Molecular Divergence in Allopatric *Ceratosolen* (Agaonidae) Pollinators of Geographically Widespread *Ficus* (Moraceae) Species

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Ann. Entomol. Soc. Am. 103(6): 1025–1037 (2010); DOI: 10.1603/AN10083

ABSTRACT Speciation in pollinating seed predators such as fig wasps (Hymenoptera: Agaonidae) is likely to have been influenced by a combination of ecological and geographical isolating mechanisms, but recent molecular analyses of fig wasps have focused on pollinator specialization as the main factor driving speciation. This study investigates the contribution of geographic modes of speciation such as dispersal, vicariance, and isolation by distance. We sampled haplotypes of mitochondrial cytochrome oxidase I from *Ceratosolen* pollinators of six geographically widespread Australasian fig (Moraceae: *Ficus*) species, including four species spanning Wallacea. Phylogenetic analysis investigated the extent of host conservatism and host switching accompanying divergence in *Ceratosolen*. Geographically widespread *Ceratosolen* showed deep intraspecific divergence exceeding or comparable to divergence between named sister species. Maximum parsimony and Bayesian analyses supported species monophyly in five of six cases, whereas results for a sixth species were equivocal. Bayesian divergence time estimation suggested dispersal across Wallacea during the Miocene epoch, after the collision of Australian and Asian continental plates. Cryptic species were evident in all six focal taxa. Because the deep mitochondrial divergence within these taxa is regionally distributed, allopatric divergence provides a simple explanation for the existence of these cryptic lineages pollinating widespread fig species. We found little evidence of divergence accompanied by host switching. The ancient origin of cryptic and geographically isolated species suggests that long-distance dispersal may be rare in *Ceratosolen* and that host associations are generally conserved during range expansion.

KEY WORDS phylogeography, Wallacea, mitochondrial DNA

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 cospeciation 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. 2004a,b; 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), provides 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

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0013-8746/10/1025–1037$04.00/0 © 2010 Entomological Society of America

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, also could produce such a pattern. Phylogeographic data are needed to clarify 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 *Ficus rubiginosa* Desf. ex Vent. in Australia but no evidence of host switching. Su et al. (2008) found a different situation in Mexico, where cryptic wasp species pollinating *Ficus petiolaris* Kunth did not form a monophyletic group, suggesting repeated and independent colonization of the host by different pollinator lineages.

Unequal dispersal rates 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 (Kerdelhue 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 with 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 (Fig. 1). The two plates came into contact ≈50 mya and brought distinct flora and fauna into 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 breakup 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, range expansion, or both.

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 nonmonophyly, 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). We also included a pair of sister species, *Ceratosolen pygmaeus* Grandi and *Ceratosolen nanus* Wiebes, 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 nonoverlapping regions of Wallacea (Berg and Corner 2005) to divergence within species distributed across Wallacea. In addition, geographically isolated populations of *Ceratosolen abnormis* Wiebes, a New Guinea endemic, were sampled 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 before 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 (University of Minnesota, St. Paul, MN).

In addition to *Ceratosolen* pollinators of six widespread *Ficus* species (Table 2), phylogenetic analyses included cytochrome oxidase I (COI) sequences from 32 *Ceratosolen* species (Table 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

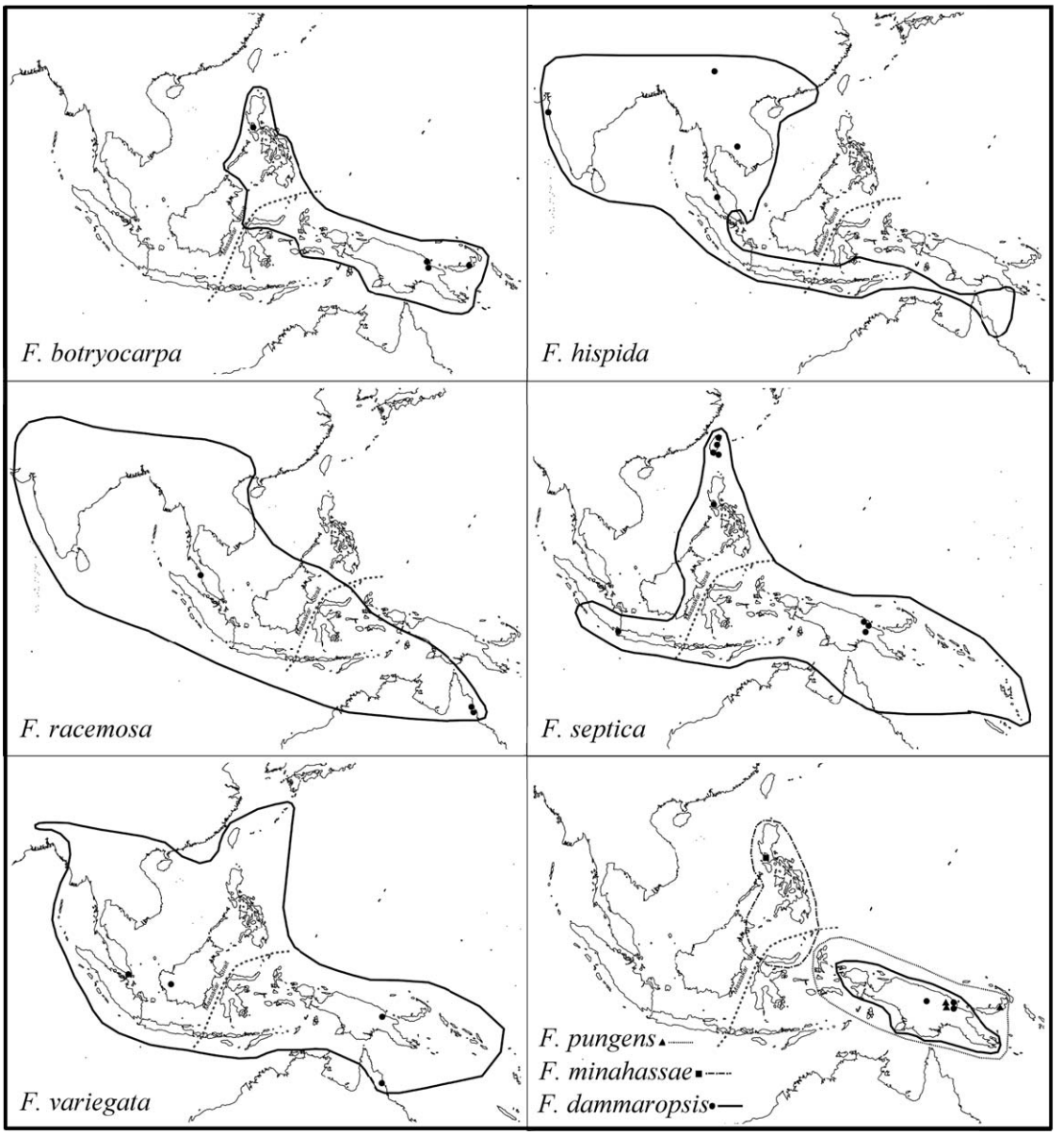


Fig. 1. Maps of Wallacea illustrating geographic distributions of focal *Ficus* species and sampling localities. The Wallace Line (dashed) is a major biogeographical transition zone that marks the contact of the Sunda shelf (Borneo, Java, and mainland Asia) to the Sahul shelf, including New Guinea and Australia. Geographic ranges (solid lines) and sampling localities (circles) are shown for five widespread taxa (*F. botryocarpa*, *F. hispida*, *F. septica*, *F. racemosa*, and *Ficus variegata* Blume). The map on the lower right map illustrates distributions of three locally endemic taxa sampled for comparison with widespread species: *Ficus dammaropsis* Diels (solid line and circles), *Ficus minahassae* (Teijsm. & de Vriese) Miq. (dashed line and square), and *Ficus pungens* Reinw. ex Blume (dotted line and triangles).

pollinators of widespread hosts are not monophyletic. In addition, outgroup sequences were obtained from GenBank (Table 4). For distance, parsimony and Bayesian analyses, the outgroup included two *Kradibia* species, sister group to *Ceratosolen* (Cruaud et al. 2010). In the case of divergence time estimation, the outgroup was expanded to include representative sequences from 14 genera of pollinating fig wasps to improve temporal calibration of the phylogeny.

DNA Extraction, Polymerase Chain Reaction (PCR), and Sequencing. Sequences of 410–801 bp of COI were obtained directly from individual wasps or from GenBank accessions. DNA was extracted using a DNeasy tissue kit (QIAGEN, Valencia, CA). We amplified ≈500 bp of mitochondrial COI by using primers SW2618 and Pat (Simon et al. 1994, Machado 1998). Amplification was performed on a Mastercycler thermocycler (Eppendorf North America, New York, NY)

Table 1. Focal *Ceratosolen* species and localities sampled

Pollinator	Host	Host section	Samples	Haplotypes	Localities[a]
C. corneri	*F. botryocarpa*	*Sycocarpus*	10	9	New Guinea (8), New Britain (1), Philippines (1)
C. bisulcatus	*F. septica*	*Sycocarpus*	14	14	New Guinea (5), Indonesia (2), Taiwan (6), Philippines (1)
C. solmsi	*F. hispida*	*Sycocarpus*	5	4	Cambodia (1), Malaysia (1), India (2), China (1)
C. appendiculatus	*F. variegata*	*Sycomorus*	10	5	New Guinea (6), Singapore (2), Indonesia (1), Australia (1)
C. fusciceps	*F. racemosa*	*Sycomorus*	4	3	Malaysia (1), Australia (3)
C. abnormis	*F. dammaropsis*	*Adenosperma*	12	10	New Guinea lowland (9), New Guinea highland (3)
C. nanus	*F. pungens*	*Bosscheria*	11	11	New Guinea (11)
C. pygmaeus	*F. minnahassaed*	*Bosscheria*	1	1	Philippines (1)

[a] Numbers in parentheses indicate number of samples from each locality.

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 version 3.1 Cycle Sequencing Ready reaction kit (Applied Biosystems, Foster City, CA) on a Mastercycler thermocycler with 1 min at 96°C followed by 26 cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C, and then they were analyzed on a Prism 377 DNA sequencer (Applied Biosystems).

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 Akaike 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, 2004). Under maximum parsimony criterion, a heuristic search was performed with 10,000 sequence addition replicates. Parsimony bootstrap analysis was performed with 1,000 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 4,000 trees from two simultaneous runs of four chains over 2×10^6 generations of Markov chain Monte Carlo 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* (Grandi) and *Kradibia tentacularis* (Grandi), the latter formerly *Liporrhopalum* (Cruaud et al. 2010). 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. 2010, 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 ($\delta = 619.8$ $P < 0.001$). We used Bayesian methods and a 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. 2010, Lopez-Vaamonde et al. 2009).

The tree was calibrated by assigning strong prior distributions to three nodes within *Ceratosolen* and to one clade of nonpollinating fig wasps. A clade endemic to New Guinea was given normal prior distribution with a mean of 40 my and a SD 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*, also should 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 SD 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 SD 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.

Table 2. GenBank accession numbers and locality information for focal *Ceratosolen* species

Haplotype	Pollinator species	Locality	Collection no. or citation[a]	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]
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]
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]

[a] Where collection numbers were unavailable, the source publication is listed.

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 sequences 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 with 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

Table 3. GenBank accession numbers and locality information for nonfocal *Ceratosolen* species

Pollinator species	Locality	Collection no.[a]	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]
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]

[a] Where collection numbers were unavailable, the source publication is listed.

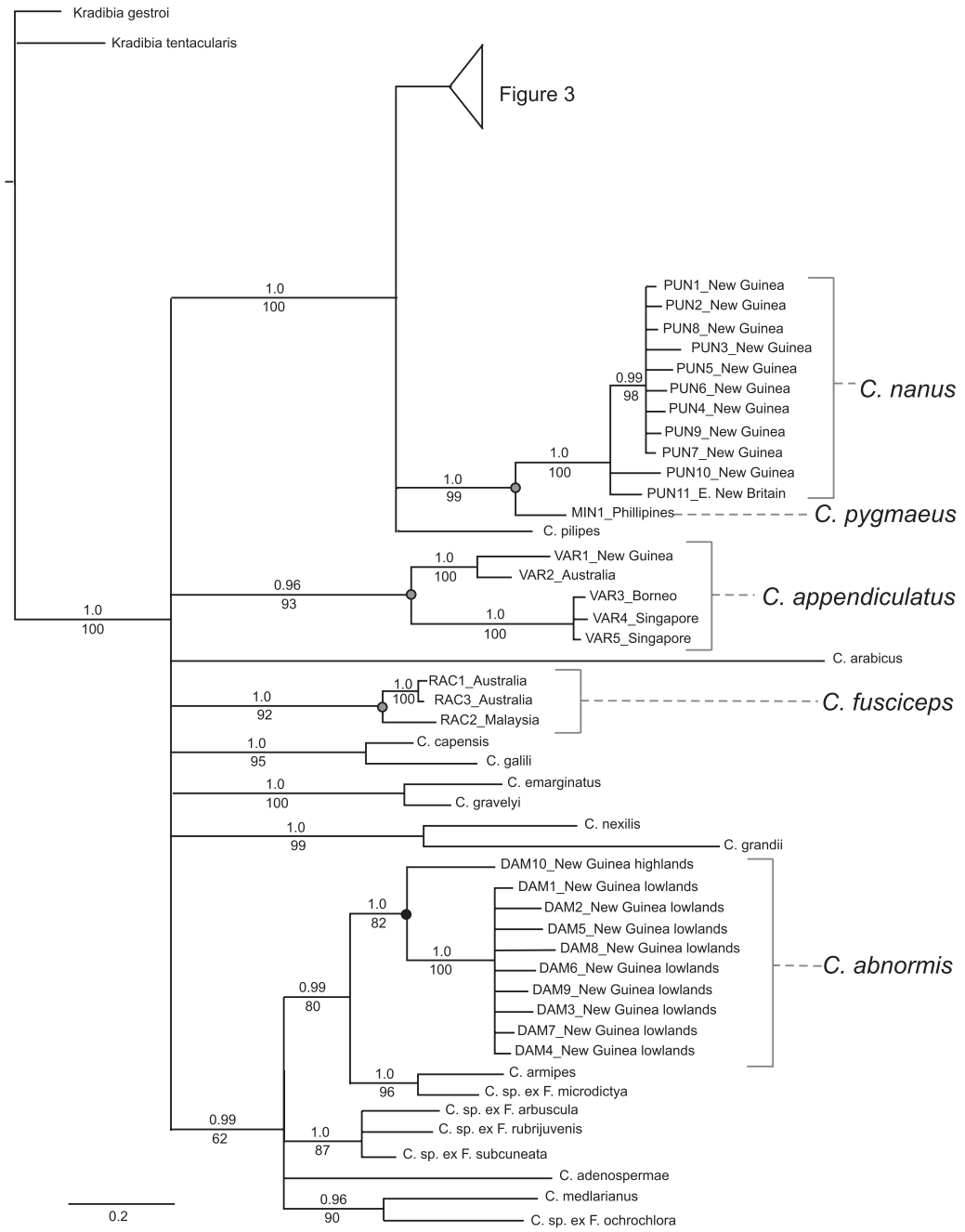
divergence within named taxa provides evidence for the existence of unnamed species, which we provisionally call cryptic species. Large genetic distances (up to 18.8%) within named species were associated with geographic isolation. For example, lowland and highland samples of *C. abnormis* populations 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 *Ceratosolen fusciceps* Mayr was 7.8–8.5% divergent from Australian samples. *Ceratosolen appendiculatus* Mayr was 15.7–18.8% divergent between a New Guinean clade and an Indo-Malayan clade. *Ceratosolen corneri* Wiebes showed 9.3–11.4% divergence between New Guinea samples and Philippine samples. Three cryptic species within *Ceratosolen solmsi* Mayr showed deep divergence among India, China, and Southeast Asia (15.8–17.8%). *Ceratosolen bisulcatus* Mayr 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.

Table 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]

Pollinators of each focal *Ficus* species were monophyletic across all analyses except *C. bisulcatus* (Figs. 2 and 3). The relationship among monophyletic *C. corneri* and three *C. bisulcatus* lineages, corresponding to black Australian, black Asian, and yellow Asian morphotypes, was unresolved (Fig. 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).

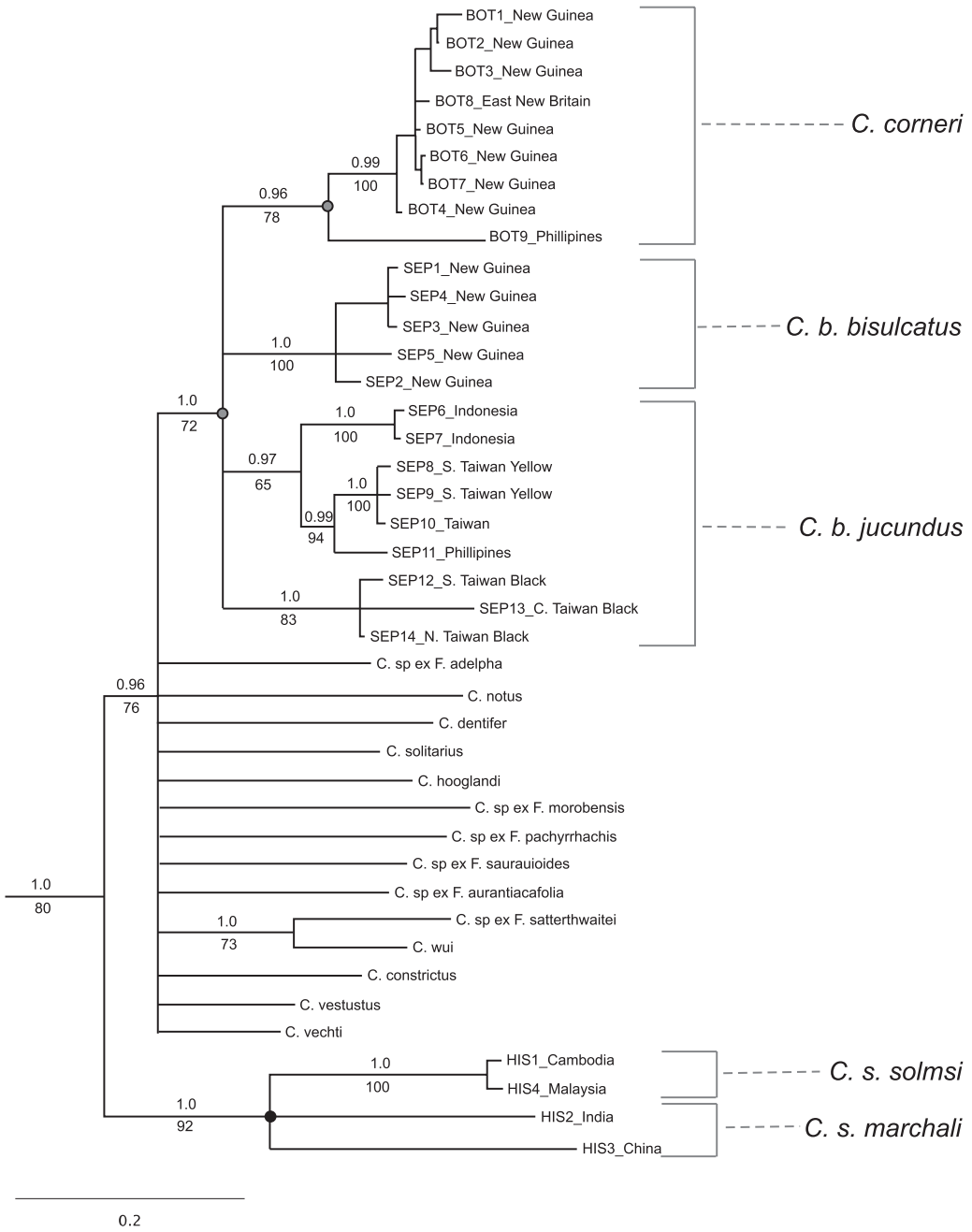
Molecular dating identified several independent dispersal events across the Wallace Line in a time interval between 8.4 and 18.8 mya (Fig. 4). The split between Wallacean sister species *C. nanus* (New Guinea) and *C. pygmaeus* (Philippines) occurred at least 14.9 mya. Cryptic species diverged at a minimum 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).



Figs. 2 and 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.

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



Figs. 2 and 3. Continued.

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 (Fig. 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 sam-

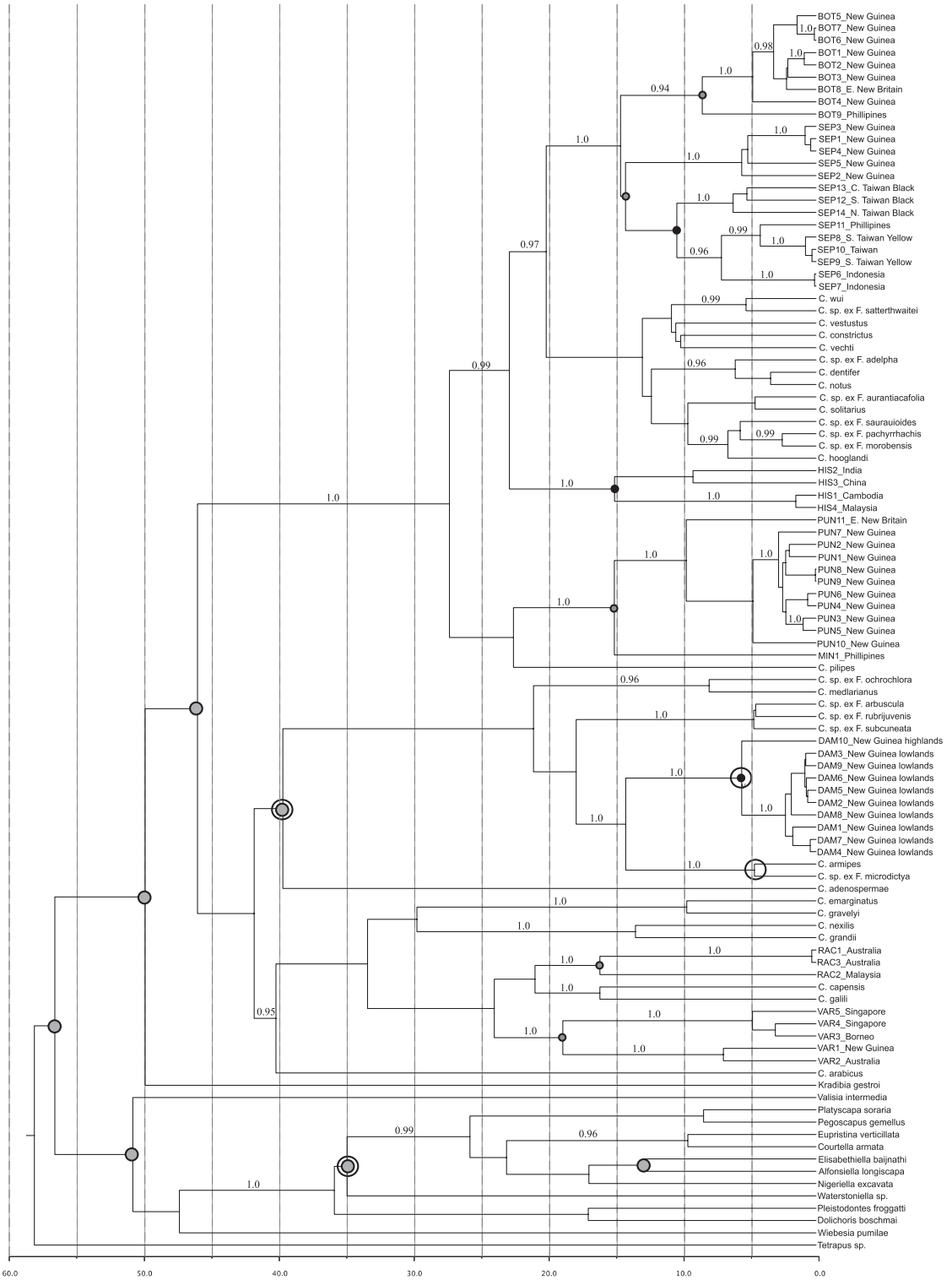


Fig. 4. Chronogram for *Ceratosolen* according to Bayesian divergence time estimation. The horizontal axis corresponds to million years before present. Small, shaded circles mark dispersal events associated with the Wallace Line. Small, solid circles mark deep divergence within named species. Large, 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 Materials and Methods.

pling? 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.

C. 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 *Ficus septica* Burm.f. in southern Taiwan (Lin et al. 2008) 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 (Fig. 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 (Fig. 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* seems not to have been associated with the colonization of novel host species during range expansion. On the contrary, host associations seem 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 seem to have been maintained in each case of ancient dispersal across Wallacea (Fig. 4). *C. 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 reassessment of *Ceratosolen* species limits, where morphological characters distinguishing divergent lineages may yet be found. In *C. solmsi* and *C. bisulcatus*, named subspecies (Wiebes 1982) might be elevated in rank, but new names also will be needed to recognize, for example, divergent lineages of Taiwanese *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 ± 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 *Ceratosolen*, divergence between sister species was similar to hymenopteran data with 7.6–20.4% divergence and an average of 11.3 (±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 many new species. In *C. abnormis*, New Guinea highland and lowland populations also could 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 >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 <500 km, or highland and lowland *C. abnormis* separated by <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 also may 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. 2008). However, geographic variation in fig morphology is well known and subspecies are recognized in the taxonomic literature. *Ficus botryocarpa* Miq., for example, is divided into subspecies *botryocarpa* in the Philippines and subspecies *subalbidoramea* in New Guinea (Berg and Corner 2005) that happen to coincide with the divergent lineages of *C. corneri. Ficus hispida* Blanco and *Ficus racemosa* L. include many regional synonyms with no currently recognized subspecies, but each are considered "highly variable" (Berg and Corner 2005). *F. septica* varieties include a widespread variety *septica* and a Philippine endemic variety *salicifolia* (Corner 1965). Might sympatric *C. bisulcatus* in Taiwan have resulted from local pollinator specialization on variety *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 covariation in fig and pollinator population genetic structure.

In conclusion, 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 seem 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. 2004b, Machado et al. 2005, Su et al. 2008), the divergence of *Ceratosolen* mitochondrial DNA haplotypes seems 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.

Acknowledgments

We thank Brus Isua, Lin Rong-Chien, and Monika Zavodna for providing fig wasp samples; Nina Ronsted for cytochrome oxidase sequences; and two anonymous reviewers for comments. This work was supported by National Science Foundation grant DEB-0841885 and the David and Lucille Packard Foundation.

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Received 20 May 2010; accepted 2 September 2010.