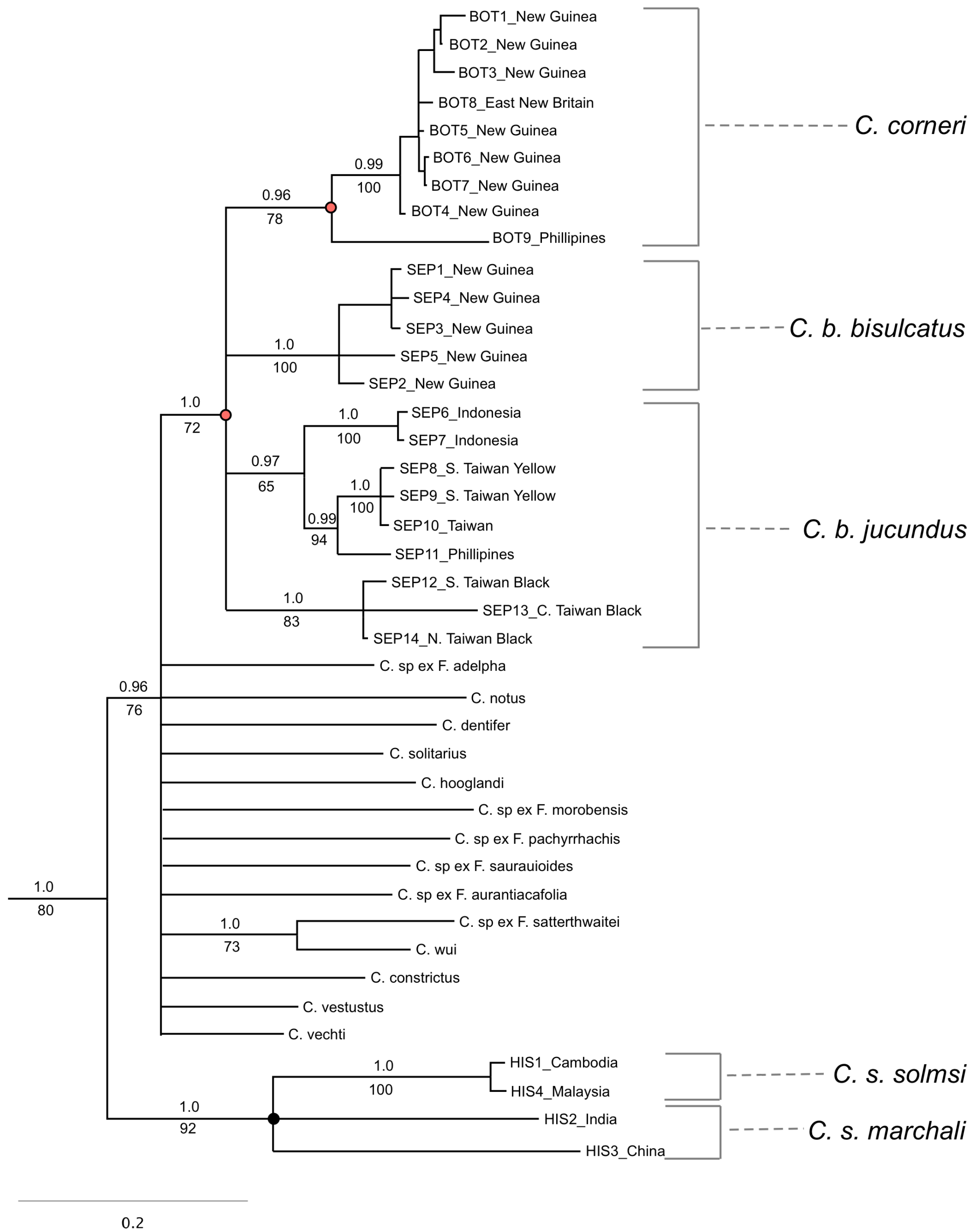
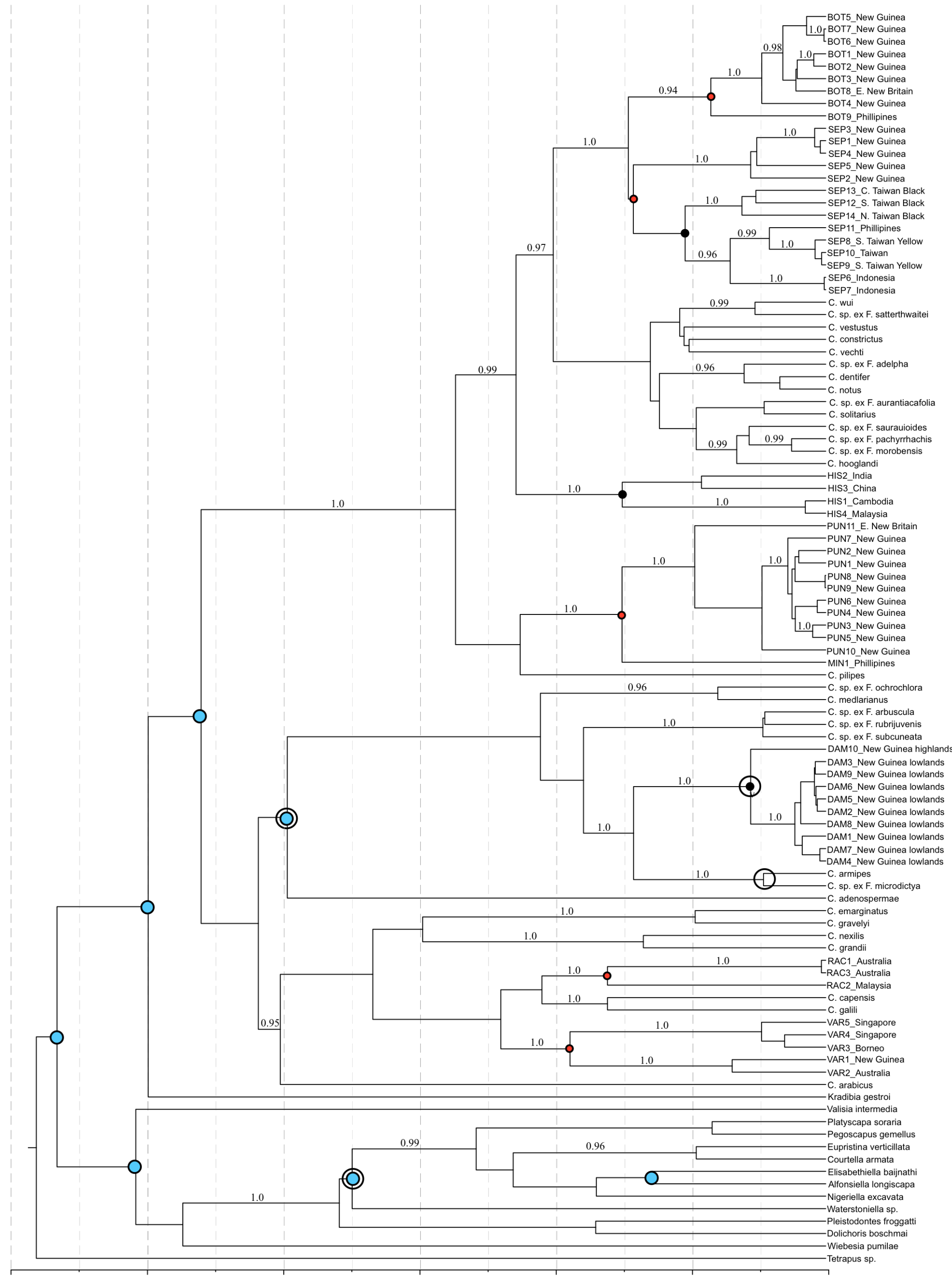

BOT1_New Guinea
BOT2_New Guinea
BOT3_New Guinea
BOT8_East New Britain
BOT5_New Guinea
BOT6_New Guinea
BOT7_New Guinea
BOT4_New Guinea
BOT9_Phillipines
C. corneri
0.99
100
0.96
78
SEP1_New Guinea
SEP4_New Guinea
SEP3_New Guinea
SEP5_New Guinea
SEP2_New Guinea
1.0
100
C. b. bisulcatus
1.0
72
SEP6_Indonesia
SEP7_Indonesia
1.0
100
0.97
65
SEP8_S. Taiwan Yellow
SEP9_S. Taiwan Yellow
SEP10_Taiwan
1.0
100
0.99
94
SEP11_Phillipines
C. b. jucundus
SEP12_S. Taiwan Black
SEP13_C. Taiwan Black
SEP14_N. Taiwan Black
1.0
83
C. sp ex F. adelpha
0.96
76
C. notus
C. dentifer
C. solitarius
C. hooglandi
C. sp ex F. morobensis
C. sp ex F. pachyrrhachis
C. sp ex F. saurauioides
1.0
80
C. sp ex F. aurantiacafolia
C. sp ex F. satterthwaitei
1.0
73
C. wui
C. constrictus
C. vestustus
C. vechti
HIS1_Cambodia
HIS4_Malaysia
1.0
100
C. s. solmsi
1.0
92
HIS2_India
HIS3_China
C. s. marchali
0.2

FIGURE 1.4. Chronogram for *Ceratosolen* according to Bayesian divergence time estimation. The horizontal axis corresponds to million years before present. Small, shaded circles mark dispersal events associated with the Wallace Line. Small, solid circles mark deep divergence within named species. Large, shaded circles mark nodes that were constrained as monophyletic in the Bayesian analysis. Large, open circles mark nodes calibrated with fossils and geographic events as explained in the methods.

FIGURE 1.4.



BOT5_New Guinea
BOT7_New Guinea
BOT6_New Guinea
BOT1_New Guinea
BOT2_New Guinea
BOT3_New Guinea
BOT8_E. New Britain
BOT4_New Guinea
BOT9_Phillipines
SEP3_New Guinea
SEP1_New Guinea
SEP4_New Guinea
SEP5_New Guinea
SEP2_New Guinea
SEP13_C. Taiwan Black
SEP12_S. Taiwan Black
SEP14_N. Taiwan Black
SEP11_Phillipines
SEP8_S. Taiwan Yellow
SEP10_Taiwan
SEP9_S. Taiwan Yellow
SEP6_Indonesia
SEP7_Indonesia
C. wui
C. sp. ex F. satterthwaitei
C. vestustus
C. constrictus
C. vechti
C. sp. ex F. adelpha
C. dentifer
C. notus
C. sp. ex F. aurantiacafolia
C. solitarius
C. sp. ex F. saurauioides
C. sp. ex F. pachyrrhachis
C. sp. ex F. morobensis
C. hooglandi
HIS2_India
HIS3_China
HIS1_Cambodia
HIS4_Malaysia
PUN11_E. New Britain
PUN7_New Guinea
PUN2_New Guinea
PUN1_New Guinea
PUN8_New Guinea
PUN9_New Guinea
PUN6_New Guinea
PUN4_New Guinea
PUN3_New Guinea
PUN5_New Guinea
PUN10_New Guinea
MIN1_Phillipines
C. pilipes
C. sp. ex F. ochrochlora
C. medlarianus
C. sp. ex F. arbuscula
C. sp. ex F. rubrijuvenis
C. sp. ex F. subcuneata
DAM10_New Guinea highlands
DAM3_New Guinea lowlands
DAM9_New Guinea lowlands
DAM6_New Guinea lowlands
DAM5_New Guinea lowlands
DAM2_New Guinea lowlands
DAM8_New Guinea lowlands
DAM1_New Guinea lowlands
DAM7_New Guinea lowlands
DAM4_New Guinea lowlands
C. armipes
C. sp. ex F. microdictya
C. adenospermae
C. emarginatus
C. gravelyi
C. nexilis
C. grandii
RAC1_Australia
RAC3_Australia
RAC2_Malaysia
C. capensis
C. galili
VAR5_Singapore
VAR4_Singapore
VAR3_Borneo
VAR1_New Guinea
VAR2_Australia
C. arabicus
Kradibia gestroi
Valisia intermedia
Platyscapa soraria
Pegoscapus gemellus
Eupristina verticillata
Courtella armata
Elisabethiella baijnathi
Alfonsiella longiscapa
Nigeriella excavata
Waterstoniella sp.
Pleistodontes froggatti
Dolichoris boschmai
Wiebesia pumilae
Tetrapus sp.
60.0
50.0
40.0
30.0
20.0
10.0
0.0

CHAPTER 2

POLLINATOR SHARING IN DIOECIOUS FIGS

(*FICUS*, MORACEAE)

Pollinators visit flowers in order to obtain pollen, nectar, oils, brood sites, or perceived mating opportunities. The potential for gene flow among sympatric plant species and the maintenance of species integrity depends on the degree to which pollinators consistently visit flowers of the same species vs. different species (Waser and Ollerton 2006). Pollinator specificity can act as a pre-zygotic mechanism of reproductive isolation among plant species (Grant 1949, 1994) and is a product of behavioral, morphological and chemical interactions. Such pollinator-mediated reproductive isolation has been observed in diverse angiosperm lineages with varying degrees of sympatry and of gene flow in hybrid zones. Classic examples include *Aquilegia* (Grant 1952; Fulton and Hodges 1999), *Mimulus* (Schemske and Bradshaw 1999; Ramsey et al. 2003), *Ipomopsis* (Campbell et al. 2003; Campbell 2004), *Penstemon* (Kimball 2008) and *Asclepias* (Kephart and Theiss 2004). Pollinator specificity is thought to be strongest in obligate pollinator-nursery mutualisms (Ollerton 2006) in which pollination services are exchanged for brood sites. The best studied among these mutualisms are those between yucca plants-yucca moths (Pellmyr 2003), fig plants-fig wasps (Janzen 1979) and *Glochidion* trees-*Epicephala* moths (Kato et al. 2003). Studies have shown plant volatile cues to play a significant role in pollinator-mediated plant reproductive isolation (Grison-Pige et al. 2002b; Okamoto et al. 2007). The morphological match between host and pollinator are also known to contribute to reproductive isolation (van Noort and Compton 1996; Godsoe et al. 2008; Smith et al. 2009).

Fig plants (Moraceae: *Ficus*) and their wasp pollinators (Hymenoptera: Agaonidae) represent an extreme along the continuum of plant-pollinator specialization continuum where specificity has chemical and morphological components. The life cycle of the agaonid wasp begins and ends within an enclosed inflorescence, or syconium, of its host *Ficus* plant (Weiblen 2002). After maturation and mating within the fig, a female wasp emerges, carrying pollen, and searches for another fig in which to lay her eggs. Volatile chemical signals attract her to a receptive fig of her host (Song et al. 2001; Grison-Pige et al. 2002a; Chen and Song 2008; Proffit et al. 2008). She gains access to her new host fig through a tight, bract-covered opening called the ostiole. Specialized appendages on her head help her to pry her way through the bracts. As she oviposits into the styles of the tiny flowers within the fig, she deposits pollen that she has carried from her birth fig onto the stigmas. Some flowers will produce seed and others will house the developing wasp larvae. In monoecious figs, both flower types are found within the same fig. In dioecious figs, seed producing and wasp/pollen producing figs are located on separate plants. Pollinator visitation of a fig depends on both a foundress wasp locating and recognizing her specific host via chemical cues (Bronstein 1987; van Noort et al. 1989) as well as morphological match between wasp and fig (van Noort and Compton 1996; Weiblen 2004), allowing access to flowers within the enclosed inflorescence. Successful pollination additionally depends on a number of other factors, including wasp behavior and pollen compatibility. The purpose of this study was to examine the effectiveness of pre-zygotic isolating mechanisms associated with pollinator visitation. We examined pollinator specificity by identifying wasp species that successfully located, accepted, and gained access to the syconia of sympatric and dioecious fig species. A previous study examining monoecious fig pollinator visitation found that a single wasp species comprised 99% of pollinators attracted to *F. pertusa*, and only this species gained access to *F. pertusa* syconia (Bronstein 1987).

However, the last decade of study on the fig-fig wasp pollination mutualism and the proliferation of molecular data has changed our view of fig-fig wasp specificity from a highly species specific 1:1 association to a more complex model that includes two or more pollinator species associated with particular fig species (Molbo et al. 2003; Haine et al. 2006; Marussich and Machado 2007; Jackson et al. 2008; Peng et al. 2008; Su et al. 2008; Zhang et al. 2008; Compton et al. 2009) and pollinator sharing among multiple fig species (Molbo et al. 2003; Machado et al. 2005; Marussich and Machado 2007; Su et al. 2008). Molecular evidence of hybridization among fig species has also been documented (Parrish et al. 2003; Machado et al. 2005; Renoult et al. 2009). However, the ubiquity of such events in the fig-fig wasp system is unclear. Pollinator sharing has implications for plant species integrity, the delimitation of *Ficus* species, and the importance of host switching in shaping the evolutionary history of the mutualism. As floral constancy of pollinators in other systems are often context-dependent and variable among populations (Moeller 2005; Aldridge and Campbell 2007; Hersch and Roy 2007; Aldridge and Campbell 2009), it is important to ask whether the patterns seen in these studies are universal across the fig-fig wasp mutualism. Does the frequency of pollinator sharing differ across fig lineages, associated wasp lineages, geographically, or among plant breeding systems?

Although pollinator sharing and host switching have been invoked to explain molecular and phylogenetic patterns among figs and their pollinating wasps (Herre et al. 2008; Renoult et al. 2009), few studies have reported direct evidence of a single species of wasp associated with more than one species of fig. A recent review (Herre et al. 2008) reported pollinator sharing in monoecious neotropical *Ficus* subgenera *Urostigma*

(Molbo et al. 2003; Machado et al. 2005; Marussich and Machado 2007; Su et al. 2008) and *Pharmacosycea* (Su et al. 2008). The studies of *Urostigma* found shared pollinators in 10 out of 25 *Ficus* species using molecular methods of wasp identification. In contrast, (Weiblen et al. 2001) reared only unique pollinators from each of 14 dioecious, paleotropical *Ficus* species examined. This study involved morphological identification of fig wasps reared from 8-19 crops per fig species. Could the different findings of these studies be explained by morphological versus molecular species concepts? Or are they a result of biological differences between the two study systems?

This study examined pollinator sharing in dioecious, Paleotropical *Ficus* through molecular and morphological identification of agaonid wasps capable of accessing flowers within syconia. We focused on identifying the pollinators visiting dioecious fig species in New Guinea, a center of *Ficus* diversity where up to 70 species of figs are known to co-occur in lowland rainforests.

MATERIALS AND METHODS

Taxonomy and host associations. We chose to examine agaonid wasp species associated with sympatric, locally abundant and closely related *Ficus* species because these provide conditions most likely to facilitate pollinator sharing. We included five species from dioecious *Ficus* subgenus Sycomorus, section *Sycocarpus* (*Ficus hispidioides* S.Moore*, Ficus congesta* Roxb.*, Ficus morobensis* C.C.Berg*, Ficus pachyrrhachis* K.Schum. & Lauterb.*,* and two morphotypes of *Ficus bernaysii* King*,*) that met these conditions. The two morphotypes of *F. bernaysii* were first recognized by local landowners and are distinguished by the color, length and density of epidermal hairs on petioles and young shoots. Since there is not yet taxonomic recognition or information on ecological or genetic divergence between these entities, we refer to them as morphotypes “A” and “B”. We also included *Ficus copiosa* Steud. and *Ficus wassa* Roxb. (Weiblen 2000)*,* sister species from dioecious *Ficus* subgenus Ficus, section *Sycidium*.

Sycomorus figs make up a clade of predominantly dioecious plants estimated to have diversified approximately 45 million years ago (Ronsted et al. 2005). The focal species in section *Sycocarpus* are a monophyletic, but highly unresolved subset (due to lack of sequence diversity) of this clade estimated to have originated at least 15 mya (Silvieus et al. 2008). Members of *Sycocarpus* are morphologically similar, but differentiated mainly by syconium, leaf and epidermal hair characteristics (Berg and Corner 2005). *Sycocarpus* species are pollinated by *Ceratosolen* wasps, the largest genus of Australasian agaonids (Wiebes 1994). The *Ceratosolen* pollinators of *Sycocarpus* are monophyletic and appear to have codiversified with their hosts (Lopez-Vaamonde et al. 2009). The known pollinators of section *Sycidium* are *Kradibia,* a close relative of the *Ceratosolen* (Lopez-Vaamonde et al. 2009). Among 234 herbarium specimens of *F. copiosa* and *F. wassa* collected by the authors, two were identified as hybrids, one from New Guinea and the other from New Britain (GD Weiblen unpubl. data). Germination of seed from the New Guinea individual supports this identification as the backcross displays a wide range of morphotypes spanning the continuum of variation between *F. copiosa* and *F. wassa* (AM Moe unpubl. data).

Foundress collection. Foundresses were collected from a four km^2 area of lowland rainforest surrounding Ohu village, in Madang Province, Papua New Guinea (Lat. 5˚ 13’

38” S Long. 145˚ 40’ 44”E). Up to 25 receptive figs were removed from each of 10 trees of each focal species from section *Sycocarpus* and section *Sycidium* and from a single *F. copiosa x F. wassa* individual. Figs were immediately opened and live foundresses were placed in airtight collection tubes containing silica gel and a cotton plug. Dried foundresses were stored at -80°C. A leaf voucher was taken for each tree from which foundress wasps were collected. In total, 372 *Ceratosolen* pollinators of *Ficus* section *Sycocarpus* and 210 *Kradibia* pollinators of *Ficus* section *Sycidium* were identified using DNA barcodes. Before DNA extraction, the head of each wasp was removed and mounted as a voucher for morphological identification.

DNA extraction, PCR, and sequencing. Sequences from the 3’ end of cytochrome oxidase I (COI) were obtained directly from individual wasps. DNA was extracted using a Dneasy Tissue Kit (Qiagen). We amplified approximately 546 base pairs of mitochondrial COI using primers SW2618 and Pat (Simon et al. 1994; Machado 1998).

Amplification was performed on an Eppendorf mastercycler thermocycler with 1 min at 94˚C followed by 36 cycles of 30 s at 94˚C, 1 min at 45˚C, 30 s at 68˚C, followed by a final extension of 5 min at 72˚C. The amplified PCR products were purified using a Qiaquick PCR purification kit (Qiagen). Sequencing reactions were performed using a BigDye Terminator v3.1 Cycle Sequencing Ready reaction kit on an Eppendorf mastercycler thermocycler with 1 min at 96˚ C followed by 26 cycles of 10 s at 96˚ C, 5 s at 50˚ C, 4 min at 60˚ C, then visualized on a ABI Prism 377 DNA Sequencer. Sequences were edited in Sequencher and manually aligned.

Molecular identification. Molecular analyses were performed separately for *Ceratosolen* and *Kradibia* samples. *Ceratosolen* sequences served as an outgroup for *Kradibia* analyses and vice-versa. In order to detect potential pollinator sharing among focal and non-focal *Ficus* species, COI sequences from all *Sycocarpus*-pollinating *Ceratosolen* and *Sycidium*-pollinating *Kradibia* available in GenBank were included in the analyses. Haplotype frequencies were noted and redundant haplotypes removed from further analysis. Uncorrected p-distances were calculated in PAUP 4.0 (Swofford 2001) to facilitate comparison with barcoding literature (Hebert et al. 2003; Hebert et al. 2004) and neighbor-joining trees were constructed to examine clustering of genetic haplotypes. Maximum parsimony and Bayesian methods were used to construct best estimates of phylogeny and assess monophyly of genetic clusters. Under maximum parsimony, a heuristic search was performed in PAUP with 10,000 random addition sequence replicates. Parsimony bootstrap analysis was performed with 10,000 replicates using the "fast step-wise addition" option. Bayesian estimates of phylogeny with posterior probabilities were obtained with Mr. Bayes 3.1.2 (Huelsenbeck and Ronquist 2001) by sampling 2,000 trees from two simultaneous runs of four chains over 1×10^6 generations of MCMC analysis and a GTR + G + I model of evolution. The final standard deviation of split frequencies was 0.026 in *Ceratosolen* and 0.008 in *Kradibia*, indicating that separate runs had converged onto a stationary distribution.

Morphological identification. Slide mounted wasp heads were cleared in 10% KOH solution and examined under a compound microscope to determine whether genetic clusters corresponded to published descriptions of named species (Wiebes 1963, 1980a).

Figures 2.1 and 2.2 show diagnostic characters used to identify wasp species, including the prominence of mandibular teeth and glands, the shape of the projection in the epistomal margin, and the number of lamellae on the mandibular appendage. All pollinators found in *Ficus* species other than their predominant host were examined to confirm their identity and rule out the possibility of DNA contamination or other laboratory errors.

Pollinator Sharing Ratio. For each pollinator species, the number of individuals encountered with each of several sympatric fig species was counted to calculate a pollinator sharing ratio. The pollinator sharing ratio (PS) is defined as v'/(v + v') where v is the number of sampled foundresses visiting the predominant host and v' is the number of sampled foundresses from all other host species. A pollinator sharing ratio of zero indicates complete fidelity to a single host. A ratio of 0.5 indicates that pollinators are just as likely to visit an alternative host as they are to visit their predominant host. As the PS ratio approaches 1.0, visitation of the most common host declines in proportion to the number of alternative host species and the frequency of visits to alternative hosts. We used a binomial test to compare our pollinator sharing ratios for these dioecious figs with a pollinator sharing ratio obtained from rearing data from monoecious figs reported in Molbo et al. (2003). *Pegoscapus gemellus* sp. A wasps were reared from both *Ficus popenoei* Standl. and *Ficus bullenei* I.M. Johnst. Twenty eight of 32 observations of *P. gemellus* sp. A came from *F. popenoei*, whereas the rest were from *F. bullenei,* resulting in a pollinator sharing ratio of 0.125. This is likely to be an underestimate of the true pollinator sharing ratio because it assumes: (1) every pollinator sharing event resulted in a brood, (2) each brood is the progeny of a single foundress, and (3) shared host species were sampled equally.

RESULTS

Alignment of a 532 bp segment of COI yielded 155 *Ceratosolen* haplotypes and 31 *Kradibia* haplotypes (Tables 2.1 and 2.2). Pairwise uncorrected p-distances (Figure 2.3) among named species (average = 11.7% ± SD = 2.2%) were markedly greater than pairwise p-distances within species (average = 0.8% ± SD = 0.6%). Substantial intraspecific divergence was observed in *C. hooglandii* (average distance between divergent clades = 3.8% ± SD = 0.5%), *C. wassae* (4.0% ± 0.2%) and *C. copiosae* (7.2% ± 0.4%).

Neighbor-joining trees (Figures 2.4 and 2.5) illustrate discrete genetic clusters of pollinator haplotypes characterized by large distances among clusters and small distances within clusters. Parsimony and Bayesian analysis confirmed the monophyly of these genetic clusters corresponding to named pollinator species with high bootstrap support and posterior probabilities. Each pollinator species was predominantly associated with a single *Ficus* host species (Figures 2.4 and 2.5). Additionally, clades of divergent *C. hooglandii* haplotypes showed strong associations with different *F. bernaysii* morphotypes. Among *Ceratosolen* pollinators, two of the five focal species (*C. dentifer* and *C.* sp. ex *F. morobensis*) were found visiting more than one species of fig, but encounters with alternate host species were rare (Table 2.1). *Ceratosolen dentifer* and *Ceratosolen* sp. ex *F. morobensis* had pollinator sharing ratios of 0.032 and 0.018, respectively. Pollinator sharing ratios for *C. hooglandii* clades visiting the *F. bernaysii* morphotypes ranged 0.018-0.031 (Table 2.1). In section *Sycidium,* no pollinator sharing was detected between *F. copiosa* and *F. wassa* (Table 2.2). However, both *K. copiosae* and *K. wassae* visited a single *F. copiosa* x *F. wassa* hybrid with a biased ratio of 1:19 for *K. copiosae*: *K. wassae*.

Binomial tests showed each *Ceratosolen* pollinator sharing ratio to be significantly less than that estimated for *Pegoscapus* (p= 0.005- 0.011). Given the likely underestimation of the pollinator sharing ratio for *P. gemellus*, the observed difference seems likely to hold with comparable sampling of *P. gemellus* and its hosts.

Examination of pollinator head morphology (Figures 2.1 and 2.2) confirmed the identity of described species (Wiebes 1963, 1980a) corresponding to well supported clades of pollinator haplotypes. All pollinator sharing events were confirmed by head morphology. However, no consistent morphological differences in female heads were found to distinguish between intraspecific clades of *C. hooglandii*, *K. copiosae* or *K. wassae* haplotypes.

DISCUSSION

Low pollinator sharing ratios in *Ceratosolen* and *Kradibia* suggest that pollinators of dioecious fig species are highly discriminatory among hosts as indicated by the predominance of a single host species over rare alternate hosts. These results confirm previous findings in dioecious *Ficus* based on morphological species concepts (Weiblen et al. 2001; Silvieus 2007). Nonetheless, pollinators do occasionally visit fig species other than their predominant host. In section *Sycocarpus*, between four and six of 372 pollinators visited an alternate host, depending on whether *C. hooglandii* is considered a single species or two, resulting in a pollinator sharing ratio of 0.008-0.016. In section *Sycidium,* pollinator sharing was not observed directly between *F. copiosa* and *F. wassa*. We did not estimate a pollinator sharing ratio between parent and hybrid trees in section *Sycidium* because of highly unequal sampling among hosts (Table 2.2) given the rarity of hybrid trees at less than 1% of the *Sycidium* population. Estimates of parent and hybrid relative abundance would allow normalization of the pollinator sharing ratio according to the relative abundance of the respective tree species. Nonetheless, rare hybrids are evidence that pollinator sharing has occurred between the parental species in the past. Furthermore, the fact that pollinators of both parental species visited the hybrid is evidence of some variation in host fig choice. Asymmetric hybrid visitation, characterized by a predominance of *K. wassae*, may be due to differences in pollinator body size and the ability to negotiate a hybrid ostiole. The size of the ostiole is likely correlated with the fig diameter and ripe figs of *F. copiosa* are 4.4-5.6 cm in diameter, whereas those of *F. wassa* are only 1.0-1.5 cm (Berg and Corner 2005; Weiblen et al. 2010). The intermediate diameter of *Ficus wassa x copiosa* hybrid figs, 2.7-3.4 cm, (GD Weiblen unpubl. data) could explain the preponderance of *K. wassae* over *K. copiosae* visitors. Body size of *K. copiosae* and *K. wassae* averages 1.8 mm and 1.5 mm respectively (Wiebes 1980b), and head width averages 0.58 mm and 0.46 mm respectively (AM Moe unpubl. data). Smaller *K. wassae* pollinators are more likely to successfully navigate hybrid ostioles than *K. copiosae* and this would tend to promote asymmetrical introgression from species with large figs to species with small figs.

Although pollinator sharing ratios reported here are significantly lower than neotropical monoecious figs, pollinator sharing in dioecious figs may be biologically significant. Hundreds or thousands of wasps may emerge from any given fig and thousands of figs are pollinated by wasps at any given time in these forests. On this scale, even events as rare as 0.8-1.6% could translate into a substantial number of interspecific pollination events. The reproductive outcome of pollinator sharing events is unknown. These events could result in fig hybridization, as has been suggested by molecular studies in African *Ficus* section *Galoglychia* (Renoult et al. 2009). Pollinator sharing may result in poor pollination services or even parasitism, as was found in African *Sycomorus* figs that share *Ceratosolen* pollinators (Kerdelhué et al. 1999). Two pollinator species were shared between *Ficus sycomorus* and *F. mucuso* but only one pollinator effectively pollinated *F. sycomorus* whereas the other pollinated *F. mucoso*. Pollinator sharing could lead to the colonization of new host species and host switching, as suggested by wasp rearing and incongruent host-pollinator co-phylogenies from neotropical monoecious figs (Molbo et al. 2003; Machado et al. 2005; Marussich and Machado 2007), or could be a reproductive dead end for pollinators, a possibility yet to be investigated. Cross-pollination experiments are needed to compare the fitness consequences of interspecies pollinations for figs and pollinators to those of intraspecies pollinations.

Differences in the extent of pollinator sharing between monoecious figs and dioecious figs might be attributed to fundamental differences in breeding system influencing the evolution of host specificity and inferred cophylogenetic patterns. Highly congruent phylogenies of dioecious figs and pollinators have been interpreted as evidence of cospeciation (Weiblen and Bush 2002; Silvieus et al. 2008), whereas the highly incongruent phylogenies of monoecious figs and pollinators suggest the opposite (Machado et al. 2005; Marussich and Machado 2007; Su et al. 2008). We propose two possible explanations for these differences. First, pollinators of functionally dioecious figs may be selected for a strong tendency to colonize figs that are highly similar to their birth fig, namely a gall fig as opposed to a seed fig. Choosing a seed fig as a host has an absolute fitness cost, as the pollinators of seed figs fail to reproduce (Valdeyron and Lloyd 1979; Kjellberg et al. 1987). Therefore, pollinators of dioecious figs may be selected to discriminate between functionally male gall figs and functionally female seed figs, which they do to some extent in certain host species (Anstett et al. 1998) but not in others (Patel et al. 1995). A strong discriminatory behavioral response to variation in fig volatile attractants among host species could be a by-product of adaptations associated with seed fig avoidance. On the other hand, figs might be selected for specialized nursery function as it relates to the male component of fitness if pollen is limiting. A functionally female tree can achieve reproductive success with visits from only a few pollinators carrying conspecific pollen whereas a functionally male tree depends on successful rearing of wasps with behavior affecting conspecific pollination. Therefore, floral morphology or chemistry that restricts rearing to faithful pollinators may be under positive selection. Such selection pressures may be absent or weaker in monoecious figs, where individual trees perform both male and female functions. Cross-pollination experiments in the field are needed to test these hypotheses.

Mitochondrial DNA divergence within three named wasp species could indicate cryptic or incipient speciation (Figure 2.3), but it is difficult to tell without expanded sampling of geographically distributed populations (Haine et al. 2006; Lin et al. 2008; Moe and Weiblen 2010). In the case of *K. wassa*, the rarity of individuals belonging to *K. wassa* clade 2 at our locality could be explained by phylogeography. Divergent *K.*

copiosa haplotypes were more or less equally abundant in sympatry, and perhaps the depth of mtDNA divergence is evidence of cryptic species or incipient speciation. Divergence in *C. hooglandii* could be a case of ecological speciation, with each clade specializing on a different *F. bernaysii* morphotype. Differences between morphotypes are very subtle and had local naturalists not pointed out epidermal hairs, we might have concluded that two cryptic species pollinate the same fig species. Many recent studies have discovered and described multiple cryptic wasp species pollinating a single species of fig (Molbo et al. 2003; Machado et al. 2005; Haine et al. 2006; Peng et al. 2008; Su et al. 2008; Moe and Weiblen 2010). A closer examination of morphology and population genetics of host taxa from which cryptic wasp species are reported could shed light on speciation in progress or lack thereof.

Named *Ceratosolen* and *Kradibia* form reciprocally monophyletic groups based on COI haplotypes, which correlate with morphology. Species identification using COI appears to be straightforward in these fig wasps such that DNA barcoding can serve as a useful diagnostic tool for detection of “cryptic” species as has been broadly proposed for Lepidoptera (Hebert et al. 2009) and birds (Tavares and Baker 2008). Characters of the female head, which are highly variable among species and implicated in accessing the fig (van Noort and Compton 1996), were sufficient to identify named species, but not to discriminate between deeply divergent clades within named species, or putative cryptic species.

CONCLUSIONS

Dioecious fig pollinators *Ceratosolen* and *Kradibia* showed high levels of host specificity and low pollinator sharing ratios. Pollinator sharing in dioecious fig lineages appears to be less common than what has been reported for neotropical monoecious fig lineages. However, even low rates of pollinator sharing could be evolutionarily significant. The impact of rare pollinator sharing on fig species delimitation and on coevolution in the fig and fig wasp mutualism will depend on the reproductive consequences of pollinator sharing, which can be examined through direct experimentation and manipulative pollination in the field. Although molecular and morphological species concepts could yield different numbers of pollinator species associated with particular fig species, rates of pollinator sharing in New Guinea were not sensitive to such differences. Deep mitochondrial divergence of lineages within named species of pollinators suggests incipient speciation. Divergent clades are strongly associated with different host morphotypes in the case of *Ceratosolen hooglandii*, which points to the possibility of plant-pollinator codivergence. Broader geographic sampling and molecular characterization of *F. bernaysii* is needed to further investigate this possibility.

TABLE 2.1. Frequency of associations between sympatric New Guinea *Ceratosolen* species and *Ficus* section *Sycocarpus* species. Data include numbers of foundresses collected (n_f), numbers of foundress haplotypes (n_h), numbers of foundresses visiting the predominant host (v), numbers of foundresses visiting other fig species (v'). The pollinator sharing ratio, (PS = v'/v+v') * indicates significant difference from monoecious fig expectations derived from Molbo et al. (2003).

pollinator species	**n_f**	**n_h**	**v**	**v'**	**common host**	**alternate host**	**PS ratio**
C. dentifer	63	14	61	2	*F. hispidioides*	*F. bernaysii* B *F. congesta*	0.032*
C. hooglandii clade 1	64	32	62	2	*F. bernaysii* A	*F. bernaysii* B	0.031*
C. hooglandii clade 2	57	19	56	1	*F. bernaysii* B	*F. bernaysii* A	0.018*
C. notus	75	27	75	0	*F. congesta*	-	NA
C. sp. ex *F. morobensis*	55	24	54	1	*F. morobensis*	*F. bernaysii B*	0.018*
C. sp. ex *F. pachyrrhachis*	58	8	58	0	*F. pachyrrhachis*	-	NA
TOTAL	372	124	366	6			

TABLE 2.2. Focal *Kradibia* species and their visitation of fig hosts. n_f indicates the number of foundresses collected, n_h indicates the number of foundress haplotypes, v indicates the number of foundresses visiting their common host, v' indicates the number of foundresses visiting the *F. copiosa* x *F. wassa* hybrid. PS ratio is not applicable as it was not observed in samples from host species, but only in a rare hybrid. Disproportionate sampling of the hybrid violates a key assumption of the PS ratio.

pollinator species	**n_f**	**n_h**	**v**	**v'**	**common host**	**alternate host**
K. copiosae clade 1	35	6	32	3	*F. copiosa*	*F. copiosa* x *F. wassa*
K. copiosae clade 2	30	6	28	2	*F. copiosa*	*F. copiosa* x *F. wassa*
K. wassae clade 1	142	18	47	95	*F. wassa*	*F. copiosa* x *F. wassa*
K. wassae clade 2	3	1	3	0	*F. wassa*	-
TOTAL	210	31	110	100		

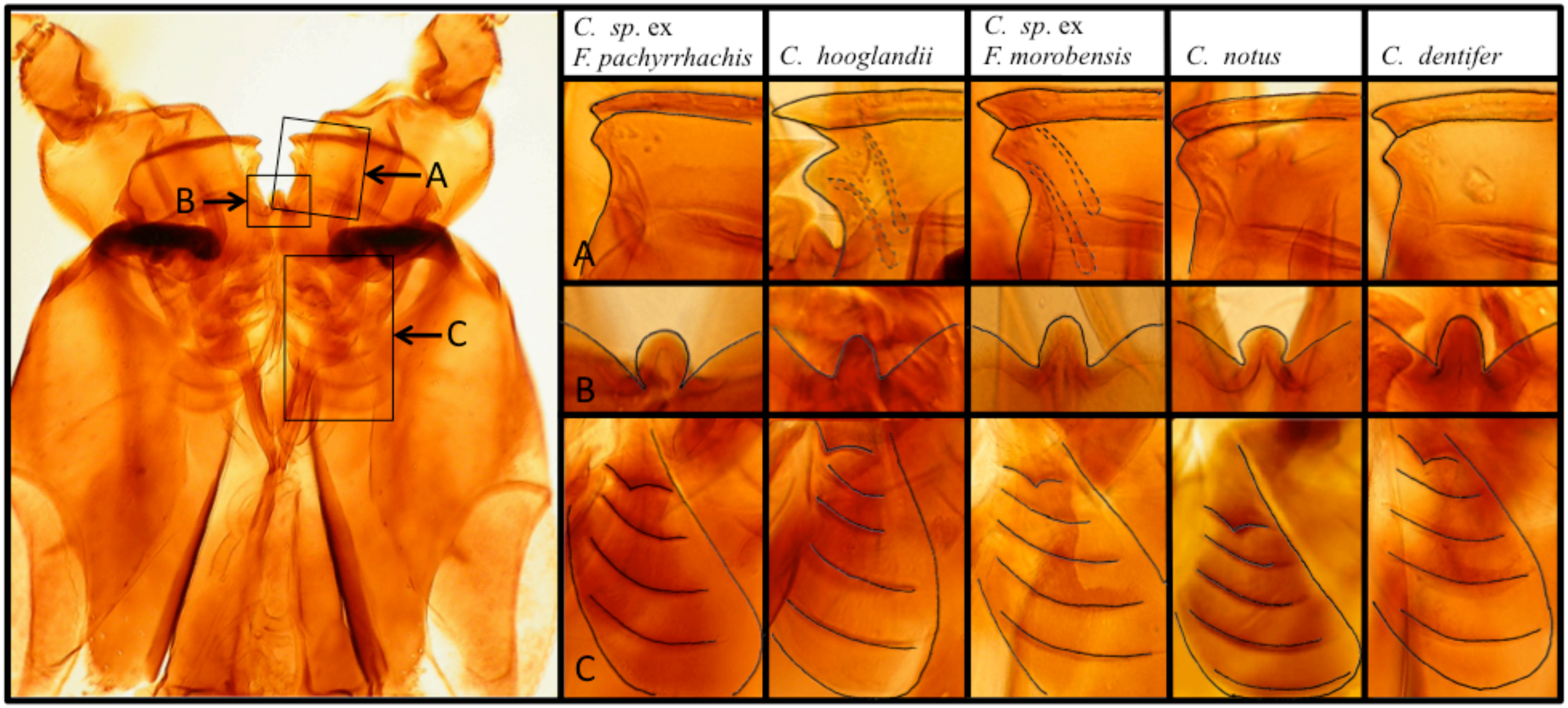


FIGURE 2.1. Diagnostic characteristics for *Ceratosolen* species. A) Mandible. *Ceratosolen hooglandii* is distinguished by a large, over-extended upper tooth on the mandible that all other species lack. *Ceratosolen* sp. ex *F. morobensis* and *C. hooglandii* are distinguished by two large, prominent mandibular glands. B) Epistomal projection. *Ceratosolen notus* is distinguished by a "knob-like" projection in the epistomal margin, which is at least as wide as it is long. C) *Ceratosolen* sp. ex *F. pachyrrhachis* is distinguished by four lamellae on the mandibular appendage.

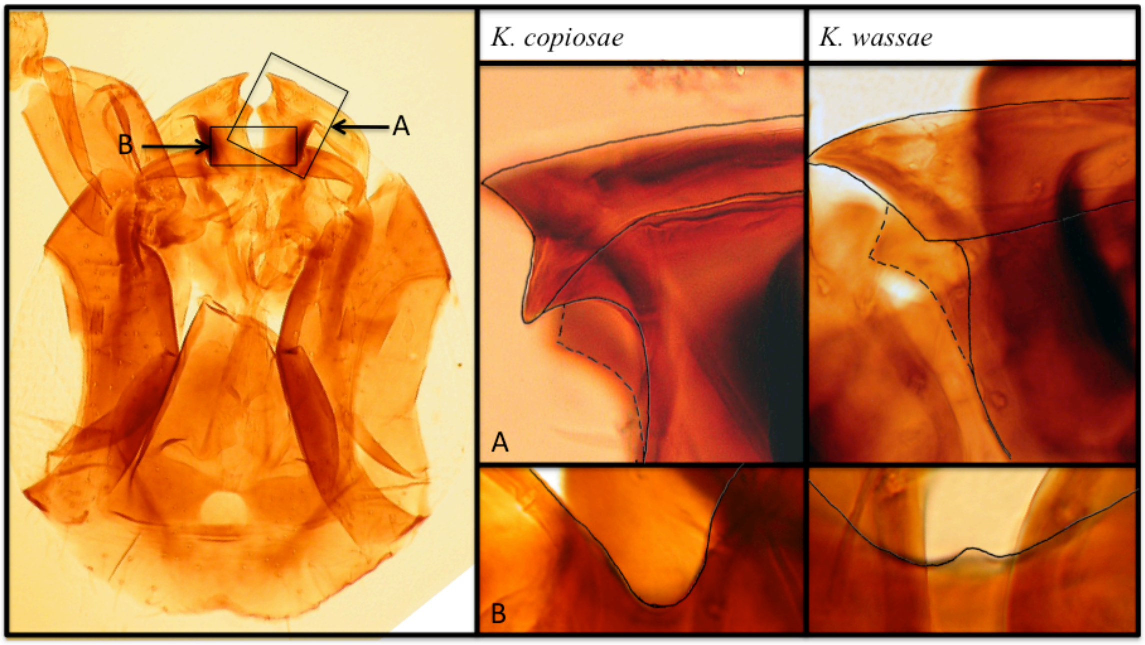


FIGURE 2.2. Diagnostic characteristics for *Kradibia* species. A) *Kradibia copiosae* has two strong teeth on the mandible. *Kradibia wassae* has a strong upper tooth and weak lower tooth on the mandible. B) *K. copiosae* has a deeply bilobed epistomal margin with no projection and *K. wassae* has a shallowly bilobed epistomal margin with a slight projection.

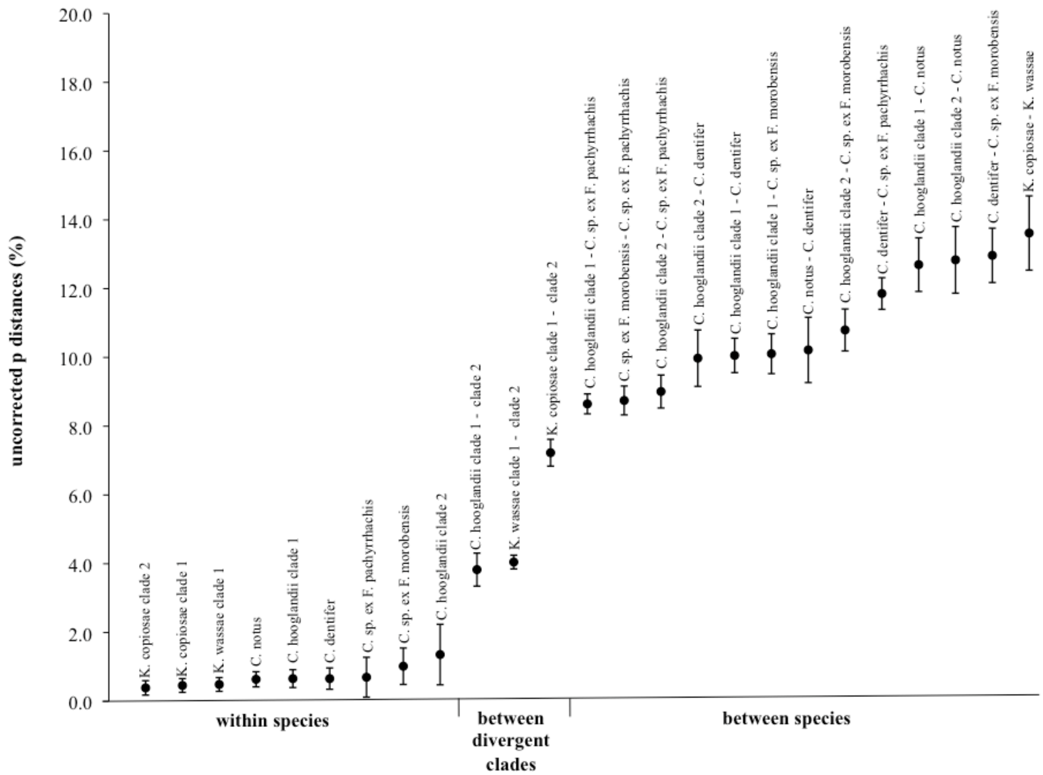


FIGURE 2.3. Pairwise uncorrected p-distances among foundress wasp haplotypes.

FIGURE 2.4. Cytochrome oxidase I neighbor-joining tree of *Ceratosolen* pollinator haplotypes from *Ficus* section *Sycocarpus.* Branches are labeled with bootstrap support/Bayesian posterior probabilities. Taxon symbols correspond to host *Ficus* species from which the pollinator haplotypes were collected.

FIGURE 2.4

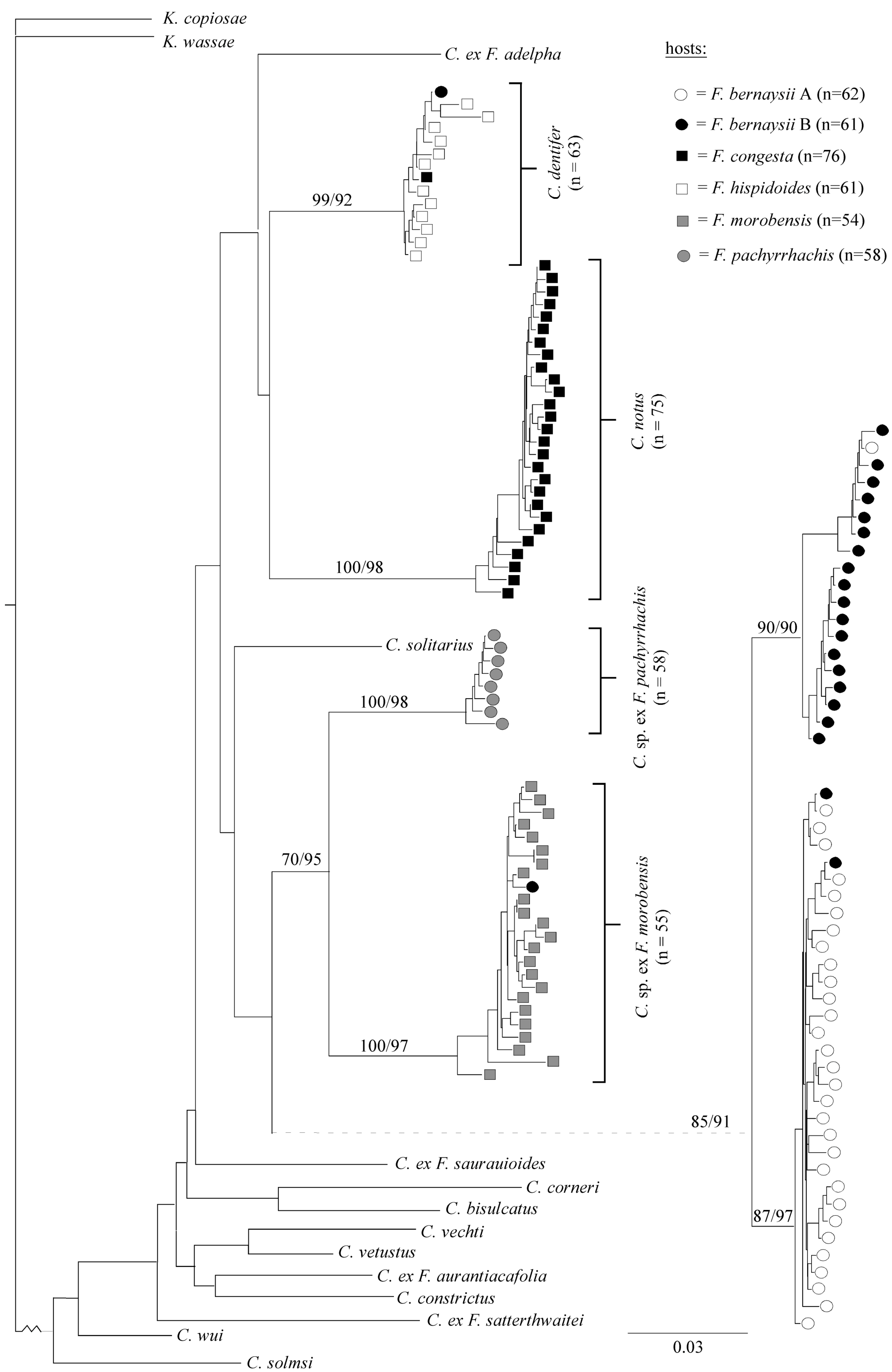
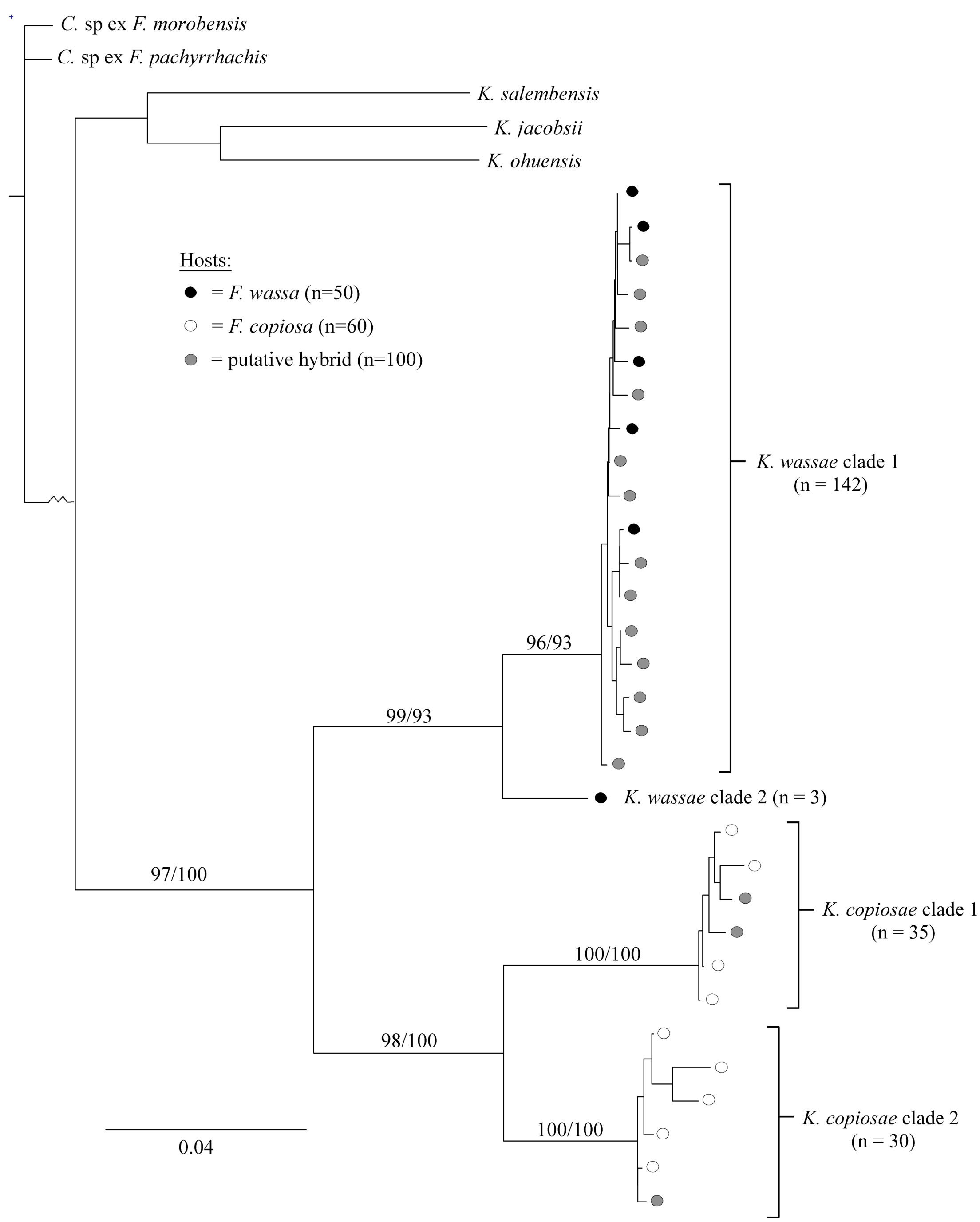

K. copiosae
K. wassae
C. ex F. adelpha
hosts:
= F. bernaysii A (n=62)
= F. bernaysii B (n=61)
= F. congesta (n=76)
= F. hispidoides (n=61)
= F. morobensis (n=54)
= F. pachyrrhachis (n=58)
99/92
C. dentifer
(n = 63)
C. notus
(n = 75)
100/98
C. solitarius
100/98
C. sp. ex F. pachyrrhachis
(n = 58)
70/95
C. sp. ex F. morobensis
(n = 55)
100/97
90/90
C. hooglandii clade 2
(n = 57)
C. hooglandii clade 1
(n = 64)
85/91
87/97
C. ex F. saurauioides
C. corneri
C. bisulcatus
C. vechti
C. vetustus
C. ex F. aurantiacafolia
C. constrictus
C. ex F. satterthwaitei
C. wui
C. solmsi
0.03

FIGURE 2.5. Cytochrome oxidase I neighbor-joining tree of *Kradibia* pollinator haplotypes from *Ficus* section *Sycidium.* Branches are labeled with bootstrap support/Bayesian posterior probabilities. Taxon symbols correspond to host *Ficus* species from which the pollinator haplotypes were collected.

FIGURE 2.5


C. sp ex F. morobensis
C. sp ex F. pachyrrhachis
K. salembensis
K. jacobsii
K. ohuensis
Hosts:
= F. wassa (n=50)
= F. copiosa (n=60)
= putative hybrid (n=100)
K. wassae clade 1
(n = 142)
96/93
99/93
K. wassae clade 2 (n = 3)
97/100
K. copiosae clade 1
(n = 35)
100/100
98/100
K. copiosae clade 2
(n = 30)
100/100
0.04

CHAPTER 3

POLLINATOR MEDIATED REPRODUCTIVE ISOLATION AMONG DIOECIOUS FIG SPECIES (*FICUS*, MORACEAE)

The observation that some closely related plants co-occur as distinct species, even in the presence of hybridization, has long motivated the study of reproductive isolating mechanisms (Stebbins 1950). Whether reproductive isolation in plants involves pre-pollination mechanisms, such as phenological, mechanical and ethological barriers, or post-pollination mechanisms, such as pollen incompatibility, genetic incompatibility, or hybrid inferiority (Grant 1971), can help to explain the origin and evolution of plant species diversity. Diverse plant lineages with particularly specialized pollination syndromes involving animals have invited speculation that coevolution might play a role in diversification (Wiebes 1979; Waser and Ollerton 2006). Among plant-pollinator mutualisms, those characterized by extreme specialization and interacting reproductive traits have been identified as models for investigating co-diversification (Kiester et al. 1984). However, the study of strict-sense coevolution (Janzen 1980) or co-adaptation due to reciprocal selection has contributed little to our understanding of the evolution of reproductive isolation in plants (Clayton et al. 1999; Thompson et al. 2002; Benkman et al. 2003; Brodie and Ridenhour 2003; Gomulkiewicz et al. 2003; Nuismer et al. 2003; Ridenhour and Nuismer 2007; Pauw et al. 2009; Nuismer et al. 2010) but see (Yoder and Nuismer 2010).

Pollinators may act as agents of reproductive isolation by means of species-specific floral constancy in foraging behavior (Esfeld et al. 2009; Oyama et al. 2010). Additionally, trait mismatching in heterospecific visitation can prevent heterospecific pollen deposition or the collection of pollinator rewards (Campbell et al. 1997; Kephart and Theiss 2004; Kay 2006; Anderson et al. 2010). When reproduction of pollinators and host is interdependent, selection for specialization may be intense, especially where similar hosts occur in sympatry. Indeed, the evolution of extreme pollinator specialization is observed in cases where plants provide “nursery” rewards for pollinators (Waser and Ollerton 2006). A recent natural experiment on the obligate pollination mutualism between Joshua trees and yucca moths (Smith et al. 2009) revealed that phenotype matching plays a role in reproductive isolation of parapatric host tree varieties. In spite of hybridization between varieties in a contact zone, fitness differences between pollinators ovipositing in the host variety from their native range compared to the host variety from outside their range selects for specificity and serves as a mechanism of reproductive isolation between Joshua tree varieties.

The obligate pollination mutualism between figs (*Ficus*, Moraceae) and fig wasps (Agaonidae, Hymenoptera) is similarly specialized (Janzen 1979; Weiblen 2002; Cook and Rasplus 2003; Waser and Ollerton 2006). Life cycles of pollinating fig wasps and their *Ficus* host plants are completely interdependent. Volatile signals attract female fig wasps to a receptive fig of a new host (Song et al. 2001; Grison-Pige et al. 2002a; Chen and Song 2008; Proffit et al. 2008) where they gain access to the enclosed flowers through a narrow, bract-covered opening, or ostiole. Females oviposit in flowers and either actively or passively deposit pollen in the process. In monoecious figs, the unisexual flowers either set seed, form galls that nourish pollinator offspring, or produce pollen that eclosing wasps later transport to other receptive figs. In dioecious species, male and female reproductive functions are separated between plants bearing either seed-producing inflorescences (seed figs) or inflorescences producing wasps and pollen (gall figs).

Recently, much attention has focused on the evolutionary implications of host specificity in pollinating fig wasps (Herre et al. 2008). A pattern of one-to-one species specificity was generally assumed (Wiebes 1979) until departures from this simple model of host associations came to light. Observations of agaonid wasps visiting multiple fig species (Molbo et al. 2003; Machado et al. 2005; Marussich and Machado 2007; Su et al. 2008) and molecular evidence of fig hybridization (Parrish et al. 2003; Renoult et al. 2009) suggest that deviations from the one-to-one model are evolutionarily important. Reports of hybrids, (Ramirez 1970; Parrish et al. 2003; Machado et al. 2005), in some cases, when species are cultivated beyond their native range (Ware and Compton 1992; Ramirez 1994; Harrison 2007), indicate that hybridization might be common in the genus. The extent of reproductive isolation among sympatric fig species, depending on the ubiquity and fitness consequences of pollinator sharing could pose challenges for *Ficus* species delimitation.

Most studies of this system have either examined patterns of host conservatism or inferred host switching from phylogenetic patterns (Lopez-Vaamonde et al. 2002; Weiblen and Bush 2002; Jousselin et al. 2003; Machado et al. 2005; Marussich and Machado 2007; Jackson et al. 2008; Ronsted et al. 2008a; Azuma et al. 2010; Moe and Weiblen 2010). Little has been done by way of experimentation to investigate ecological mechanisms and evolutionary processes that could produce such patterns. Given that the nature and extent of reproductive isolating mechanisms among sympatric fig species depends on the reproductive consequences of pollinator host choice, experiments are needed to estimate fitness for both mutualistic partners. Lack of experimental work is partly due to the logistical challenges of manipulating such closely intertwined life cycles in the field. However, the direct linkage of life cycles also provides the opportunity to simultaneously estimate the fitness of interacting individuals, and to compare it among different kinds of interaction.

This study investigated mechanisms of reproductive isolation among sympatric fig species by experiment and the population genetic signature of such processes by inference from molecular markers. We developed a new method for introducing pollinators to novel hosts. Bypassing the ostiole allowed us to control access to the fig interior, and to separate host choice behavior from subsequent pollination and oviposition behaviors. We used this method to compare fig and pollinator reproduction following conspecific visitation versus heterospecific visitation in the field. We then examined genetic differentiation in the same community of sympatric fig species and applied Bayesian clustering methods to identify putative natural hybrids and estimate rates of heterospecific gene flow.

METHODS

Study system. Six named species of dioecious *Ficus* subgenus *Sycomorus* section *Sycocarpus* were used in this study (*Ficus bernaysii* King, *Ficus congesta* Roxb., *Ficus hahliana* Diels, *Ficus hispidioides* S. Moore, *Ficus morobensis* C.C. Berg *and Ficus pachyrrhachis* K. Schum. & Lauterb.). Members of *Sycocarpus* are morphologically similar, but are differentiated by fig, leaf and epidermal hair characteristics (Berg and Corner 2005). The focal species belong to a clade of dioecious species estimated to have originated at least 15 mya (Silvieus et al. 2008). They co-occur in the lowland rainforests of New Guinea and are often found at high density in patches of secondary re-growth resulting from local slash and burn agriculture. All species have abundant, cauliflorous figs, growing along the length of the trunk, such that figs can be collected, manipulated and observed from the ground. Considering fig size and abundance per tree *Ficus hispidioides* was chosen as the pollen acceptor for all experiments. Relatively large figs (3-4 cm diameter at receptivity) eased the manipulation and introduction of pollinators and the presence of numerous figs per tree in accessible locations enabled experimental replication.

Local landowners at the study site recognized two morphotypes of *F. bernaysii*, distinguished by the length and density of epidermal hairs on young shoots and the persistence of stipules. These entities were referred to as *F. bernaysii* morphotypes A and B in a previous publication on pollinator sharing (Moe et al. 2011). However, recent examination of *Ficus* type specimens determined that the species referred to as *F. bernaysii* in numerous publications (Weiblen 2000, 2001; Weiblen et al. 2001; Novotny et al. 2002; Weiblen and Bush 2002; Weiblen 2004; Weiblen et al. 2006; Weiblen et al. 2010) and then, subsequently referred to as *F. bernaysii* A (Moe et al. 2011), is actually *F. hahliana*. Likewise, wasps collected from these specimens are not *Ceratosolen hooglandii,* but *Ceratosolen* sp. ex *F. hahliana.* The less common and less frequently collected *F. bernaysii* was previously referred to as *F. bernaysii* B (Moe et al. 2011). As mature *F. bernaysii* individuals were rare in the forest, only the five other species were used as pollen donors in cross pollination experiments, but all six species were included in genetic analyses.

Sampling. Methods for this study were developed over eight months and two field seasons in 2007-2008. The data presented here are from a set of experiments performed May-August of 2009 at Ohu village in the Madang district of Madang Province, Papua New Guinea (Lat. 5˚ 13' 38" S Long. 145˚ 40' 44" E). The area is heavily populated and the forest surrounding the village is mostly a patchwork of secondary re-growth of varying ages, but includes a small (102 ha) conservation area of old growth forest. Experimental trees, both pollen donors and pollen acceptors, were located within an area surrounding the Ohu village of approximately 400 ha in size (Appendix 3). This area contained at least 75 trees of each species included in the study. Fifty individuals of each focal species were sampled for population genetic analyses. Young leaf tissue was collected from each individual and immediately dried over silica gel, and later stored at -80˚C for DNA extraction. A voucher specimen was also collected from each individual, alcohol preserved, and later dried for long-term storage.

Preliminary measurements. Before beginning experiments, we collected phenological and life history data on *F. hispidioides* in order to appropriately design pollination experiments. We wanted to know the average number of foundresses naturally pollinating receptive figs of *F. hispidioides* so that we could introduce a comparable number of wasps into experimental figs. We collected a total of 250 receptive figs from 10 *F. hispidioides* trees and found an average of five foundresses per fig (SD = 4.06). We chose to introduce six pollinators per experimental fig, expecting that some wasps would not survive introduction.

We also wanted to know the minimum diameter at which figs are receptive to pollinators to ensure that any fig included in experiments had not already been naturally pollinated. We collected 120 figs, measured their diameter and classified them as pre-receptive (no pollinators inside), receptive (live pollinators inside) or post-receptive (dead pollinators inside and/or developing seed or galls inside). The average diameter of receptive figs was 33. 8 mm (range 30-39.5). However, the smallest post-pollination fig had a diameter of 25 mm. Although this was an outlier, we chose a conservative maximum diameter of 24 mm as the criterion for selecting figs to include in our experiments.

Pre-receptivity treatment. Seven functionally female and seven functionally male trees of *F. hispidioides* were identified as suitable experimental trees, having large clusters of young, unreceptive figs growing at eye level and below. Figs with diameters measured at 24 mm or less were tagged loosely around the peduncle and treated to exclude natural pollinators. A ring of Tanglefoot pest barrier was applied around the ostiole of the young fig and a square of organza mesh pressed into the glue, sealing the opening of the fig (Figure 3.1A&B). This treatment allowed fluid to escape from the fig interior and for expansion of the fig during development while excluding pollinators from entering the ostiole (Figure 3.1C). Ten figs on each tree were tagged but received no treatment in order to determine the effect of treatment on fig development. Fig diameters were measured and recorded every two days and the seal around the ostiole checked for integrity. Fresh Tanglefoot and mesh were applied as needed. If a seal was discovered to be broken and an ostiole exposed for any period of time, the fig was considered potentially contaminated and removed from the experiment. Figs were considered receptive when they reached a diameter above 30 mm and masses of pollinating wasps were trapped at the ostiole by Tanglefoot.

Receptivity treatment. When figs reached receptivity, a small section of the fig wall running perpendicular to the shoot apex was cored using a 3 mm hypodermic needle and a small section of Pasteur pipet plugged with cotton was inserted into the hole (Figure 3.1D). After two days, experimental figs that survived coring were treated with pollinators. Figs from all the pollen donor species were collected and brought to the experimental tree (Figure 3.1E). First, we tried introducing naive wasps emerging from ripe, functionally male figs. However, these wasps refused to enter experimental figs. Out of 894 trials, none of the winged wasps demonstrated taxis along the length of the pipet toward the fig interior, but rather taxis in the opposite direction was observed. We then tried introducing foundress wasps that had already entered receptive figs and were engaged in pollination behavior (Figure 3.1F). These foundress wasps readily entered figs after being introduced through the pipet (Figure 3.1G). After six wasps had actively entered a fig, the pipet was re-plugged with cotton. The number of figs treated with pollinators from each focal species was determined by the number and species of receptive figs available on the day of treatment and the number of pollinating wasps found within them. Control figs received no pollinator introduction. Fig diameters were measured every two days until figs aborted or reached maturity. The experiments lasted approximately six weeks from the tagging of pre-receptive figs to harvesting of mature figs.

Fig processing. Aborted and mature figs were collected, opened and checked for seed or gall development. Whenever possible, foundresses were counted to make sure they did not exceed the number of foundresses that were experimentally introduced. However, in the case of many mature figs, foundresses were too decomposed to be counted. Seed samples were taken and gall contents were examined to determine whether wasp larvae had developed to maturity. A three mm wide longitudinal section of each fig was cut and flowers were counted as developed (seed or gall) or undeveloped under a dissection microscope. The remainder of the fig was dried in a solar drier, stored over silica gel, and eventually frozen at -80 ˚C.

Seed viability. To determine the viability and survivorship of the various experimental crosses in comparison with non-hybrid *F. hispidioides* seed/seedlings, we measured germination, growth and survival rates for each pollen donor treatment. As the focal species are "pioneer species" and naturally establish in forest gaps, growth rate could be an important aspect of fitness. Seed from experimental figs was germinated in a light chamber with 40-100 seeds from each fig in a separate petri-dish lined with wet filter paper. Total counts and proportion of seeds germinated per dish/fig were recorded. Germinated seeds were assigned, using a random number generator, to planters in 12 x 6 arrays. The seedlings were grown in a growth chamber at 26.6˚C with 12 hours of light per day. Growth rate was characterized by length of the longest leaf, measured once a week, and seedling height, measured once a month for six months.

Analyses. The control treatment measured a baseline level of natural pollinator contamination in the experiment. To test whether the development of galls in experimental figs was significantly higher than the baseline level of contamination, a series of pairwise 2x2 contingency tests, using counts of developed and undeveloped galls, were performed to test if the proportion of figs that initiated gall development for each pollen donor species was significantly higher than the control. A second set of pairwise 2x2 contingency tests compared the proportion of figs that produced mature galls with developing pollinator larvae against the control. These tests were not needed in experimental seed figs because microsatellite genotyping of seedlings either confirmed or rejected their expected identity.

Another series of pairwise 2x2 contingency tests compared the proportion of figs that initiated gall and seed development in each non-conspecific treatment against development in the conspecific treatment (*F. hispidioides* pollen donor).

One-way ANOVAs were performed to determine (1) the effect of pollen donor species on seed set and percent gall formation and (2) the effect of pollen donor species on seed germination rates. Each experimental fig was a replicate for these measurements. One-way ANOVAs were performed to test for the effect of pollen donor species on the two measures of growth rate (height and length of longest leaf). Seedling survivorship after 160 days was compared between non-hybrids and each hybrid cross with pairwise 2x2 contingency tests.

Microsatellite amplification and scoring. DNA was extracted from each 15-20 mg of dried plant tissue from each individual using a Qiagen DNeasy Plant Tissue extraction kit. In total, we amplified and genotyped individuals at 14 loci (Table 3.1). Among the microsatellite loci used, four primer pairs had been developed for *Ficus montana* (FM4-15 and FM3-64) and *Ficus septica* (FS4-11 and FS3-31) by Zavodna et al. (2005), four had been developed for *Ficus racemosa* (Frac86) and *Ficus rubiginosa* (Frub29, Frub38 and Frub436) by Crozier et al. (2007) and six were developed for *F. hahliana* (B30, B47, B83) and *F. pachyrrhachis* (P164, P211, P215) by Moe and Weiblen (2011). Amplification of microsatellite loci was performed on an Eppendorf Mastercycler in a total volume of 10 μL using 0.2 mM fluorescent end-labeled forward primer and unlabeled reverse primer, 0.2 mM buffer solution, 0.2 mM of each dNTP, 0.8 mM BSA, 0.3 units of TaKaRa Ex Taq polymerase (TAKARA BIO inc.) and 20-50 ng template DNA. PCR conditions are indicated in Table 3.1. Microsatellite alleles were visualized using an ABI 377 Sequencer along with a ROX 500 (Applied Biosystems) size standard and scored using Genotyper 2.5 software (Applied Biosystems).

Microsatellite analyses. Tests of Hardy-Weinberg equilibrium and linkage disequilibrium can be affected by sampling substructure, such as the presence of sibling groups. To identify sibling groups in our samples, we performed kinship analysis using Kinalyzer (Berger-Wolf et al. 2007; Ashley et al. 2009). One individual from each sibling group was randomly chosen and included for analyses using GENEPOP on the web (Raymond and Rousset 1995; Rousset 2008). We tested for linkage disequilibrium and deviations from Hardy-Weinberg equilibrium. Sequential Bonferroni corrections for multiple tests (Holm 1979) were used to assess the significance of these tests. In order to describe genetic differentiation among focal species, we 1) calculated Fst, a measure based on allele identity (Weir and Cockerham 1984) 2) calculated Rho (Valdes et al. 1993), a measure based on allele size and an estimate of Rst (Slatkin 1995), and 3) tested for significant genic differentiation between all species pairs using Fisher's method (Fisher 1922). All measures of differentiation were calculated using all 14 loci. Additionally, the private allele method (Barton and Slatkin 1986) was implemented in GENEPOP to estimate the number of migrants per generation among all the focal species.

All individuals, regardless of their sibling group membership were included in Bayesian clustering analyses implemented in STRUCTURE (Pritchard et al. 2000). This method assigns individuals to one or more ancestral populations based on their allelic genotypes. Hybrids can be identified by their partial assignment to more than one ancestral population. To examine whether morphological species concepts reflected genetically distinct clusters, we ran five independent iterations in STRUCTURE with ancestral population number (K) set for K=4, K=5, K=6, and K=7 and without using a priori species identification information. Each Markov chain included a 10,000 generation burn-in and ran for 10^6 additional generations. We used an admixture model and allowed for correlated allele frequencies among clusters. The K value of six had the highest average likelihood and resulted in the most consistent cluster assignments over five iterations. These results along with the observation of six morphological species suggest that the mostly likely number of ancestral populations is six (Pritchard et al. 2000). Assuming K=6, we used prior information on species assignment, based on morphological identification, and ran the analysis again at three values of interspecies migration rate, v= 0.01, 0.05 and 0.10. STRUCTURE estimated the posterior probabilities of each individual being 1) a non-hybrid, but with an incorrect *a priori* species assignment, 2) an F1 hybrid or 3) an F2 hybrid. Eight individuals having a significant posterior probability of being either misidentified or a hybrid were singled out for re-examination. Leaf vouchers from these individuals were checked for misidentifications. DNA was also re-extracted from these individuals and a subset of loci was amplified and genotyped to make sure admixture was not a result of cross-contamination or incorrect scoring of microsatellite alleles. Analyses were run again at K=6 with corrected genotypes and one misidentified individual reassigned to the correct species. The STRUCTURE results reported are from this second round of analyses.

An additional analysis identifying hybrid individuals was implemented in BayesAss (Wilson and Rannala 2003) to compare against results from STRUCTURE analysis. BayesAss uses Bayesian and Monte Carlo Markov chain methods to estimate recent migration rates among populations and estimate each individual's ancestry. Individuals are classified as an immigrant from a specific population, a non-immigrant, or the offspring of an immigrant and a non-immigrant (hybrid). The program assumes unlinked loci and a relatively low rate of migration among populations (less than 1/3), but allows for deviations from H-W equilibrium. A 10,000 iteration burn-in, followed by 3,000,000 iterations and default delta values were used. Individuals assigned as hybrids were noted. The mean and 95% confidence interval for estimated pairwise migration rates between focal species were recorded.

RESULTS

Pollination experiments. A total of 563 functionally male (gall) figs and 345 functionally female (seed) figs were included in the experiment. Between tagging and the introduction of pollinators, there was an abortion rate of 17.9% in gall figs and 31.3% in seed figs. Most of these abortions occurred after the coring and insertion of the Pasteur pipet. A total of 410 gall figs (Table 3.2) and 216 seed figs (Table 3.3) survived the pre-receptivity treatment to receive pollinator introductions or to be left as controls without pollinator introductions. Comparison of fig growth among treated and untreated figs on a single male tree and single female tree showed the coring treatment and insertion of the Pasteur pipet inhibited and/or slowed the growth of the experimental figs (Figure 3.2). On average, experimental gall figs reached only 85% the size of mature untreated gall figs, regardless of whether or not they developed to maturity. Experimental seed figs reached 99% the size of untreated seed figs only if they developed to maturity. Development of all treated experimental figs lagged behind the development of untreated figs by an average of six days.

Galls initiated development in some proportion of figs in all treatments (Table 3.2 & Figure 3.3A), including a small fraction of the control figs (Table 3.2). While all non-resident pollinator species induced gall formation in a significantly lower proportion of figs than the resident pollinator (2x2 contingency test $p<0.01$), only figs treated with pollinators from *F. hispidioides*, *F. congesta* and *pachyrrhachis* initiated gall formation in a significantly greater number of figs than controls (2x2 contingency test $p<0.01$). Galls reached maturity to produce wasp offspring only in figs that received the natural pollinators of *F. hispidioides*.

Seeds initiated development in some proportion of figs in all treatments (Table 3.3), and nearly all the seed that initiated development reached maturity (Figure 3.3B). Seed also developed in a small proportion of the control figs (Table 3.3). Microsatellite genotyping of offspring grown from experimentally produced seed allowed us to discriminate between hybrid and non-hybrid seed. Since more than two parental alleles at each locus were represented in non-hybrid offspring, we are confident that non-hybrid seed development in figs receiving a interspecific pollination treatment was not the result of agamospermy, but rather, the unsuccessful exclusion of natural pollinators. The data shown in Table 3.3 and Table 3.4 and Figure 3.3B & D have excluded data from eight figs that developed non-hybrid seed as a result of natural pollinator contamination.

Within each gall fig, the percent of flowers that initiated gall development was not significantly different (ANOVA p=0.297) among treatments (Figure 3.3C). Among all treatments that produced seed, the percent of flowers that developed seed was not significantly different (p= 0.621). However, percent seed set and percent gall formation are greatly reduced in the experimental figs, compared to previous estimates in untreated *F. hispidioides* (Tables 3.2 & 3.3).

Germination and growth. Seed resulting from all successful crosses had above 50% germination and there were no significant differences (ANOVA p=0.557) among the seed types (Table 3.4 & Figure 3.4A). Non-hybrid *Ficus hispidioides* had the highest survivorship among germinated seeds (Table 3.4 & Figure 3.4B) and *F. hispidioides* x *F. morobensis* and *F. hispidioides* x *F. pachyrrhachis* seed types had significantly lower survivorship (2x2 contingency test p<0.01). Survivorship of seedlings was generally low across all seedling types due to fungal infections, followed by overly dry conditions in the growth chamber.

Seedlings of all types grew at comparable rates (Figure 3.4C&D). The height of seedlings was not significantly different among seedling types at 160 days (ANOVA $p=0.955$). The length of the longest leaf was not significantly different among seedling types at 160 days (ANOVA $p=0.209$).

Molecular differentiation. Kinship analysis revealed a large number of siblings in our samples. This reduced the sample size of each species from 50 to 17-23 individuals (Table 3.5) for the calculation of measures of genetic differentiation, tests of H-W equilibrium and linkage disequilibrium. After sequential Bonferroni multiple test corrections, linkage disequilibrium was not found between any loci across all species. The lowest p-values were 0.055 (P211 & Frub29) and 0.133 (FS3-31 and FM4-15). In four of the six focal species (*F. hahliana*, *F. congesta*, *F. hispidioides*, *F. pachyrrhachis*), a single locus was found to be significantly heterozygote deficient after multiple test corrections. However, a different locus was heterozygote deficient in each species (Table 3.5), which suggests the presence of null alleles in these populations. All tests of genic differentiation among species pairs were highly significant ($p<0.001$). Mean Fst values for each species ranged 0.1875-0.2346 (Table 3.5). The mean Rho values for each species ranged 0.2349-0.4227 (Table 3.5). Pairwise Fst and Rho values are shown in Table 3.6.

Bayesian Clustering. The average Ln probability of the data over five iterations was

-12766 for K=4, -12231 for K=5, -11650 for K=6 and -11667 for K=7. A value of K=6 consistently resulted in clusters corresponding to the morphological identification of individuals and very little admixture was observed across all iterations. Results from one of the five iterations are pictured in Figure 3.5. When K=4, each iteration resulted in a different grouping of individuals. *F. hahliana* individuals were grouped either with *F. hispidioides*, *F. congesta*, or *F. morobensis* and the latter three species grouped together at least once in all possible combinations. *Ficus pachyrrhachis* and *F. bernaysii* never grouped with another species. When K=5, one of two patterns was observed over the five iterations. All individuals identified as *F. hahliana* were grouped with *F. congesta* or all *F. hahliana* individuals grouped with *F. morobensis.* When K=7, one of two patterns was observed over the five iterations. Either the *F. hahliana* cluster was divided into two clusters or a seventh cluster was formed from 1-3 individuals from each species.

Hybrid identification. After re-extraction and genotyping of admixed individuals confirmed their genotypes, STRUCTURE identified seven individuals out of 300 as having a high posterior probability of hybrid ancestry (either F1 or F2) with an assumed migration rate of 0.10 or less (Table 3.7). Only two (MOR6 and PAC 51) of these seven individuals had a high posterior probability of hybrid ancestry at all tested migration rates (0.01, 0.05 and 0.10), of which only one (MOR6) was identified as a first generation hybrid. These same two individuals, plus an additional individual (MOR21) were identified as putative F1 hybrids by BayesAss analysis.

Migration/hybridization rates. Pairwise migration rates estimated in BayesAss were very low (Table 3.8). The highest estimated migration rates were from *F. pachyrrhachis* into *F. morobensis* at 0.41% (95% CI = <0. 01% - 2.26%), and from *F. morobensis* into *F. pachyrrhachis* at 0.64% (95% CI = <0. 01% - 2.50%). All other pairwise migration rate estimates were below 0.3% with upper bounds of 95% confidence intervals below 1.6%. The private allele method implemented in GENEPOP estimated 1.54 immigrants per generation among all species.

DISCUSSION

Reproductive consequences of pollinator sharing. Pollination experiments bypassing the mechanism by which pollinators are attracted to figs and gain access to flowers, resulted in hybridization. Comparable seed set in *F. hispidioides* from intraspecific crosses and crosses involving three of four close relatives provides evidence against post-pollination barriers to interspecific fertilization or embryogenesis. Although germination rates of hybrid and non-hybrid seed were similar and growth rates were comparable, survivorship of *F. morobensis* x *F. hispidioides* and *F. pachyrrhachis* x *F. hispidioides* was lower than non-hybrid seedlings. Bayesian clustering analysis of microsatellite genotypes suggested little admixture among sympatric species populations and that naturally occurring hybrids are few in number. Approximately 1-2% of the sampled genotypes could be regarded as putative hybrids assuming a liberal migration rate of 10%. With a more conservative migration rate of 1%, which aligns more closely with estimates from Bayesian and private allele methods, only two individuals out of 300 (0.67% of the sample) would be considered hybrids. That the majority of individuals were assigned to only one ancestral population with high probability and estimated migration rates were low suggest limited backcrossing and introgression. The frequency of introgression will depend to some extent on the abundance of hybrids reaching reproductive age, the attraction of pollinators to hybrids, and the reproductive success of pollinators visiting hybrid figs.

The only other study examining natural hybridization among dioecious figs also found hybrids to be rare (Parrish et al. 2003). In Krakatau, Indonesia, out 64 collections of putative parents, *Ficus septica* Burm. f., *Ficus hispida* L. f. and *Ficus fistulosa* ex. Blume, and 22 specifically sought out putative hybrid collections, only nine individuals with hybrid amplified fragment length polymorphisms (AFLPs) were found. The authors went on to suggest that, although hybrids appeared to be fertile, “hybridization should have little effect on species integrity”.

Although hybridization among dioecious figs appears to be rare (Ramirez 1994; Parrish et al. 2003), backcrossing and introgression could occur in theory and yet we found little evidence of gene flow among sympatric species populations. This situation may be explained by the fitness consequences for specialized pollinating wasps choosing novel or hybrid host figs.

Reproductive consequences of pollinator host choice. That cross-pollinating wasps induced gall development in a novel host, but offspring did not reach maturity suggests selection for host specificity. Although the physiological mechanism of abortion of cross-pollinated galls is unknown, the initiation of galls at least indicates that cross-pollinating wasps were capable of oviposition in the novel host. Though non-resident wasp larvae could not develop to maturity in *F. hispidioides*, gall formation was initiated in some proportion of all treatments. The chemosensory attraction and discriminatory behavior of wasps, therefore, only occurs prior to oviposition and indeed prior to entering a fig.   This makes sense in light of the fact that the antennal segments bearing sensillae are torn from the wasp's head in the process of entering the fig. Morphological mismatches between the wasp ovipositors and style lengths cannot explain why galls initiate but fail to mature (Weiblen 2004). Rather, some subsequent mechanism is the likely cause, such as the failure of larvae in a novel host to stimulate gall growth or feed upon the proliferating nucellus required for their diet.

Reproductive isolation of dioecious fig species. The primary reproductive isolating mechanism among sympatric dioecious figs appears to be the extreme species-specificity of pollinator host choice. The failure of pollinators to colonize a novel host suggests selection for species-specific recognition of suitable hosts. Even the newly emerged female resident pollinators *of F. hispidioides* refused to enter experimental figs through a Pasteur pipet, whereas females removed from receptive figs while in the act of pollination readily entered experimental figs. This suggests two important aspects of wasp behavior. First, attraction to fig flowers and the associated oviposition and pollination behaviors are conditional upon having passed through an ostiole. Second, after a wasp has passed through, it does not discriminate among host species. The extension of this observation is that the most important behavioral component of reproductive isolation among these dioecious figs occurs before pollinators enter the syconia. This idea is supported by the low number of non-resident wasps found within the syconia of these sympatric dioecious figs (Weiblen et al. 2001; Moe et al. 2011).

The role of volatile cues in pollinator host choice has been investigated experimentally (Ware et al. 1993; Ware and Compton 1994; Chen et al. 2009; Proffit et al. 2009). Studies have shown that fig volatile cues at receptivity are species-specific (Ware et al. 1993; Grison-Pige et al. 2002b; Proffit et al. 2009) and wasps are attracted to the volatile bouquet of particular hosts (Bronstein 1987; Ware and Compton 1994; Chen et al. 2009). Recently, Lu et al. (2009) found molecular evidence of selection on a gene influencing olfactory reception in *Ceratosolen solmsi*, the pollinator of dioecious, *Ficus hispida*. Species-specific chemical signals might serve to reinforce reproductive isolation among sympatric species whose hybrids are less fit. For example, in polyploid system *Heuchera grossulariifolia*, pollinator preferences for either diploids or tetraploids (Thompson and Merg 2008) and the divergence of flowering times (Nuismer and Cunningham 2005), are likely results of reinforcement-like processes that minimize pollen flow between plants that are genetically incompatible. Alternatively, pollinator behavior in response to variation in chemical signals may be one of few mechanisms maintaining species diversity among closely related sympatric species, which implies that pollinator specificity could potentially play a role in diversification, as was modeled by Kiester et al. (1984). This study determined that sympatric fig species can produce viable hybrid seed and hybrids can establish in nature, and therefore, we suggest that pollinator specificity in response to volatile chemical cues may play some role in the diversification of figs. However, estimates of hybrid fitness are needed to evaluate the importance of reinforcement processes in shaping pollinator behavior.

Hybridization and speciation. Although experimental evidence suggests pre-reproductive isolation among dioecious fig species and population genetic evidence suggests that hybrids are rare, pollinator sharing could potentially play a significant role in diversification. Rare events can have disproportionately large impacts on the evolution of systems (e.g. beneficial mutations). Rare hybridization events could lead to the establishment of new evolutionary lineages. Linnaeus was first to propose a model of speciation by hybridization (Linné 1774). Hybridization was recognized as a potential source of evolutionary diversification by botanists in the early 20th century. Much of extant plant diversity is proposed to be of polyploid origin (Stebbins 1950; Grant 1971). More recently, emphasis has been placed on the potential for homoploid hybrids to found new evolutionary lineages (Gross and Rieseberg 2005). Novel genetic combinations generated through occasional hybridization may allow hybrids to invade new ecological niches inaccessible to either parent (Arnold 1997; Rieseberg 1997; Gross and Rieseberg 2005). Strong pre-mating barriers to hybridization could then facilitate ecological divergence of a new hybrid lineage. However, few examples of homoploid hybrid speciation are known (Gallez and Gottlieb 1982; Rieseberg 1991; Arnold 1993; Wolfe et al. 1998; Brochmann et al. 2000). In only one system have the mechanism of ecological divergence been identified as pollinators (Wolfe et al. 1998). Although a handful of *Ficus* species are known to be 2n=52 tetraploids, the majority of species examined in cytological studies, including *F. congesta*, are 2n=26 diploids (Condit 1964; Löve 1969). In theory, resident pollinators might occasionally colonize rare diploid hybrids. If the volatile bouquet of the hybrid is sufficiently different from either parent, a colonizing lineage could be selected for specific attraction to the extent that pre-zygotic reproductive isolation evolves within a few generations.

Nonetheless, the results of this study would suggest that pollinator specificity has played a larger role than hybridization in the diversification of our focal group of dioecious figs. Until focal fig species with putative hybrid origins are identified, the relative importance of hybridization as a mechanism of fig diversification cannot be fully evaluated.

Considerations for coevolutionary studies. The last decade of work on fig pollination has contributed several valuable lessons with respect to the study of coevolution. Machado et al. (2005) critically reviewed host specificity in the fig-fig wasp system and called for examination of the long-held understanding of the assumption that fig pollination is a strictly species-specific mutualism. Most studies approached the question through DNA barcoding, phylogenetic and co-phylogenetic analyses (Haine et al. 2006; Marussich and Machado 2007; Anderson et al. 2008; Jackson et al. 2008; Jousselin et al. 2008; Su et al. 2008; Renoult et al. 2009; Azuma et al. 2010; Moe et al. 2011). Findings can be summarized with three points: (1) Multiple wasp species may pollinate the same fig species (Haine et al. 2006; Marussich and Machado 2007; Jackson et al. 2008; Lin et al. 2008; Peng et al. 2008; Su et al. 2008; Zhang et al. 2008; Compton et al. 2009). (2) Individual wasp species may pollinate multiple fig species (Molbo et al. 2003; Machado et al. 2005; Marussich and Machado 2007; Su et al. 2008). (3) Figs may hybridize (Parrish et al. 2003; Machado et al. 2005; Renoult et al. 2009).

These patterns invite explanation, but we must bear in mind that multiple processes can produce similar patterns and a particular process may result in diverse patterns (Irwin 2002; Revell et al. 2008; Cavender-Bares et al. 2009; Crisp and Cook 2009). Co-phylogenetic studies of the fig-fig wasp system have generated working hypotheses on host specificity and hybridization that require independent testing through other lines of inquiry, experimentation, and analysis.

Until now, evidence of gene flow among *Ficus* species has been interpreted as evidence of host switching and low pollinator specificity (Renoult et al. 2009). Our cross-pollination experiments point to a mechanism for gene flow among sympatric species populations that does not require the successful colonization of a new host or the evolution of a broad host range. This finding highlights the importance of examining ecological context and the reproductive consequences for both mutualistic partners, in concert with patterns of co-phylogeny and contemporary host-pollinator associations. Further, the results of our examination of pollinator sharing and fig hybridization can provide potential explanations for patterns examined in previous studies. Shallow incongruence among fig-fig wasp co-phylogenies (Weiblen and Bush 2002) could be explained by limited introgression among closely related and/or sympatric *Ficus* species (Machado et al. 2005). Introgression might also explain the difficulty resolving *Ficus* phylogenies (Ronsted et al. 2008a; Ronsted et al. 2008b; Silvieus et al. 2008). To our knowledge, hybridization among fig wasps has been observed directly in only one study (Molbo et al. 2004), in which there was no evidence of further introgression. If one mutualistic partner lineage has diversified by evolving reticulate processes and the other partner has not, we might question whether co-phylogenetic approaches to evaluating host specificity are informative. In any case, the extent of hybridization and its consequences for diversification in the fig system as a whole remains generally unclear.

The power of examining both mutualist partners is illustrated by recent work in the yucca moth and Joshua tree study system. Two yucca moth species with non overlapping ranges were discovered pollinating *Yucca brevifolia* (Pellmyr and Segraves 2003). A later study found morphological differences between Joshua trees pollinated by different moth species (Godsoe et al. 2008) and a recommendation was made to recognize tree types as different species (Lenz 2007). Although recent studies have reported the existence of multiple “cryptic” or previously undescribed fig wasp species pollinating a particular fig species (Lopez-Vaamonde et al. 2002; Molbo et al. 2003; Haine et al. 2006; Lin et al. 2008; Moe and Weiblen 2010), few studies have examined the genetic diversity of the corresponding figs. In previous work on our focal fig species, two “cryptic” sister species of fig wasp within *C. hooglandii* were identified, each showing a strong preference for one of two *F. bernaysii* "morphotypes" (Moe et al. 2011). Subsequent examination of *Ficus* type specimens indicated that the two "morphotypes" had been recognized and named as separate species. The similarity of the two species led Corner to note that *F. hahliana* may be a form or variety of *F. bernaysii* and that "field study will dispose of the problem" (Corner 1955). While field studies alone did not, molecular data has disposed of the problem. Our population genetic data shows that *F. bernaysii* and *F. hahliana* are genetically distinct entities (Figure 3.5) pollinated by genetically distinct wasps (Moe et al. 2011). Without careful molecular and morphological examination of specimens identified as *F. bernaysii* and *Ficus* type specimens, the discovery of its two “cryptic” pollinators would be considered yet another departure from the 1:1 model of species-specificity, when in fact the combined evidence point to pollinator specificity.

A challenge to coevolutionary studies is that simple pairwise interactions between organisms are complicated by webs of interactions (Bascompte and Jordano 2007; Gomez et al. 2011). The strength and direction of selection can change given different ecological contexts in which the interaction is found (Rudgers and Strauss 2004; Strauss and Irwin 2004), or different evolutionary histories (Dupas et al. 2009). The fig-wasp pollinator mutualism provides a unique opportunity to begin disentangling the effect of ecological, environmental and life history variables on the strength and direction of selection pressures on figs and their wasp pollinators. For example, the strength of selection for host specificity may differ between monoecious and dioecious lineages of *Ficus*. Pollinator sharing among multiple fig hosts appears to be less common in dioecious fig species than in monoecious fig species (Weiblen et al. 2001; Molbo et al. 2003; Machado et al. 2005; Lin et al. 2008; Su et al. 2008; Moe et al. 2011). Studies of co-phylogenetic patterns (Weiblen and Bush 2002; Machado et al. 2005; Ronsted et al. 2005; Marussich and Machado 2007; Jackson et al. 2008; Azuma et al. 2010) further suggest that host switching and hybridization may have played a larger role in the evolutionary history of monoecious lineages than in dioecious lineages. It has been suggested that the highly species-specific discriminatory behavior seen in pollinators of dioecious figs could be a by-product of selection on wasp for avoidance of female figs, a reproductive dead end for pollinators (Moe et al. 2011). Our study on a dioecious lineage has identified high host specificity as a reproductive isolating mechanism among sympatric figs in New Guinea. Similar studies on monoecious lineages may inform the questions of how breeding system may influence selection for host-specificity in the plant-pollinator mutualisms. The presence and variability of other interacting organisms, such as frugivore dispersers (Dumont et al. 2004; Lomascolo et al. 2010) and parasites (Elias et al. 2007; Dunn et al. 2008; Zhang et al. 2009), provide further opportunity to investigate the context dependence of host specificity in the fig-pollinator wasp mutualism.

Perhaps the most important implication of our findings is that patterns of host specificity and co-diversification in a system which has been long touted as a textbook example of coevolution need not necessarily be the result of reciprocal selection. The predominant 1:1 species association observed in the focal group (Weiblen et al. 2001; Moe et al. 2011) as well as patterns of codivergence in *Ficus* section *Sycomorus* (Weiblen and Bush 2002; Silvieus et al. 2008) can be explained by strong selection on the pollinating wasps, imposed by host figs, without selection on the host fig, imposed by the pollinating wasp. Reproductive isolation of the fig hosts is a product of highly specific wasp behavior, but as far as this study can discern, not a result of fitness differences among individual figs attracting conspecific pollinators or heterospecific pollinators. There may be fitness differences that this study is not able to detect, such as infertility of hybrids, or reduced attractiveness of hybrids to pollinators. However, in theory, these differences need not exist in order to explain the high specificity of pollinators, rarity of hybrids and co-diversification of fig and pollinating wasp species. The development of evolutionary models of unidirectional selection imposed by one organism on another, where lifecycles of the interacting organisms are interdependent is needed to evaluate whether a model of coevolution (or reciprocal selection) is needed to explain what we assume to be coevolutionary patterns in model systems like figs and their pollinating wasps.

CONCLUSIONS

This study identifies selection against cross-pollinating behavior in wasps as an important mechanism of reproductive isolation in a clade of sympatric dioecious figs. The finding provides an explanation for the low incidence of pollinator sharing among these fig species (Moe et al. 2011) and is consistent with a low number of hybrids in natural populations. Hybridization among the focal sympatric dioecious fig species appears to be extremely rare. Hybrid rarity can be attributed to the observed high specificity of pollinators, and not to inviability of hetero-specific pollination as experimentally produced hybrid seed developed, germinated and grew at rates comparable to non-hybrid seed. In combination, these results suggest that unidirectional selection could explain some patterns of host specificity and co-diversification that have been attributed to coevolution, (reciprocal selection).

However, this study does not examine selection on adult hybrids, which may play a role in maintaining species diversity. Predictive models are needed to incorporate information on fig community composition, pairwise levels of pollinator sharing, and the probability of hybrid establishment to test whether observed levels of pollinator sharing alone are sufficient to explain the observed frequency of hybrids. Additionally, information on pollinator visitation of natural hybrids and experiments examining the reproductive consequences of hybrid visitation for both fig and pollinators can independently test for selection against natural hybrids.

We suggest that hybridization among the dioecious fig species is rare and less important to the diversification of the focal group in comparison to processes leading to co-divergence. However, rare events can have evolutionarily significant effects. Renoult et al.'s 2009 study, in which *Ficus* phylogenies based on mitochondrial and nuclear DNA sequence were significantly incongruent, is the first evidence that hybridization may have played a role in *Ficus* diversification. Specific hypotheses regarding hybrid origins of focal *Ficus* lineages or species and their putative parent lineages should be tested through detailed examination of nuclear and mitochondrial discordance at species and population levels, cytological studies and direct experimentation.

TABLE 3.1. Microsatellite loci included in analyses. The total number and length of alleles observed across all species, PCR conditions: annealing temperature (T_a) and number of cycles , and the original primer note references are shown. * indicates number of touchdown cycles starting 10 ˚C above the annealing temperature.

Primer	No. of alleles	Length (bp)	T_a	No. of Cycles	Reference
FM3-64	9	267-291	54	10*+20	Zavodna et al. 2005
FM4-15	18	232-298	53	30	Zavodna et al. 2005
FS3-31	8	219-243	54	10*+20	Zavodna et al. 2005
FS4-11	11	279-357	54	10*+20	Zavodna et al. 2005
Frac86	10	141-183	50	15*+20	Crozier et al. 2007
Frub29	6	179-199	54	10*+20	Crozier et al. 2007
Frub38	25	172-132	50	15*+20	Crozier et al. 2007
Frub436	15	97-131	53	30	Crozier et al. 2007
B30	45	215-347	60	10*+20	Moe & Weiblen 2010
B47	16	171-219	53	30	Moe & Weiblen 2010
B83	16	165-195	53	30	Moe & Weiblen 2010
P164	18	227-288	60	10*+20	Moe & Weiblen 2010
P211	15	99-127	53	30	Moe & Weiblen 2010
P215	18	212-244	53	30	Moe & Weiblen 2010

TABLE 3.2. Experimental gall fig treatments. The species of pollinator introduced, the species of pollen carried by the pollinator, number of *F. hispidioides* figs treated, number of treated figs that initiated gall development, number of treated figs that produced mature wasp larvae, average % flowers galled per fig, and standard deviation of % flowers galled per fig. *values were calculated by Weiblen, Yu and West (2001). Control figs were treated for pollinator exclusion and received no pollinator introduction. Untreated figs received no treatment and were naturally pollinated, but were measured for growth.

introduced pollinator	pollen donor	n treated	n initiated	n matured	avg % flowers galled	SD % flowers galled
C. dentifer	*F. hispidioides*	90	51	25	10.55%	11.99%
C. notus	*F. congesta*	81	13	0	8.82%	17.00%
C. sp. ex *F. hahliana*	*F. hahliana*	36	2	0	2.09%	0.13%
C. sp. ex *F. morobensis*	*F. morobensis*	17	2	0	3.96%	2.60%
C. sp. ex *F. pachyrrhachis*	*F. pachyrrhachis*	124	44	0	6.09%	8.86%
control	none	62	1	0	59.00%	NA
total treated		410				
untreated	*F. hispidioides*	52	-	-	*52.30%	*19.23%

TABLE 3.3. Experimental seed fig treatments. The species of pollinator introduced, the species of pollen carried by the pollinator, number of *F. hispidioides* figs treated, number of treated figs that initiated seed development, number of treated figs that produced mature seed, average % flowers developed seed per fig, and standard deviation of % flowers developed seed per fig. *values were calculated by Weiblen, Yu and West (2001).

introduced pollinator	pollen donor	n treated	n initiated	n matured	avg % seed set	SD % seed set
C. dentifer	*F. hispidioides*	53	11	10	20.13%	13.50%
C. notus	*F. congesta*	44	4	4	22.00%	22.65%
C. sp. ex *F. hahliana*	*F. hahliana*	23	0	0	0.00%	0.00%
C. sp. ex *F. morobensis*	*F. morobensis*	16	1	1	14.20%	10.23%
C. sp. ex *F. pachyrrhachis*	*F. pachyrrhachis*	52	7	6	15.22%	7.93%
controls - pollinators excluded	none	28	1	1	19.32%	8.56%
total treated		216				
untreated – natural pollinator	*F. hispidioides*	21	-	-	*85.60%	*10.61%

TABLE 3.4. Germination and survival totals of hybrid and non-hybrid seeds. Totals presented are pooled over all experimental figs/replicates that developed seed.

Pollen donor	Total n seeds collected	Total n seeds germinated	Total n germinated seeds planted	Total n seedlings survived
F. congesta	177	115	82	36
F. hahliana	0	0	0	0
F. hispidioides	371	327	80	37
F. morobensis	59	37	46	3
F. pachyrrhachis	278	245	136	42

TABLE 3.5. Overall species differentiation. Focal species, number of individual trees sampled (n), number of sibling groups (n_s), mean Fst and Rho values across all loci, and loci that differ significantly from H-W equilibrium in each sample are shown.

Species	n	n_s	mean Fst	mean Rho	Heterozygote deficient loci
Ficus bernaysii	50	21	0.22	0.41	none
Ficus congesta	50	19	0.21	0.23	P164 ($p<0.001$)
Ficus hahliana	50	23	0.19	0.34	B47 ($p<0.001$)
Ficus hispidioides	50	19	0.21	0.28	P211 ($p<0.001$)
Ficus morobensis	50	18	0.19	0.30	none
Ficus pachyrrhachis	50	17	0.23	0.42	B83 ($p<0.001$)

TABLE 3.6. Pairwise species differentiation - Fst and Rho values.

Fst/Rho	*Ficus bernaysii*	*Ficus congesta*	*Ficus hahliana*	*Ficus hispidioides*	*Ficus morobensis*	*Ficus pachyrrhachis*
Ficus bernaysii		0.34	0.20	0.37	0.43	0.48
Ficus congesta	0.23		0.19	0.14	0.11	0.38
Ficus hahliana	0.45	0.20		0.34	0.19	0.52
Ficus hispidioides	0.22	0.19	0.13		0.30	0.27
Ficus morobensis	0.20	0.21	0.16	0.21		0.47
Ficus pachyrrhachis	0.24	0.22	0.26	0.28	0.17	

TABLE 3.7. Posterior probabilities of hybrid species assignment from Bayesian clustering analysis (K=6) using *a priori* species identity information with migration rate priors of 0.01, 0.05 and 0.10 are shown. Bold type values indicate the ancestry assignment with the highest posterior probability. Bold type individuals are most likely of hybrid ancestry at all tested migration rates. *indicates individuals identified as hybrids in BayesAss analysis.

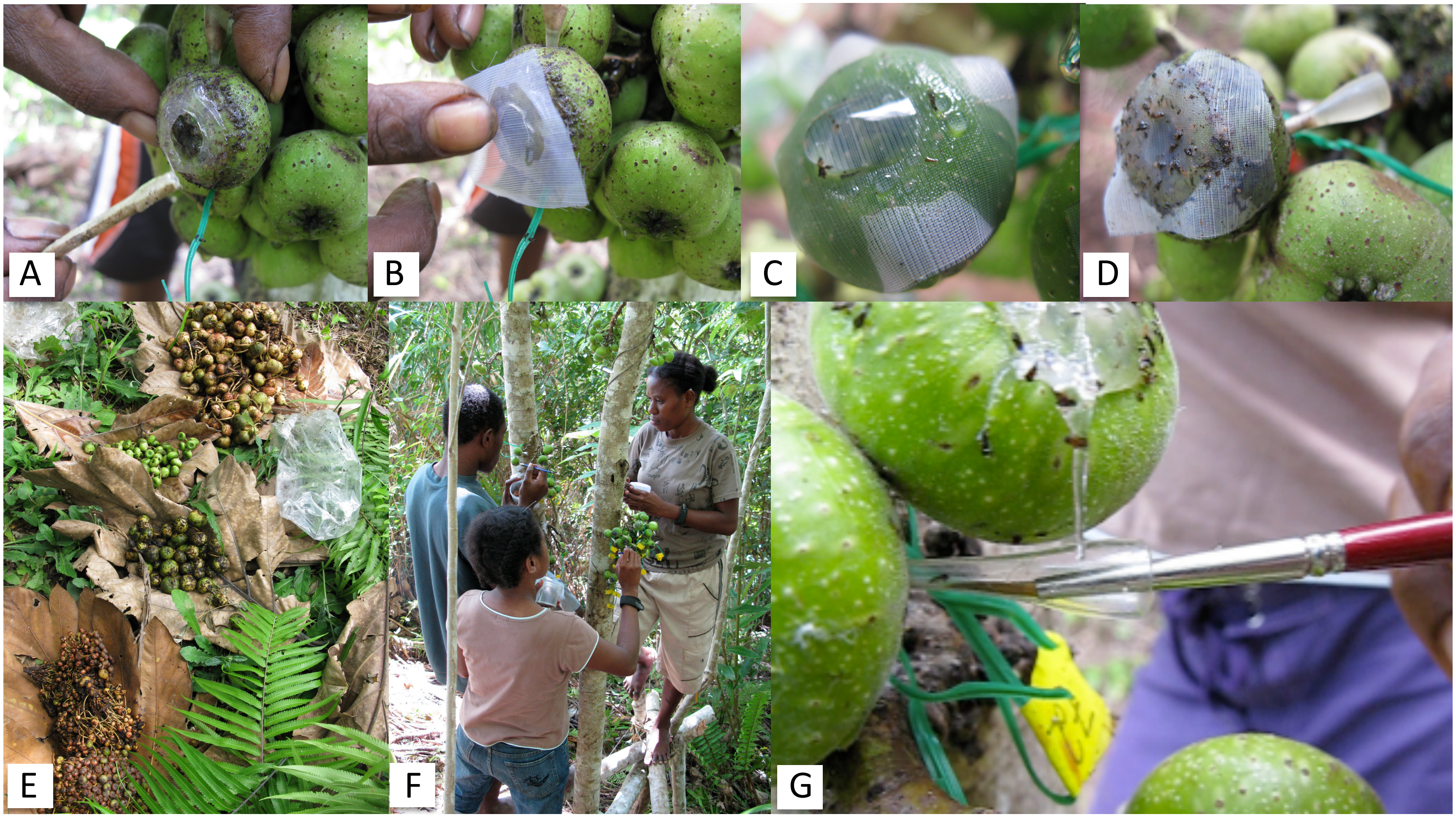
Individual	hybrid species assignment	migration rate	non-hybrid	F1 hybrid	F2 hybrid
BERA23	*Ficus hahliana* x *Ficus bernaysii* F2 hybrid	0.01	**0.931**	0.006	0.063
		0.05	0.**668**	0.027	0.305
		0.10	0.410	0.051	**0.536**
BERB16	*Ficus hahliana* x *Ficus pachyrrhachis* F2 hybrid	0.01	**0.507**	0.004	0.489
		0.05	0.145	0.007	**0.848**
		0.10	0.066	0.007	**0.927**
MOR6*	*Ficus morobensis* x *Ficus pachyrrhachis* F1 hybrid	0.01	0.027	**0.899**	0.074
		0.05	0.003	**0.926**	0.071
		0.10	0.001	**0.930**	0.069
MOR21*	*Ficus morobensis* x *Ficus pachyrrhachis* F2 hybrid	0.01	**0.920**	0.008	0.071
		0.05	**0.658**	0.034	0.307
		0.10	0.470	0.051	**0.521**
MOR52	*Ficus morobensis* x *Ficus hahliana* F2 hybrid	0.01	**0.933**	0.011	0.054
		0.05	**0.676**	0.057	0.267
		0.10	0.433	0.105	**0.462**
PAC24	*Ficus morobensis* x *Ficus pachyrrhachis* F2 hybrid	0.01	**0.784**	0.000	0.216
		0.05	0.386	0.001	**0.612**
		0.10	0.222	0.002	**0.776**
PAC51*	*Ficus morobensis* x *Ficus pachyrrhachis* F2 hybrid	0.01	0.452	0.003	**0.545**
		0.05	0.128	0.005	**0.867**
		0.10	0.062	0.005	**0.933**

TABLE 3.8. Pairwise migration rates between species. Means and (95% confidence intervals). Values <0.0001 are rounded to 0. Bolded values indicate the largest migration rates, having a mean greater than 0.4% and confidence interval upper bound greater than 2.0%.

FROM/INTO	*Ficus bernaysii*	*Ficus congesta*	*Ficus hahliana*	*Ficus hispidioides*	*Ficus morobensis*	*Ficus pachyrrhachis*
Ficus bernaysii		0.0015 (0-0.0108)	0.0012 (0-0.0087)	0.0011 (0-0.0070)	0.0018 (0-0.0105)	0.0019 (0-0.0117)
Ficus congesta	0.0015 (0-0.0115)		0.0013 (0-0.0093)	0.0013 (0-0.0086)	0.0020 (0-0.0124)	0.0017 (0-0.0101)
Ficus hahliana	0.0015 (0-0.0097)	0.0017 (0-0.0105)		0.0013 (0-0.0090)	0.0027 (0-0.0148)	0.0018 (0-0.0100)
Ficus hispidioides	0.0013 (0-0.0094)	0.0017 (0-0.0113)	0.0012 (0-0.0094)		0.0018 (0-0.0108)	0.0018 (0-0.0110)
Ficus morobensis	0.0015 (0-0.0095)	0.0024 (0-0.0157)	0.0013 (0-0.0097)	0.0013 (0-0.0090)		**0.0064 (0-0.0250)**
Ficus pachyrrhachis	0.0014 (0-0.0100)	0.0014 (0-0.0099)	0.0013 (0-0.0086)	0.0014 (0-0.0090)	**0.0041 (0-0.0226)**	

FIGURE 3.1. Controlled pollination experimental method. A-D Pre-receptivity treatment. Excludes natural pollinators and provides entrance for controlled pollinator introductions. E-G Receptivity treatment. Foundress wasps are collected from receptive figs of the focal species and inserted into experimental figs via a section of pasteur pipet.

FIGURE 3.1



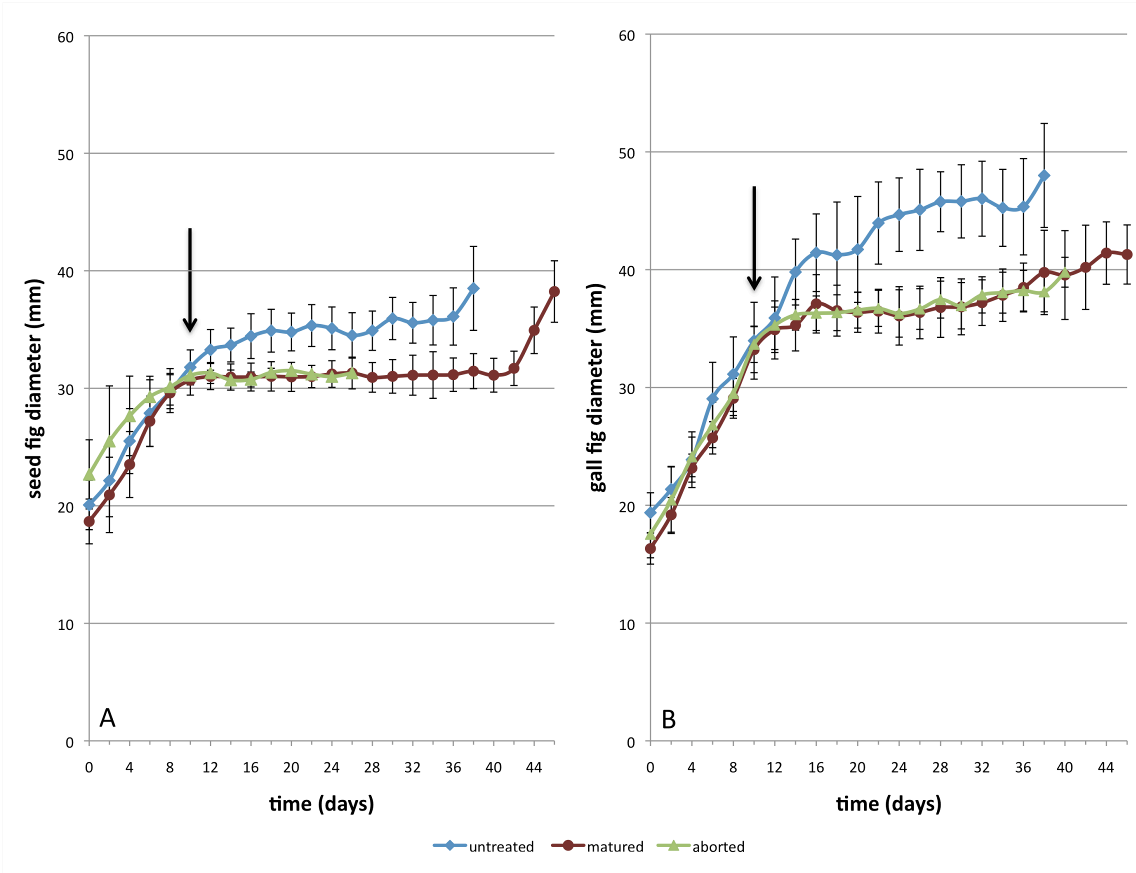
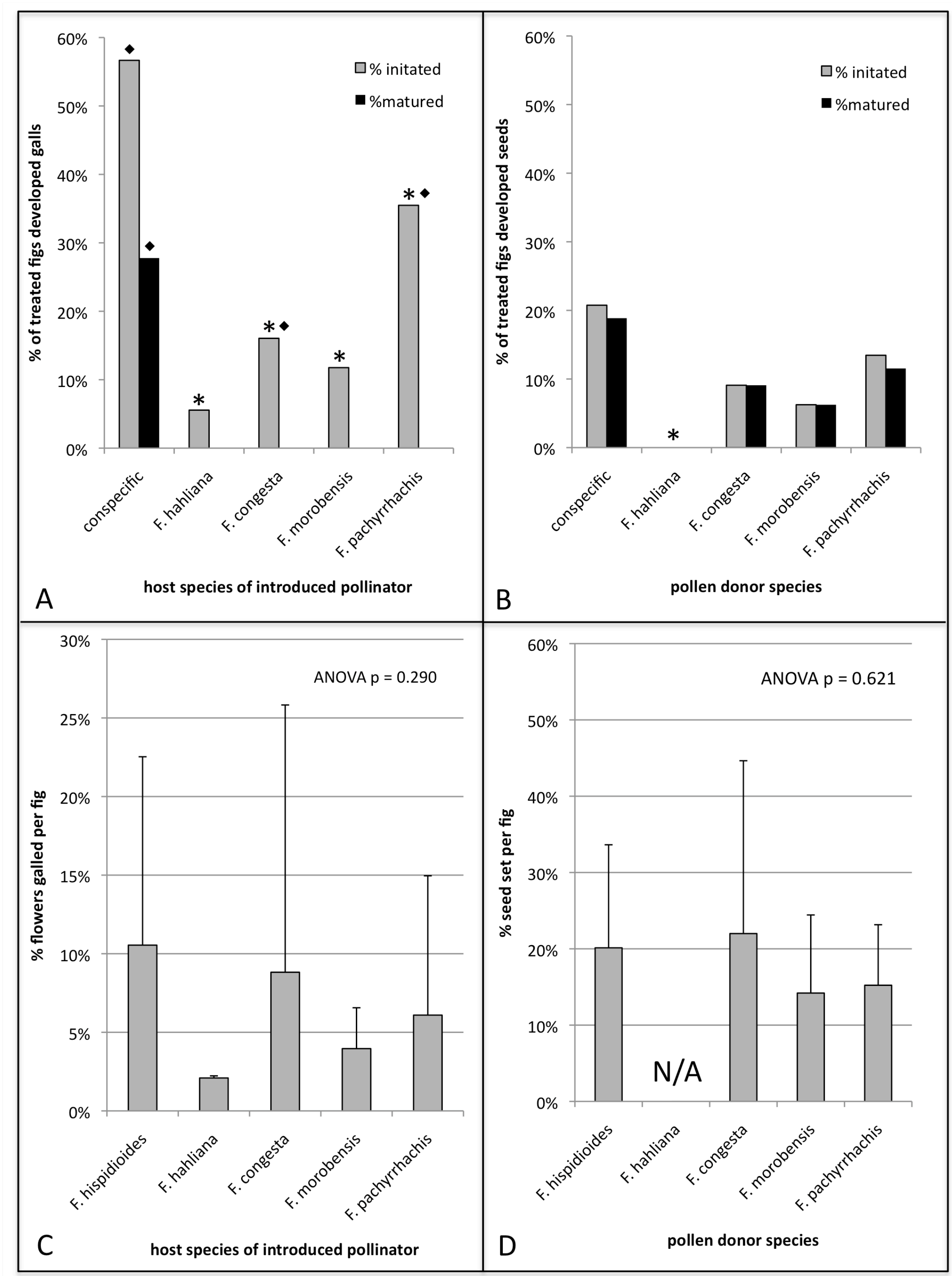


FIGURE 3.2. Fig development from (A) a single experimental female tree and (B) a single experimental male tree illustrates the treatment effect. Plots show the growth of untreated figs (triangles), and figs treated with *C. dentifer* that developed to maturity (circles) or aborted (squares). Arrows indicate the time at which figs were cored and pollinators introduced.

FIGURE 3.3. Gall and seed development in experimental figs. A) Total proportion of treated gall figs in which gall development initiated (gray bars) and galls matured (black bars) B) Total proportion of treated seed figs in which seed development initiated (gray) and seeds developed to maturity (black). C) Average percent of flowers that developed galls within a single fig. D) Average percent of flowers that developed seed within a single fig. Error bars are one standard deviation. * indicates significant difference from *F. hispidioides* pairwise 2x2 contingency test $p < 0.01$. w indicates a significant difference from the proportion of control figs (not shown) pairwise 2x2 contingency test $p<0.01$.

FIGURE 3.3.



60%
50%
40%
30%
20%
10%
0%
% initated
%matured
% of treated figs developed galls
conspecific
F. hahliana
F. congesta
F. morobensis
F. pachyrrhachis
A
host species of introduced pollinator
% of treated figs developed seeds
B
pollen donor species
ANOVA p = 0.290
30%
25%
15%
5%
% flowers galled per fig
F. hispidioides
C
ANOVA p = 0.621
% seed set per fig
N/A
D

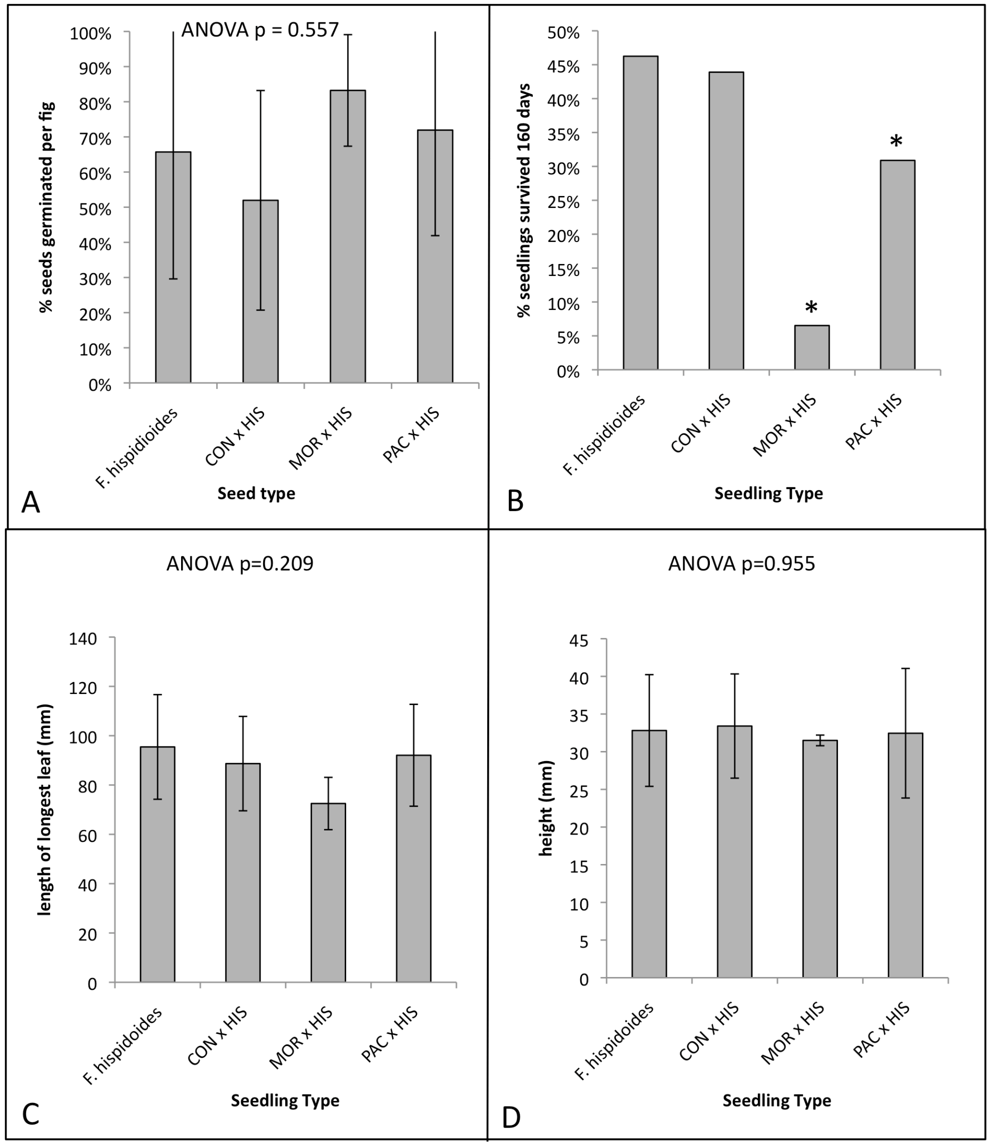


FIGURE 3.4. Seed germination, seedling survival and growth. A) Average proportion of seed that germinated from an individual fig. B) Proportion of seedlings that survived 160 days in a growth chamber. C) Average seedling height at 160 days after planting. D) Average length of the longest leaf at 160 days after planting. Error bars are one standard deviation. * indicates significant difference from *F. hispidioides* pairwise 2x2 contingency test $p < 0.05$.

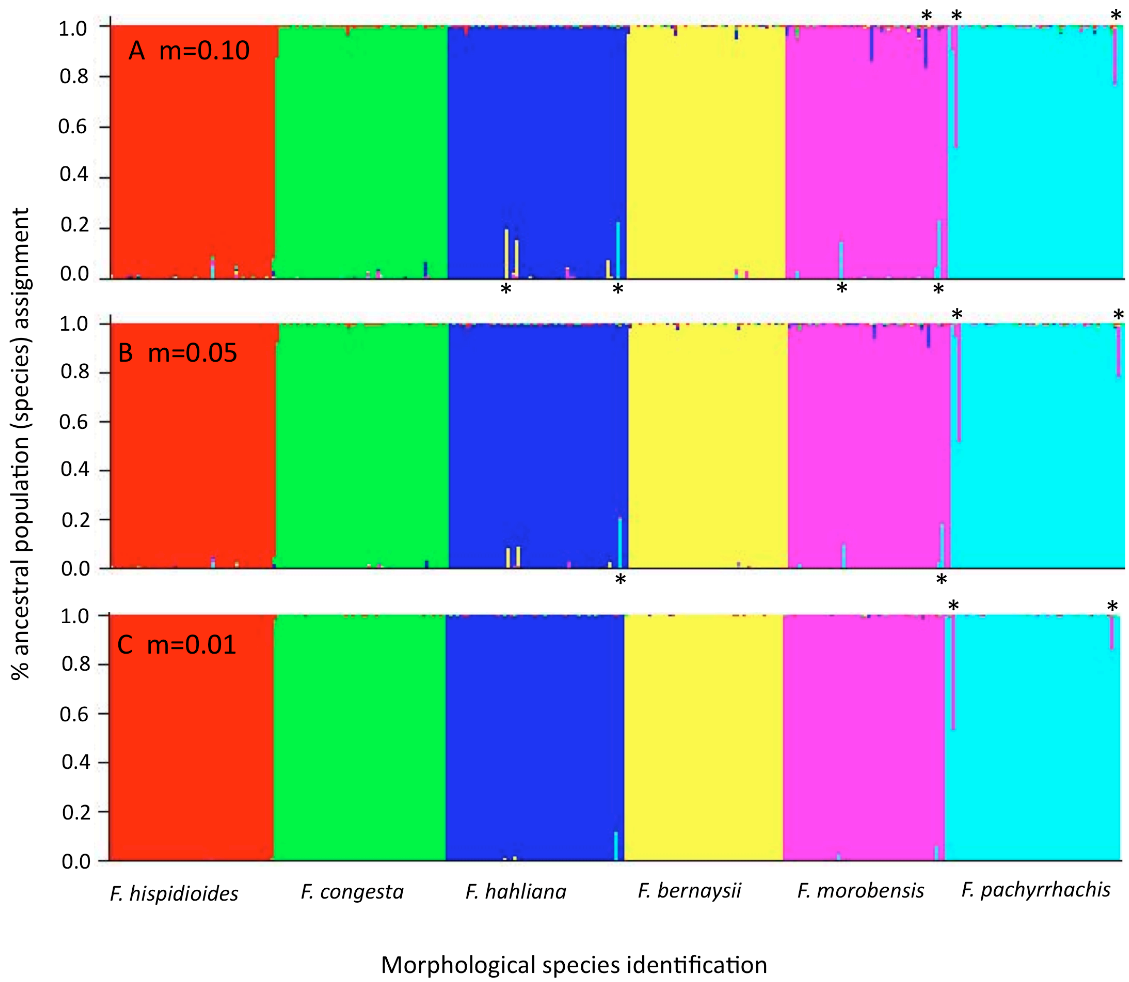


FIGURE 3.5. Barplots of ancestral population assignments for 300 individuals from Bayesian clustering analysis assuming six ancestral populations (K=6). Priors were used on population information based on morphological identification and an assumed migration rate if A) 0.10. B) 0.05 and C) 0.01 Asterisks indicate individuals identified with the highest probability as either first or second generation hybrids.

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APPENDICES

APPENDIX I: Development and characterization of microsatellite loci in dioecious figs (*Ficus*, Moraceae).

The genus *Ficus* (Moraceae), which contains over 750 species of tropical woody plants, is perhaps best known for its obligate mutualism with agaonid wasp pollinators. Molecular phylogenetic analyses have provided insight into evolutionary relationships among major *Ficus* lineages (Ronsted et al. 2008a), but relationships among recently diverged species remain obscure due to low sequence variation among close relatives (Ronsted et al. 2008b; Silvieus et al. 2008) and questions regarding hybridization among figs (Machado et al. 2005). The development and characterization of rapidly evolving markers such as microsatellites is needed to provide information on population genetics and species-level phylogeny in *Ficus*.

Here, we report the development and characterization of six microsatellite loci from *Ficus bernaysii* and *Ficus pachyrrhachis* (section *Sycocarpus*) and four microsatellite loci from *Ficus copiosa* (section *Sycidium*). These two sections represent the most diverse lineages of dioecious figs (Weiblen 2000).

METHODS AND RESULTS

Leaf tissue for the study was collected from 50 individuals each of five species in section *Sycocarpus* (*F. bernaysii* King, *F. congesta* Roxb., *F. hispidioides* S. Moore, *F. morobensis* C.C. Berg and *F. pachyrrhachis* Laut. & K. Schum.) and two species in section *Sycidium* (*F. copiosa* Steud. and *F. wassa* Roxb.) from populations located in a 4 km^2 area of lowland rain forest surrounding Ohu village in Madang Province, Papua New

Guinea (Lat. 5˚ 13’ 38” Long. 145˚ 40’ 44”). Leaf samples were dried in silica gel immediately upon collection and stored at -20 C until extraction. Specimen vouchers were deposited at the University of Minnesota Herbarium (Table A1.1). Genomic DNA was extracted from dried leaf tissue using the DNeasy Plant Mini Kit (QIAGEN). Microsatellite loci were isolated from a subset of the focal species, *F. copiosa*, *F. bernaysii* and *F. pachyrrhachis*, using the Dynabeads 2003 enrichment protocol of Glenn and Schable (2002). Genomic DNA of one individual per species was enriched using a set of five microsatellite motifs. *Ficus copiosa* and *F. pachyrrhachis* enrichments used Motif Set 1 [$AAAT_8$, $AACT_8$, $AAGT_8$, $ACAT_8$, $AGAT_8$] and the *F. bernaysii* enrichment used Motif Set 2 [AAC_6, ACT_{12}, AAT_{12}, ATC_8, AAG_8]. Enriched fragments were used as a template for amplification in a polymerase chain reaction (PCR) and PCR products were cloned using the TOPO TA cloning kit (Invitrogen) according to the manufacturer’s protocol. Bacterial colonies were amplified directly using M13 primers and screened on agarose gel. PCR products of positive clones were purified with Exonuclease I (New England Biolabs) and Shrimp Alkaline Phosphatase (SAP; US Biochemical Corp.) according to the Glenn and Schable protocol (Glenn and Schable 2005). DNA sequencing was performed with M13 primers using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing was performed on an ABI PRISM 3730 DNA Analyzer.

Based on 95 sequenced clones from *F. bernaysii*, primers were developed for seven loci containing eight or more perfect repeats imbedded in sufficient flanking sequence to allow for primer design. These were screened for scorability in 16 individuals of *F. bernaysii*. The three that amplified and could be consistently scored are reported here. Based on 150 sequenced clones from *F. pachyrrhachis*, primers were designed for seven loci containing eight or more perfect repeats imbedded in enough flanking sequence to allow for primer design. These were screened for scorability in 16 individuals of *F. pachyrrhachis* and the three that amplified are reported here. Of 215 sequenced clones from *F. copiosa*, primers were developed for seven loci containing eight or more perfect repeats imbedded in enough flanking sequence to allow for primer design. These were screened for scorability in 16 individuals of *F. copiosa* and the four that amplified are reported here. Primers were developed using Primer 3 (Rozen and Skaletsky 2000).

Loci were screened and scored in 50 individuals of each of five species in section *Sycocarpus* (*F. bernaysii, F. congesta, F. hispidioides, F. morobensis* and *F. pachyrrhachis*) and two species in section *Sycidium* (*F. copiosa* and *F. wassa*). Amplification of microsatellite loci was performed on an Eppendorf Mastercycler in a total volume of 10 µL using 0.2 mM fluorescent end-labeled forward primer and unlabeled reverse primer, 0.2 mM buffer solution, 0.2 mM of each dNTP, 0.8 mM BSA, 0.3 units of TaKaRa Ex Taq polymerase (TAKARA BIO inc.) and 20-50 ng template DNA. One of three sets of PCR conditions was used for each microsatellite locus. The first PCR condition included initial denaturing at 94 C (4 min) followed by 30 cycles of 94 C (30 s), 53 C (30 s), 72 C (45 s), and a final elongation step of 72 C (10 min). Condition 2 was a touchdown protocol with initial denaturing at 95 C (5 min) followed by a 10 cycle touchdown of 95 C (15 s), 64 C-54 C (30 s) and 72 C (30 s), 20 additional cycles at a 54 C annealing temperature, followed by a final elongation step of 72 C (10 min). Condition 3 was a touchdown protocol with a higher beginning with an annealing temperature initial denaturing at 95 C (5 min) followed by a 15 cycle touchdown of 95 C (15 s), 75 C-60 C (30 s) and 72 C (30 s), 20 additional cycles at a 60 C annealing temperature, followed by a final elongation step of 72 C (10 min). Microsatellite alleles were visualized using an ABI 377 Sequencer along with a ROX 500 (ABI) size standard and scored using Genotyper 2.5 (ABI).

GENEPOP on the web (Raymond and Rousset 1995) was used to calculate number of alleles (N_a), observed and expected heterozygosity (H_o and H_e), and to test for Hardy-Weinberg equilibrium and linkage disequilibrium. The presence of sibling groups was tested using kinship analysis implemented in Kinalyzer (Ashley et al. 2009) using a broader data set of 13-14 microsatellite loci including those reported here and previously published loci (Zavodna et al. 2005; Crozier et al. 2007). Results reported in Tables A1.2 and A1.3 are based on samples of 50 individuals from single populations of each species.

Primers developed in *F. bernaysii* amplified 6-21 alleles per species in *Sycocarpus* and expected heterozygosity ranged from 0.53-0.93. Whereas, primers developed in *F. pachyrrhachis* amplified 4-13 alleles per species from section *Sycocarpus* and expected heterozygosity ranged from 0.25-0.86 (Table A1.3). Primers developed in *F. copiosa* amplified 5-15 alleles per species from section *Sycidium* and expected heterozygosity ranged from 0.68-0.87 (Table A1.3). The low heterozygosity observed in all species was due mainly to sampling of sibling groups identified in kinship analysis (Moe unpubl. data). After the removal of siblings from the analysis, populations were in Hardy-Weinberg equilibrium at most loci and had no significant evidence of linkage disequilibrium. Exceptions to Hardy-Weinberg equilibrium were most likely due to null alleles and are marked with asterisks in Table A1.3. Microsatellite loci B30, B47 and P164 also amplified in *Sycidium* species and all loci developed in *F. copiosa* (C244, C246, C281, C410) amplified in *Sycocarpus* species, but loci had low or no variability in these cross-amplifications.

CONCLUSIONS

These new microsatellite loci are highly variable within two dioecious *Ficus* sections and cross-amplify in closely related species. Several loci amplified across *Ficus* sections, but variability was reduced. Therefore, these markers may be useful for examining genetic structure within species, detecting gene flow among closely related species, and determining evolutionary relationships among recently diverged species in this ecologically important genus.

TABLE A1.1. Voucher information for taxa used in microsatellite development. All voucher specimens are deposited in MIN.

Species – Country and Locality, Accession number
Ficus bernaysii King – Papua New Guinea, Madang Province, Ohu, 920562
Ficus congesta Roxb. – Papua New Guinea, Madang Province, Ohu, 920560
Ficus copiosa Steud. – Papua New Guinea, Madang Province, Ohu, 920559
Ficus hispidioides Moore – Papua New Guinea, Madang Province, Ohu, 920557
Ficus morobensis C.C. Berg – Papua New Guinea, Madang Province, Ohu, 920561
Ficus pachyrrhachis Laut. et K. Schum – Papua New Guinea, Madang Province, Ohu, 920556
Ficus wassa Roxb. – Papua New Guinea, Madang Province, Ohu, 920558

TABLE A1.2. Microsatellite loci developed in *Ficus bernaysii* (B30, B47 and B83), *Ficus pachyrrhachis* (P164, P211 and P215) and *F. copiosa* (C244, C246, C281 and C410), primer sequences, repeat motifs, size ranges, PCR conditions, annealing temperatures and GenBank accession numbers.

Primer	Sequence (5’-3’)	Repeat motif	Size	PCR	T_a	GenBank
B30	F:TTAATTTGGCCCTGACCTTG R:CGGCGCAAATGATTCTTAAT	$(TCT)_{15}$	215-347	3	60	HQ323652
B47	F:TTTTTGTCTGGTTTTGGGTGT R:CACAATCCCCACATGATGAA	$(GAA)_{13}$	171-219	1	53	HQ323653
B83	F:CCCACCTAAAGCTGCCAATA R:TCTCCCCTTTACCCCTTTTT	$(AG)_{15}$	165-195	1	53	HQ323654
P164	F:ATCAAATCCCCACATTCCAA R:GTAGCTTGGGAGTGGAAGCA	$(CT)_{11}$	227-288	3	60	HQ323655
P211	F:CCCGTTGGAGAAATTCAAAA R:AGAATCACCGCCTTCGATTT	$(GA)_4 \ldots (GA)_{15}$	99-127	1	53	HQ323656
P215	F:ACCCCCATCATCTACTCGTG R:AACCCCATCAACAAAGAAGC	$(ATGT)_{10}$	212-244	1	53	HQ323657
C244	F:GAAGGGATTGCTCAGGCATA R:TGGGACCCACTCTTACTTGTG	$(GA)_{12}$	219-249	3	60	HQ323658
C246	F:TATCGGGAGATGGAGAGTGG R:CAAAAAGCTTCTTGAGAAACA	$(TA)_3(CATA)_{12}(TA)_3$	222-270	1	53	HQ323659

TABLE A1.2 CONTINUED

Primer	Sequence (5'-3')	Repeat motif	Size	PCR	T_a	GenBank
C281	F:ACTGTCAACTTTGAATAGAGA R:GTGACGGGTCATGCTATCCT	$(GA)_{13}$	239-261	2	54	HQ323660
C410	F:CAGCGGTTGAGATTCTAGGC R:TTCCTCCACTAACTTTTCATGTG	(GAA)12	218-269	2	54	HQ323661

TABLE A1.3. Results of initial primer screening based on a sample of 50 individuals from each species. The number of observed alleles (N_a), expected heterozygosity (H_e), and observed heterozygosity (H_o) for five species of *Ficus* section *Sycocarpus* and two species of *Ficus* section *Sycidium* are shown. Observed heterozygosity marked with an asterisk indicates significant deviation from H-W equilibrium after sibling groups were removed.

Primer	$N_a/H_e/H_o$						
	F. bernaysii	*F. congesta*	*F. hispidioides*	*F. morobensis*	*F. pachyrrhachis*	*F. copiosa*	*F. wassa*
B30	17/0.81/0.31*	14/0.91/0.50	21/0.93/0.36*	10/0.74/0.33	10/0.62/0.38		
B47	6/0.53/0.22*	8/0.76/0.74	6/0.73/0.72	7/0.81/0.81	8/0.76/0.79		
B83	11/0.90/0.70	12/0.80/0.74	10/0.68/0.48	10/0.84/0.77	10/0.74/0.40*		
P164	12/0.86/0.82	6/0.77/0.54*	7/0.48/0.46	7/0.66/0.79	4/0.66/0.75		
P211	10/0.66/0.66	6/0.61/0.50	13/0.82/0.68*	8/0.73/0.69	5/0.25/0.12		
P215	9/0.75/0.62	9/0.80/0.76	8/0.75/0.70	9/0.83/0.85	10/0.82/0.79		
C244						11/0.81/0.76	15/0.87/0.86
C246						13/0.86/0.90	9/0.74/0.53
C281						10/0.79/0.65	11/0.87/0.82
C410						5/0.68/0.37	15/0.76/0.37*

APPENDIX II: Y-tube olfactometer pollinator choice experiments

In lowland tropical forests of New Guinea, extreme fig diversity can be found in sympatry. Focal species of subgenus *Sycomorus*, section *Sycocarpus*, *F. hispidioides, F. morobensis, F. congesta, and F. pachyrrhachis,* are all found in sympatry and are closely related (Silvieus 2007). The agaonid pollinators of these species, *Ceratosolen dentifer*, *C.* sp. ex *F. morobensis, C. notus* and *C.* sp. ex *F. pachyrrhachis* respectively, are also closely related (Silvieus 2007). In chapter 2, about 1-2% of sampled pollinators were found within the syconia of a fig species other than their most common host. In chapter 3, these closely related *Sycocarpus* figs were found to be genetically distinct. The combination of these two lines of evidence indicate that pre-pollination reproductive isolating mechanisms play an important role in maintaining species integrity despite occasional gene flow. What is still unclear is whether specific attraction to divergent volatile chemical cues is responsible for reproductive isolation, or if specific morphological match between wasp morphology and fig ostiole morphology is responsible for “filtering” out non-specific pollinators.

Volatile chemical cues are known to play an important role in pollination ecology and can promote short term or long term specialization of pollinators through unique chemicals or combinations of chemicals (Raguso 2008). Previous studies of fig volatile chemicals have found that bouquets are specific to each *Ficus* species (Ware et al. 1993; Grison et al. 1999; Grison-Pige et al. 2002b; Proffit et al. 2009). Biochemical assays have shown that single compounds within the Ficus volatile bouquet may be responsible for species specific attraction of fig wasps (Chen et al. 2009). Additional studies have demonstrated that agaonid wasp pollinators are attracted to volatile bouquets of receptive figs (Hossaert-McKey et al. 1994; Song et al. 2001; Chen and Song 2008). A couple of studies using sticky traps have shown that pollinators are preferentially attracted to the volatile bouquet of a single fig species (Bronstein 1987; van Noort et al. 1989; Ware and Compton 1994). Wasp stimulation (antennation) by extracted fig volatile were also found to be species specific (Grison-Pige et al. 2002a). To my knowledge, only one study has used Y-tube olfactometer experiments to assess the preference of *Ceratosolen gravelyi* Grandi pollinators of three sympatric species of fig from subgenus Sycomorus (Chen et al. 2009). This study used isolated volatile compounds and found that *C. gravelyi* had a significant preference for the volatile bouquet of its recognized host fig *Ficus semicordata* Buch.-Ham ex Sm.

Similarly, this study uses y-tube olfactometer experiments to examine pollinator attraction to receptive figs of several closely related *Ficus* species.

METHODS

Receptive figs of *Sycocarpus* focal species, *F. hispidioides, F. morobensis, F. congesta, and F. pachyrrhachis* were collected and placed in odor-free polythene plastic bags with a carbon filter secured to the opening of the bag. A small hole is poked in the opposite end of the bag and an air-tight plastic connector connects the bag to a glass y-tube apparatus (see Figure A2.1). An empty polythene bag is set up in the same manner and connected to the second arm of the y-tube apparatus. Air is pulled through the y-tube by a small aquarium pump and the air flow kept at 0.2 L per minute using a flow meter. Light was regulated by placing the y-tube in a dark wooden box with identical lights aimed down the two arms of the y-tube. The y-tube apparatus was placed at a 20 degree incline within the wooden box. The set up was based on the personal observation that fig wasps prefer to walk upwards and toward light sources. These conditions would direct a fig wasp to the junction of the y-tube at which it must make a decision to walk toward or away from a fig volatile bouquet.

Ripe male figs were collected and placed in a plastic container, covered by fine mesh. When young pollinators emerged from the figs, they were introduced one at a time through a 3 mm diameter hole in the stem of the y-tube. The hole was then stopped with a small cork. The internal diameter of the y-tube was 5 mm, small enough that the wasp was unable to fly and forced to walk up the 10 cm stem of the y-tube to the junction. Once a wasp had passed a "finish line", marked 5 cm up one of the two arms of the y-tube, a decision was recorded as well as the time taken to reach the decision. If a wasp had not made a decision after five minutes, it was removed from the y-tube and a new wasp was introduced. Between each trial, the y-tube was washed with acetone and air-dried before introducing the next wasp. After a set of five trials, the bags attached to the ends of the y-tubes were switched to correct for any directional bias in the set up.

One hundred trials were performed on each species of pollinator to test for attraction, avoidance, or indifference to the volatile bouquet of the *Ficus* species from which they were reared. Additionally, one hundred trials were performed on pollinators reared from *F. morobensis*, *F. congesta* and *F. pachyrrhachis* to test for attraction, avoidance, or indifference to the volatile bouquet of *F. hispidioides*, a species other than their birth fig.

A chi square test was performed for each set of 100 trials with a null expectation of 1:1 decision ratio to walk toward or away from a volatile bouquet. An insignificant chi-square value indicated indifference to the volatile bouquet. A significantly high number of decisions to walk away from the bouquet indicated repulsion and a significantly high number of decisions to walk toward the volatile bouquet indicated attraction.

RESULTS

Results from the y-tube experiments (Table A2.1) showed that only *C. dentifer* was significantly attracted to the volatile bouquet of its birth fig, *F. hispidioides* (p=0.0014). *Ceratosolen* sp. ex *F. pachyrrhachis* showed significant avoidance of the volatile bouquet form its birth fig, *F. pachyrrhachis* (p=0.0455). Both *C.* sp. ex *F. morobensis* and *C. notus* demonstrated indifference to the volatile bouquets of their birth figs, *F. morobensis* and *F. congesta* respectively. *Ceratosolen notus* and *C.* sp. ex *F. pachyrrhachis* showed significant avoidance of the *F. hispidioides* volatile bouquet (p=0.0278 and 0.0027 respectively), *C.* sp. ex *F. morobensis* showed significant attraction to the *F. hispidioides* volatile bouquet (p=0.0093).

DISCUSSION AND CONCLUSION

Considering previous studies finding specific attraction of agaonid pollinators to the volatile cues of their host fig species (Bronstein 1987; Ware and Compton 1994; Grison-Pige et al. 2002a; Chen et al. 2009) and high levels of host specificity documented in chapter 2, we would expect pollinators to be attracted to the receptive figs of the same species as their birth fig. However, we observed attraction in only one of four species, and even observed avoidance of *F. pachyrrhachis* by its pollinator. The failure of our methods to detect consistent, species-specific attraction may be due to the removal of figs from trees. We suspect that wasps are repelled by fresh latex from wounded figs, which has been observed in pollinators of *Ficus pungens* (Moe unpublished). Conflicting volatile signals might have affected wasp behavior. Many of the wasps used in trials refused to make a choice in under five minutes and many moved back and forth between the two arms of the y-tube without moving past a "finish line". The experiments might need to be repeated using figs still attached to trees, which may prove logistically difficult. Y-tube experiments involving whole figs may not be the most appropriate test of pollinator attraction in the field. Isolation of volatiles from undisturbed receptive figs and subsequent y-tube experiments in using only the volatile extracts might result in more consistent pollinator behavior.

TABLE A2.1. Chi square results from y-tube experiments. *indicates significant attraction to a volatile bouquet †indicates significant avoidance of a volatile bouquet.

wasp species tested	Wasp reared from	toward birth fig species	away	Chi square	p value
C. dentifer	*F. hispidioides*	66	34	10.24	0.0014*
C. notus	*F. congesta*	48	52	0.16	0.6892
C. sp. ex *F. pachyrrhachis*	*F. pachyrrhachis*	40	60	4	0.0455†
C. sp. ex *F. morobensis*	*F. morobensis*	55	45	1	0.3173

wasp species tested	Wasp reared from	toward *F. hispidioides*	away	Chi square	p value
C. notus	*F. congesta*	35	65	9	0.0027†
C. sp. ex *F. pachyrrhachis*	*F. pachyrrhachis*	39	61	4.84	0.0278†
C. sp. ex *F. morobensis*	*F. morobensis*	63	37	6.76	0.0093*

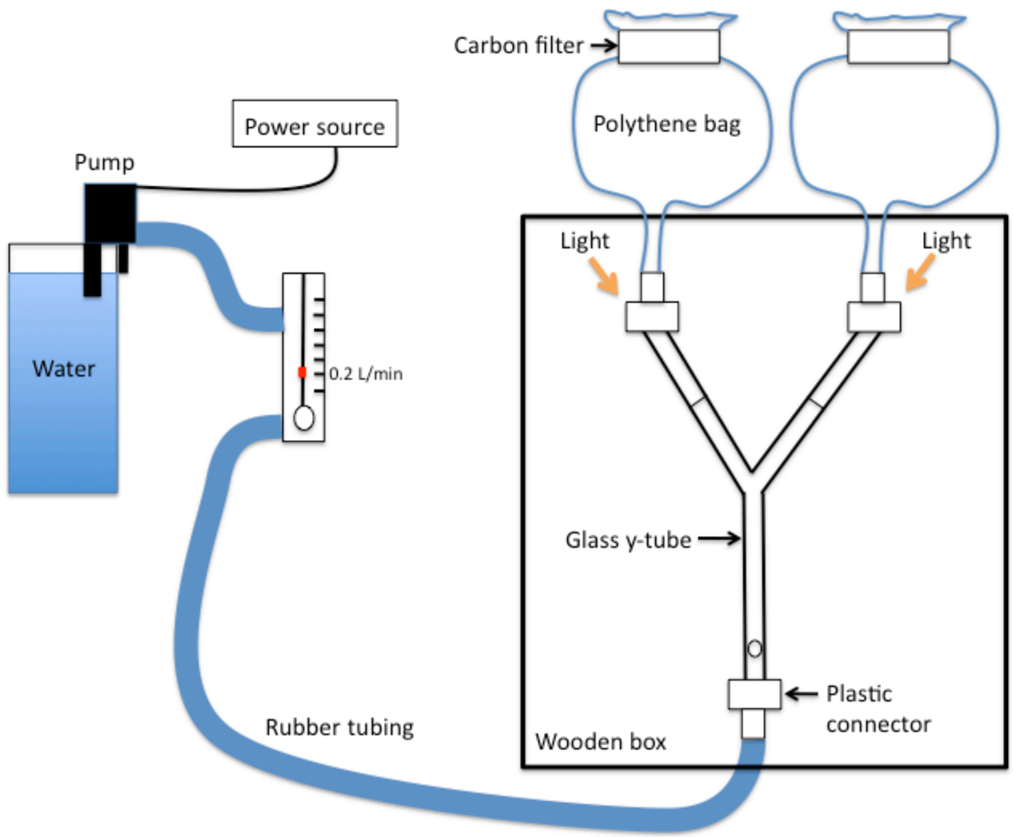

FIGURE A2.1. Experimental set-up of y-tube olfactometer experiments.

APPENDIX III. Map of collection area and sampled Ficus individuals. Ohu village, Madang district, Madang Province, Papua New Guinea. (Lat. 5˚ 13' 38" Long. 145˚ 40' 44").