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Phylogenetic Relationships of Fig Wasps Pollinating Functionally Dioecious *Ficus* Based on Mitochondrial DNA Sequences and Morphology

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Abstract.—The obligate mutualism between pollinating fig wasps in the family Agaonidae (Hymenoptera: Chalcidoidea) and *Ficus* species (Moraceae) is often regarded as an example of coevolution but little is known about the history of the interaction, and understanding the origin of functionally dioecious fig pollination has been especially difficult. The phylogenetic relationships of fig wasps pollinating functionally dioecious *Ficus* were inferred from mitochondrial cytochrome oxidase gene sequences (mtDNA) and morphology. Separate and combined analyses indicated that the pollinators of functionally dioecious figs are not monophyletic. However, pollinator relationships were generally congruent with host phylogeny and support a revised classification of *Ficus*. Ancestral changes in pollinator ovipositor length also correlated with changes in fig breeding systems. In particular, the relative elongation of the ovipositor was associated with the repeated loss of functionally dioecious pollination. The concerted evolution of interacting morphologies may bias estimates of phylogeny based on female head characters, but homoplasy is not so strong in other morphological traits. The lesser phylogenetic utility of morphology than of mtDNA is not due to rampant convergence in morphology but rather to the greater number of potentially informative characters in DNA sequence data; patterns of nucleotide substitution also limit the utility of mtDNA findings. Nonetheless, inferring the ancestral associations of fig pollinators from the best-supported phylogeny provided strong evidence of host conservatism in this highly specialized mutualism. [Coevolution; maximum likelihood; mutualism; parametric bootstrapping; pollination.]

> "... it is clear that the wasps have classified the figs better than the botanists."
>
> (Corner 1955:430)

The family Agaonidae (Hymenoptera: Chalcidoidea) includes several hundred wasp species that are closely associated with the inflorescence of *Ficus* species (Boucek, 1988). All fig wasps complete growth and development within the fig inflorescence, and their interactions with hosts may be antagonistic or mutualistic. Nonpollinating fig wasps have negative impacts on their hosts, either as gallers of figs or as parasitoids of fig pollinators. In contrast, pollinating fig wasps are obligate mutualists with morphological adaptations, extreme host specificity, and life cycles that have been regarded as products of coevolution (Wiebes, 1979). Molecular phylogenies suggest that mutualism evolved once and characterizes the subfamily Agaoninae (Machado, 1998). Mutualisms involving pollinating seed predators are also characterized by conflicts over seed resources, because in theory, the consumption of too many or too few seeds by pollinators could drive a mutualism toward extinction or parasitism (Pellmyr, 1997). In this regard, functionaly dioecious fig pollination creates a unique evolutionary conflict (Kjellberg et al., 1987; Grafen and Godfray, 1991; Anstett et al., 1997), and understanding the stability of the mutualism has been limited by the availability of phylogeny estimates for the pollinators of functionally dioecious figs.

Ficus species are either monoecious or gynodioecious, according to the arrangement of the unisexual flowers in the enclosed inflorescence, or syconium (Kjellberg et al., 1987; Grafen and Godfray, 1991; Berg and Wiebes, 1992; Weiblen, 2000). In monoecious species, all figs contain both staminate and pistillate flowers. Gynodioecious species, on the other hand, have two kinds of figs on separate plants: "gall figs" with staminate and pistillate flowers, and "seed figs" with pistillate flowers only. The pistillate flowers are dimorphic and their interactions with fig pollinators are illustrated by the example of *Ficus nodosa* and its obligate pollinator, *Ceratosolen nexilis* (Fig. 1). *C. nexilis* females are attracted to both kinds of figs. After pushing through the fig opening, or ostiole, females lay eggs and actively pollinate the pistillate flowers. Pollinator larvae feed on endosperm, which is accessible only to offspring deposited between the ovary layers

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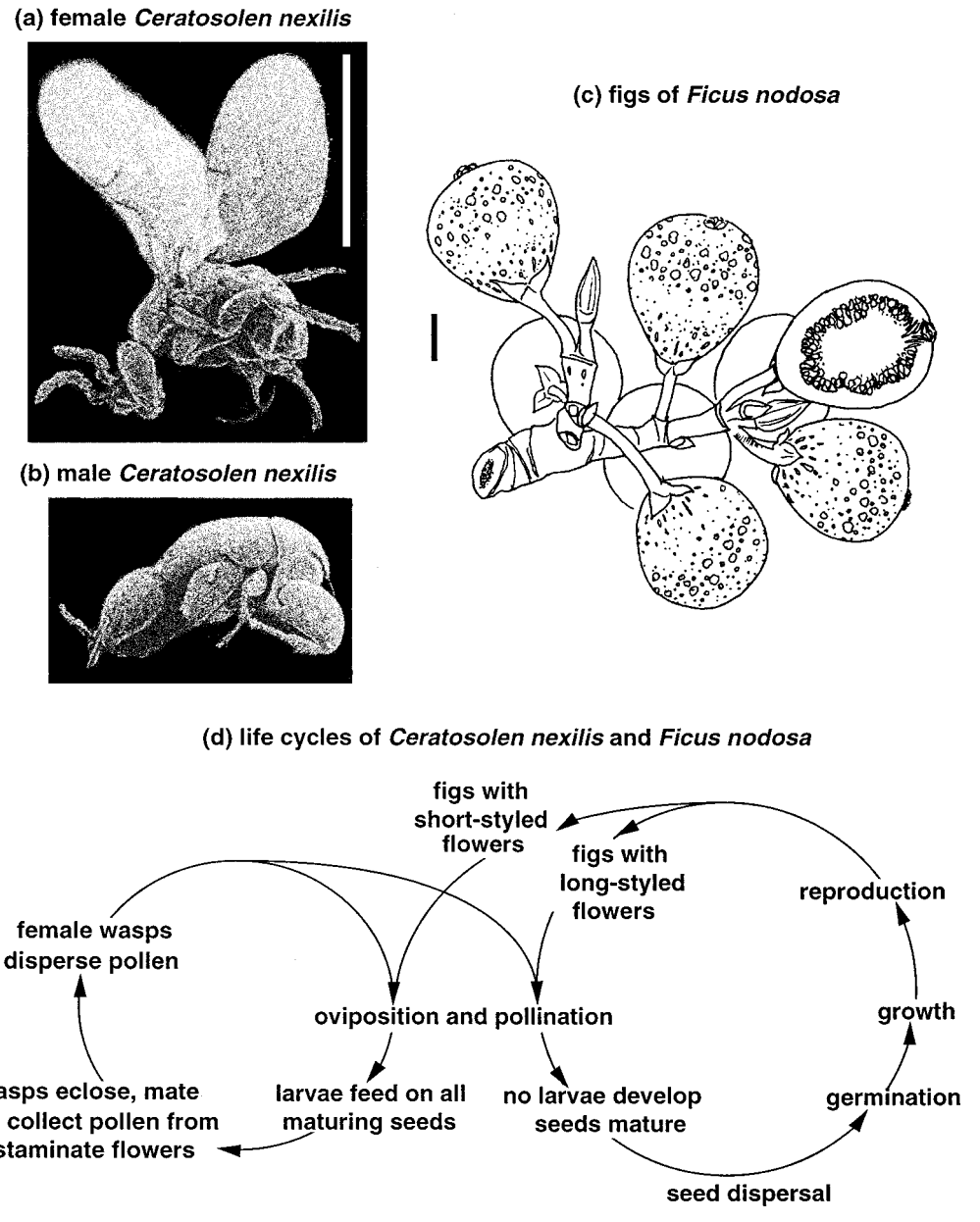

FIGURE 1. *Ceratosolen nexilis*, the pollinator of *Ficus nodosa*: (a) winged female (b) wingless male. Scale bar is 1 mm. (c) Cauliflorous figs in functionally dioecious *F. nodosa*. Fig (syconium) in cross-section shows the opening (ostiole) and flowers located inside of the receptacle. Scale bar is 1 cm. (d) Life cycle diagram illustrating the interdependence of *C. nexilis* and *F. nodosa*. Partly because of differences in the style lengths of fig flowers, the pollinators and seeds mature separately in two types of figs on separate plants. Drawing and photographs are by the author.

of short-styled flowers in gall figs. The maturation of pollinator offspring coincides with the release of pollen from staminate flowers, which the female wasps deliver to both kinds of figs (Fig. 1). In seed figs, however, pollinator ovipositors fail to penetrate the ovary layers of the long-styled flowers. Functionally, dioecious fig pollination seems paradoxical, given that the pollinators of seed figs are denied their only opportunity to reproduce, whereas the pollinators of gall figs are more fit (Grafen and Godfray, 1991). Although it would be advantageous for pollinators to avoid seed figs, this has not been observed

(Patel et al., 1995) and the existence of at least 350 functionally dioecious species suggests that this mutualism is evolutionarily stable.

The taxonomy of pollinating fig wasps has received less attention than has that of *Ficus*, but 300 species in 16 genera are currently recognized (Berg and Wiebes, 1992; Wiebes, 1994a, 1995a). Molecular phylogenetic analyses indicate that neotropical *Tetrapus* is a sister group to the rest of the pollinating Agaoninae (Machado, 1998). This position of *Tetrapus* is consistent with fossil and morphological evidence (Brues, 1910; Wiebes, 1995b) and also agrees with the phylogenetic position of their monoecious host figs in section. *Pharmacosycea* (Weiblen, 2000). The pollinators of functionally dioecious figs, on the other hand, are restricted to the Paleotropics and classified in five genera (Table 1). Their center of diversity is Indo-Australia (Wiebes, 1994a), where all five genera are represented, compared with only three in Africa (Berg and Wiebes, 1992).

The generic classification of Indo-Australian pollinators (Table 1) is incongruent with *Ficus* taxonomy (Corner, 1965) because the genus *Ceratosolen* pollinates functionally dioecious species in subgenus *Ficus* as well as monoecious species in subgenus. *Sycomorus* (Kerdelhue et al., 1999, Weiblen, 2000). Recent studies support the suggestion of Wiebes (1994a) that the pollinators of functionally dioecious figs are not monophyletic (Machado, 1998), although the clades involved have not yet received detailed phylogenetic analysis. Sampling from functionally dioecious figs was minimal in the global study (Machado, 1998), and regional studies in Panama (Machado et al., 1996), Japan (Yokoyama, 1995), and Africa (Kerdelhue et al., 1999) have focused on monoecious figs. This paper examines the phylogenetic relationships of the pollinators of functionally dioecious figs with *Ceratosolen* receiving special emphasis because of the complexity of its host associations.

Mitochondrial DNA (mtDNA) sequences have been used to reconstruct phylogeny in Hymenoptera (Cameron et al., 1992; Derr et al., 1992; Dowton and Austin, 1994) and cytochrome oxidase genes have been particularly useful for resolving fig wasp relationships (Machado, 1998). However, given that gene trees do not always reflect species phylogeny (Doyle, 1992; deQuerioz et al., 1995; Moore, 1995; Maddison, 1995, 1997; Naylor and Brown, 1998), additional characters from multiple genes and morphology may help

TABLE 1. Generic classification of Indo-Australian Agaoninae after Wiebes (1994a) and of their host figs after Corner (1965). Numbers of described species are from Wiebes (1994a). Conflicts between the botanical and entomological classifications are footnoted.

Agaoninae	No. of species	*Ficus* subgenus	*Ficus* section
Dolichoris Hill	8	*Pharmacosycea*	*Oreosycea*
Pleistodontes Saunders	18	*Urostigma*	*Malvanthera*
Platyscapa Motschoulsky	8	*Urostigma*	*Urostigma*
Deilagaon Wiebes	4	*Urostigma*	*Conosycea*
Waterstoniella Grandi	20	*Urostigma*	*Conosycea*
Eupristina Saunders	14	*Urostigma*	*Conosycea*
Blastophaga Gravenhorst	19	*Ficus*	*Ficus*[a]
Wiebesia Boucek[b]	18	*Ficus*	*Kalosyce*
		Ficus	*Rhizocladus*
Liporrhopalum Waterston	18	*Ficus*	*Sycidium*[c]
Kradibia Saunders	18	*Ficus*	*Sycidium*[c]
Ceratosolen Mayr	48	*Ficus*	*Adenosperma*
		Ficus	*Ficus*[a]
		Ficus	*Neomorphe*
		Ficus	*Sycocarpus*
		Ficus	*Sycidium*[d]
		Sycomorus	*Sycomorus*

[a] All species in section *Ficus* are pollinated by *Blastophaga* except for series *Rivulares* and series *Pseudopalmae*, which are pollinated by *Ceratosolen*.

[b] All *Wiebesia* are associated with sections *Kalosyce* and *Rhizocladus* except for *W. partita*, which pollinates *F. primaria* in section *Sycidium*.

[c] All species in section *Sycidium* are pollinated by *Kradibia* except for *Liporrhopalum*-pollinated subsection *Paleomorphe* and *F. montana*, *Ceratosolen*-pollinated *F. asperiuscula*, and two other cases[b,d].

[d] Series *Pungentes, Prostratae*, and *F. complexa* in series *Phaeopilosae* are *Ceratosolen*-pollinated.

provide informative comparisons. With regard to pollinating fig wasps, morphology has been argued to be perhaps more indicative of the functional constraints imposed by host associations than of phylogenetic relationships (Herre et al., 1996), but this assumption has not been tested. Examples of pollinator traits and the features of figs with which they are associated include (1) ovipositor lengths and fig breeding systems (Ramirez, 1980), (2) pollen pockets and the distribution of staminate flowers (Ramirez, 1978), and (3) head shape and the arrangement of ostiolar bracts (van Noort and Compton, 1994). This paper is the first to test the hypothesis that coadaptation leads to inaccurate estimates of fig wasp phylogeny. Comparable analyses examined the utility of mtDNA and morphology in reconstructing fig wasp phylogeny and in evaluating adaptive hypotheses regarding the stability of the functionally dioecious fig pollination (Ramirez, 1980; Kjellberg et al., 1987; Kerdelhue and Rasplus, 1996).

Materials and Methods

The 44 taxa sampled (Table 2) included representatives of the major groups of Indo-Australian pollinators associated with the hosts included in a phylogenetic study of functionally dioecious figs (Weiblen, in press). Thirty-five known pollinators species were identified (Berg and Wiebes, 1992; Wiebes, 1994a), and eight new species were encountered (Table 2; Appendix). Sampling included 31 pollinators of functionally dioecious figs, with at least two species from each genus. The 20 *Ceratosolen* species included representatives of the three subgenera as well as four species that pollinate monoecious figs in the Paleotropics (Kerdelhue et al., 1999). In addition, at least two representatives of each genus associated with monoecious figs in Indo-Australia were sampled except *Eupristina* and *Deilagaon*. A nonpollinating fig wasp, *Apocryptophagus spinitarsus* (Agaonidae: Sycophaginae), was included for rooting purposes because mtDNA sequences for *Tetrapus* were unavailable at the time of this study.

Sources of DNA included adult males and females preserved in 70–95% ethanol and stored at room temperature. Although useful genomic DNA was extracted from collections as much as 27 years old, the best results were obtained from specimens <1 year old. Genomic DNA was extracted from 1 to 10 pollinators reared from the same fig; these were likely to share the same mtDNA haplotype because of low founder numbers and inbreeding (Herre, 1985). Genomic DNA was isolated by using reagents from the QIAamp® Tissue Kit (QIAGEN Inc.) and an extraction protocol modified for small insects. First, trace ethanol was removed from the specimens under vacuum for 5 min. Wholly dried material was ground in Eppendorf tube mortars containing 90 μL of ATL buffer and 10 μL of proteinase K solution. Specimens in ATL buffer were incubated at 50°C for 24 hr. After 12 hr, a 10-μL aliquot of proteinase K solution was added to each tube. After incubation, specimens were vortex-mixed with 110 μL of AL buffer and incubated at 70°C for 10 min. Extracts were centrifuged at 13,000 rpm for 5 min. The supernatant was transferred to a clean tube and vortex-mixed with 110 μL of absolute ethanol. Extracts were cleaned by following the QIAamp protocol except that genomic DNA was incubated at 70°C for 5 min in 90 μL of water before elution from QIAamp spin columns. Extracts diluted 10-fold were used in the polymerase chain reaction.

Insect primers were used to amplify ~1900 bp that included mitochondrial cytochrome *c* oxidase subunit I (COI), the leucine tRNA (UUR), and part of the cytochrome *c* subunit II (COII). Three overlapping fragments (~700–900 bp) were obtained by using primer combinations Juan–Nancy, New Jerry–Pat, and sw2618–Maryln (Roehrdanz, 1993; Simon et al., 1994; Machado, 1998, respectively). The thermal conditions for amplification were as follows: denaturation at 96°C for 3.5 min; 35 cycles of denaturation at 94°C (30 sec), annealing at 45°C (60 sec), and extension at 72°C (30 sec); and final extension at 72°C for 5 min. Sequences were collected by using a 377 PRISM™ sequencer (Applied Biosystems Inc.), and all species were sequenced in both directions. Internal primers included reverse Dick (Simon et al., 1994) and sw2642 (Machado, 1998) and the new primers Marcus (5′-ATATTTAATTTTTGGAAGATGAGC-3′) and Brus (5′-GAAGMTAAAGGAGGGTA WACAG-3′). The new primers corresponded to positions S1540 and A1891, respectively, in the *Drosophila yakuba* sequence of Clary and Wolstenholme (1985).

TABLE 2. Fig wasps (Agaoninae) selected for phylogenetic analysis. Subgenera are abbreviated *(C)eratosolen, (P)arapristina, (R)othropus, (S)trepitus*, and *(V)alisia*. Host species, sections, and subgenera are also listed. Informal names of eight new species are introduced; descriptions are in preparation. Nonpollinating *Apocryptophagus spinitarsus* (Mayr) from *F. variegata* (B61, New Guinea) was included as an outgroup. GenBank accession numbers for the mtDNAs are AF200371–AF200414.

Pollinator	*Ficus* species	*Ficus* section	*Ficus* subgenus	Voucher[a], locality
Blastophaga (V.) intermedia Grandi	*F. padana* Burm. f.	*Ficus*	*Ficus*	GW1065, Java
Blastophaga (V.) malayana Wiebes	*F. grossularioides* Burm. f.	*Ficus*	*Ficus*	GW861, Borneo
Ceratosolen (C.) appendiculatus	*F. variegata* Bl.	*Neomorphe*	*Ficus*	B198, New Guinea
Ceratosolen (C.) bisulcatus (Mayr)	*F. septica* Burm. f.	*Sycocarpus*	*Ficus*	B170, New Guinea
Ceratosolen (C.) blommersi Wiebes	*F. botryoides* Baker	*Sycomorus*	*Ficus*	GW841, Madagascar
Ceratosolen (C.) capensis Grandi	*F. sur* Forssk.	*Sycomorus*	*Ficus*	GW840, Tanzania
Ceratosolen (C.) emarginatus Mayr	*F. auriculata* Lour.	*Neomorphe*	*Ficus*	RMNH2723, Malaysia
Ceratosolen (C.) fusciceps (Mayr)	*F. racemosa* L.	*Sycomorus*	*Ficus*	GW1075, Australia
Ceratosolen (C.) grandii Wiebes	*F. semivestita* Corner	*Neomorphe*	*Ficus*	GW700, New Guinea
Ceratosolen (C.) medlerianus	*F. mollior* F.v.M.	*Adenosperma*	*Ficus*	B33, New Guinea
Ceratosolen (C.) nanus Wiebes	*F. pungens* Bl.	*Sycidium*	*Ficus*	G077, New Guinea
Ceratosolen (C.) nexilis Wiebes	*F. nodosa* Teysm. et Binn.	*Neomorphe*	*Ficus*	GW829, Solomon Isl.
Ceratosolen (C.) sp. "riparianus"	*F. ochrochlora* Ridley	*Adenosperma*	*Ficus*	GW735, New Guinea
Ceratosolen (C.) cf. *nexilis* Wiebes	*F. robusta* Corner	*Neomorphe*	*Ficus*	B191, New Guinea
Ceratosolen (R.) corneri Wiebes	*F. botryocarpa* Miq.	*Sycocarpus*	*Ficus*	B135, New Guinea
Ceratosolen (R.) dentifer Wiebes	*F. hispidioides* S. Moore	*Sycocarpus*	*Ficus*	B133, New Guinea
Ceratosolen (R.) hooglandi Wiebes	*F. bernaysii* King	*Sycocarpus*	*Ficus*	G093, New Guinea
Ceratosolen (R.) vechti Wiebes	*F. lepicarpa* Bl.	*Sycocarpus*	*Ficus*	GW1086, Malaysia
Ceratosolen (S.) abnormis (Wiebes)	*F. dammaropsis* Diels	*Sycocarpus*	*Ficus*	B110, New Guinea
Ceratosolen (S.) armipes Wiebes	*F. itoana* Diels	*Sycocarpus*	*Ficus*	GW622, New Guinea
Ceratosolen (S.) sp. "kaironkensis"	*F. microdictya* Diels	*Sycocarpus*	*Ficus*	GW954, New Guinea
Ceratosolen (S.) vissali Wiebes	*F. theophrastoides* Seem.	*Sycocarpus*	*Ficus*	GW826, Solomon Isl.
Dolichoris inornata Wiebes	*F. edelfeltii* King	*Oreosycea*	*Pharmacosycea*	GW821, New Guinea
Dolichoris sp. "hombronianae"	*F. hombroniana* Corner	*Oreosycea*	*Pharmacosycea*	GW953, New Guinea
Dolichoris vasculosae Hill	*F. vasculosa* Wall.	*Oreosycea*	*Pharmacosycea*	GW1084, Malaysia
Eupristina (P.) verticillata	*F. microcarpa* L.	*Conosycea*	*Urostigma*	F2, New Guinea
Kradibia copiosae (Wiebes)	*F. copiosa* Steud.	*Sycidium*	*Ficus*	G057, New Guinea
Kradibia jacobsi (Wiebes)	*F. conocephalifolia* Ridley	*Sycidium*	*Ficus*	B154, New Guinea
Kradibia sp. "ohuensis"	*F. trachypison* K. Schum.	*Sycidium*	*Ficus*	B136, New Guinea
Kradibia sp. "salembensis"	*F. phaeosyce* Laut. et K. Sch.	*Sycidium*	*Ficus*	B179, New Guinea
Kradibia wassae (Wiebes)	*F. wassa* Roxb.	*Sycidium*	*Ficus*	B176, New Guinea
Liporrhopalum cf. *gibbosae* Hill	*F. tinctoria* Forst. f.	*Sycidium*	*Ficus*	F4, New Guinea
Liporrhopalum virgatae Hill	*F. virgata* Reinw.	*Sycidium*	*Ficus*	B166, New Guinea
Platyscapa corneri Wiebes	*F. superba* Miq.	*Urostigma*	*Urostigma*	GW851, Java
Platyscapa fischeri Wiebes	*F. prasinicarpa* Elm.	*Urostigma*	*Urostigma*	GW827, Solomon Isl.
Pleistodontes plebejus Wiebes	*F. hesperidiiformis* King	*Malvanthera*	*Urostigma*	GW624, New Guinea
Pleistodontes rieki Wiebes	*F. xylosycia* Diels	*Malvanthera*	*Urostigma*	G059, New Guinea
Pleistodontes rigisamos Wiebes	*F. destruens* C.T. White	*Malvanthera*	*Urostigma*	GW943,Australia
Waterstoniella brevigena Wiebes	*F. pellucidopunctata* Griff.	*Conosycea*	*Urostigma*	GW880, Borneo
Waterstoniella sp. "dubium"	*F. dubia* Wall.	*Conosycea*	*Urostigma*	TL1021, Borneo
Wiebesia sp. "brusi"	*F. baeuerlenii* King	*Rhizocladus*	*Ficus*	B120, New Guinea
Wiebesia sp. "frustrata"	*F. odoardi* King	*Rhizocladus*	*Ficus*	B205, New Guinea
Wiebesia punctatae Wiebes	*F. punctata* Thunb.	*Kalosyce*	*Ficus*	TL1022, Borneo

[a] Voucher specimens are deposited at the Museum of Comparative Zoology, Harvard University (MCZ), or the Rikjmuseum van Natuurlijke Historie, Leiden (RMNH). All collections were made by the author except for RMNH2723 (Corner), TL10211 and TL1022 (Laman), GW840 and GW841 (Kerdelhue), and GW1075 (Brown).

The length of the manually aligned sequence was 2083 bp but only 1932 bp were considered for analysis after excluding the 151 end positions missing from some samples. The COI portion accounted for 1602 bp of the 1932 bp. The leucine tRNA (73 bp) was located between COI and COII (257 bp). COI in fig wasps also included an insertion of variable length at the 3′ end of the molecule, which required exclusion of 174 additional positions with ambiguous alignment; this reduces the length of the analyzed sequence to 1724. Four gaps of 3, 6, or 9 bp were also present in COI and the reading frame was preserved in each instance. These and a 7 bp gap in the nontranscribed leucine tRNA were excluded.

Phylogenetic analyses were performed with PAUP* version 4.0b2 for Power Macintosh computers (Swofford, 1998). Under

maximum parsimony (MP), heuristic searches were conducted according to PAUP* default settings, except that 1,000 random addition sequence replicates were performed with MAXTREES set to increase without limit. All characters were unordered and weighted equally. Uninformative characters were excluded from all analyses. Bootstrap resampling (Felsenstein, 1985) and decay analyses (Bremer, 1988; Donoghue et al., 1992) were used to estimate clade robustness. Nonparametric bootstrapping involved heuristic searches with 5,000 replicates and a random addition sequence with $n = 1$. In addition, distance and maximum likelihood (ML) methods were used to estimate phylogenies from the mtDNA data. Because of extreme A-T bias and saturated transitions in fig wasp mtDNA sequences (Machado, 1998), genetic distances based on transversions only and corrected for multiple substitutions were used to generate a neighbor-joining tree (NJ; Tamura and Nei, 1993). NJ and MP trees were used to estimate parameters for models of nucleotide substitution, between those of Jukes and Cantor (1969; JC), Felsenstein (1981; F81), Kimura (1980), (Hasegawa et al. (1985; HKY85), Zharkikh (1994), and Rodriguez et al. (1990; GTR). Parameters for heterogeneity of substitutions across sites (Γ; Yang, 1994) and the proportion of invariant sites (I) were also estimated. Model goodness-of-fit was compared by using likelihood ratio tests (Goldman, 1993) as implemented by Posada and Crandall (1998). The model with the fewest additional parameters was used to reconstruct ML phylogeny by assuming parameter values estimated from starting trees. NJ and MP topologies were used as starting trees for branch swapping in separate heuristic searches (Swofford et al., 1996).

Alternative phylogenetic hypotheses were also evaluated by parametric bootstrapping (Huelsenbeck and Hillis, 1996). An ML heuristic search that assumed model parameters estimated from the ML topology was constrained according to a particular hypothesis of monophyly. Model parameters and branch lengths were then estimated from the constrained ML topology and used to simulate 100 replicate data sets with SEQ-GEN (Rambaut and Grassly, 1997). MP trees for each parametric bootstrap replicate were saved from heuristic searches with 10 random addition sequences replicates. The log likelihood difference of the unconstrained (best) topology and the constrained (null) topology provided a distribution under the null hypothesis that systematic errors accounted for the difference between the best topology and the true phylogeny.

Fifty-seven skeletal features were also coded as discrete characters for phylogenetic analysis (Appendix). Published descriptions cited in Weiblen (1999) provided an initial source of character states. Characters included external features of the head, antennae, mouthparts, thorax, legs, spiracles, and genitalia. The position and number of hairs, teeth, and lamellae were major sources of character states. Characters and states taken from the taxonomic literature were confirmed by examining specimens with light microscopy. In addition to specimens listed in Table 2, I consulted the type collection of J. T. Wiebes at the Rikjmuseum van Natuurlijke Historie, Leiden (RMNH) and specimens from the Bishop Museum, Honolulu (BISH). Approximately 1.4% of the resulting character matrix consisted of polymorphic character states. Also, some characters were not applicable to the outgroup and were scored as missing data. Under parsimony, morphological characters were analyzed separately and in combination with mtDNA after incongruence length difference tests (ILD; Farris et al., 1994) and Wilcoxon's sum of signed ranks tests for incongruence (Templeton, 1993; Larson, 1994) were performed. The morphological matrix and DNA sequence alignment are deposited in TreeBASE under accession number SN278 (http: www.herbaria.harvard.edu/treebase) and on the Society of Systematic Biologists website http://www.utexas.edu/ftp/depts/systbiol/.

RESULTS

Mitochondrial DNA

Complete sequences for the region including COI, the leucine tRNA, and the 5′ end of COII were obtained for 33 species. Partial sequences were obtained for another 11 species: In *C. vechti*, *C. vissali*, and *D. inornata*, the 600 bp at the 5′ end of COI could not be amplified; the 3′ end of COI was not sequenced for *W. brevigena*; and the leucine tRNA plus COII fragment was not obtained for nine species—*C. emarginatus, C. medlerianus, C.* sp. "riparianus", *C.*

cf. *nexilis*, *D.* sp. "hombronianae", *D. inornata*, *P. fischeri*, *W.* sp. "dubium", and *W. brevigena*. Mitochondrial COI and COII genes were analyzed in combination because they make up a single haplotype and do not provide independent estimates of species phylogeny (Moore, 1995). Moreover, conflict between COI and COII genes was not statistically significant ($P = 0.15$), according to an ILD test for the 33 complete sequences with 100 partition homogeneity replicates per 10 random addition replicates.

Of 1,724 positions considered for the mtDNA analysis, 381 (22%) were invariant, 325 (19%) were autapomorphic, and 1,018 (59%) were parsimony–informative. Only phylogenetically informative sites were included in parsimony analyses, whereas all sites were included in ML analyses. Overall base composition was highly A-T biased (75%), and base frequencies differed significantly among species ($\chi^2 = 191.6$, df $= 129$, $P < 0.01$) in the absence of phylogenetic autocorrelation. Base composition was similar to insect mtDNA in general (Liu and Beckenbach, 1992). First and second codon positions were less A-T biased on average (66% and 65%) and did not differ significantly among species ($\chi^2 = 105.9$, df $= 129$, n.s. and $\chi^2 = 47.8$, df $= 129$, n.s.). Third positions, however, were extremely A-T biased (80%), with significant heterogeneity among species in base frequencies ($\chi^2 = 232.3$, df $= 129$, $P < 0.01$). A comparison of nucleotide substitutions with respect to genetic distance and codon position is shown in Figure 2. The absence of visual evidence for a correlation between genetic distance and numbers of substitutions is indicative of saturated change at some sites. Transitions were relatively more saturated than transversions at all three positions but less so at first and second positions than at third positions. Transversions were most abundant at third positions and least abundant at first positions. Sequence divergence within fig wasp genera was also large. For example, between-species divergence within *Ceratosolen* ranged from 5% to 28% after correction for multiple substitutions.

Parsimony searches resulted in a single shortest tree (Fig. 3; L = 6806; CI = 0.29) based on all informative mtDNA characters.Twenty-six of 43 nodes in the MP tree were supported by >50% bootstrap support. *Dolichoris* was paraphyletic to the rest of the Indo-Australian pollinator genera after rooting the tree with nonpollinating *Apocryptophagus*. The pollinators of functionally dioecious figs fell into two weakly supported (<50%) clades, one including *Kradibia, Liporrhopalum*, and most of *Ceratosolen*, the other including the rest of *Ceratosolen, Wiebesia*, the pollinators of monoecious subgenus *Urostigma*, and *Blastophaga*.

Log likelihood ratio tests comparing models of nucleotide substitution indicated that the GTR model with a discrete approximation of the gamma distribution (GTR + Γ), provided the best fit (Table 3). Under ML, the data rejected the assumptions of equal base frequencies (JC vs. F81), an equal ratio of transition and transversion rates (F81 vs. HKY85), equal rates of transitions and transversions (HKY85 vs. GTR), and equal substitution rates across sites (GTR vs. GTR + Γ). Taking into account the proportion of invariable sites (I), however, did not significantly improve the fit of the model. Figure 4 shows the best tree obtained under ML from a heuristic search beginning with the MP topology and swapped to completion. To explore the likelihood surface more fully, the NJ topology was also used as a starting tree. Because of base composition bias and saturated transitions, Machado (1998) favored the calculation of Tamura–Nei genetic distances on transversions only; I used the same approach to obtain the NJ tree. The ML topology derived from branch-swapping on the NJ tree search was very similar to Figure 4.

Twenty-seven nodes in the ML tree were congruent with the topology obtained under MP; 15 nodes were in conflict, but only one of the conflicting nodes was supported by >50% bootstrap values under parsimony. The *Strepitus* clade with *C. abnormis* as sister to *C. armipes* plus *C.* sp. "kaironkensis" (53%) was contradicted by the placement of *C. abnormis* along a short branch with *C. grandii*, *C. medlerianus*, and *C.* sp. "riparianus". A major difference between the ML and MP trees concerned the placement of *Dolichoris vasculosae. Dolichoris* was paraphyletic to the rest of the Indo-Australian pollinators under parsimony analysis (Fig. 3), whereas *D. vasculosae* attached to *C. blommersi* under likelihood analysis (Fig. 4). The branch leading to *D.*

TABLE 3. Log likelihood ratio tests comparing models of molecular evolution for mtDNA from Indo-Australian pollinators of figs. Results are listed for the NJ topology and substitution models including JC (Jukes, and Cantor, 1969), F81 (Felsenstein, 1981), HKY85 (Hasegawa et al., 1985), and GTR (Rodriguez et al., 1990). The addition of parameters for heterogeneity of substitutions across sites (Γ; Yang, 1994) and for the proportion of invariant sites (I) were also tested. Significance was evaluated at $\alpha = 0.01$ after a Bonferroni correction for multiple tests (Posada and Crandall, 1998).

H_0	H_1	$-\ln L_0$	$-\ln L_1$	df	$-2\Lambda \log$	P
JC	F81	40578.06	39009.28	1	3137.56	<0.0001
F81	HKY	39009.28	38994.12	1	30.32	<0.01
HKY	GTR	38994.12	38104.28	4	1779.68	<0.0001
GTR	GTR + Γ	38104.28	35227.26	1	5754.01	<0.0001
GTR + Γ	GTR + Γ + I	35227.26	35223.35	1	7.81	n.s.

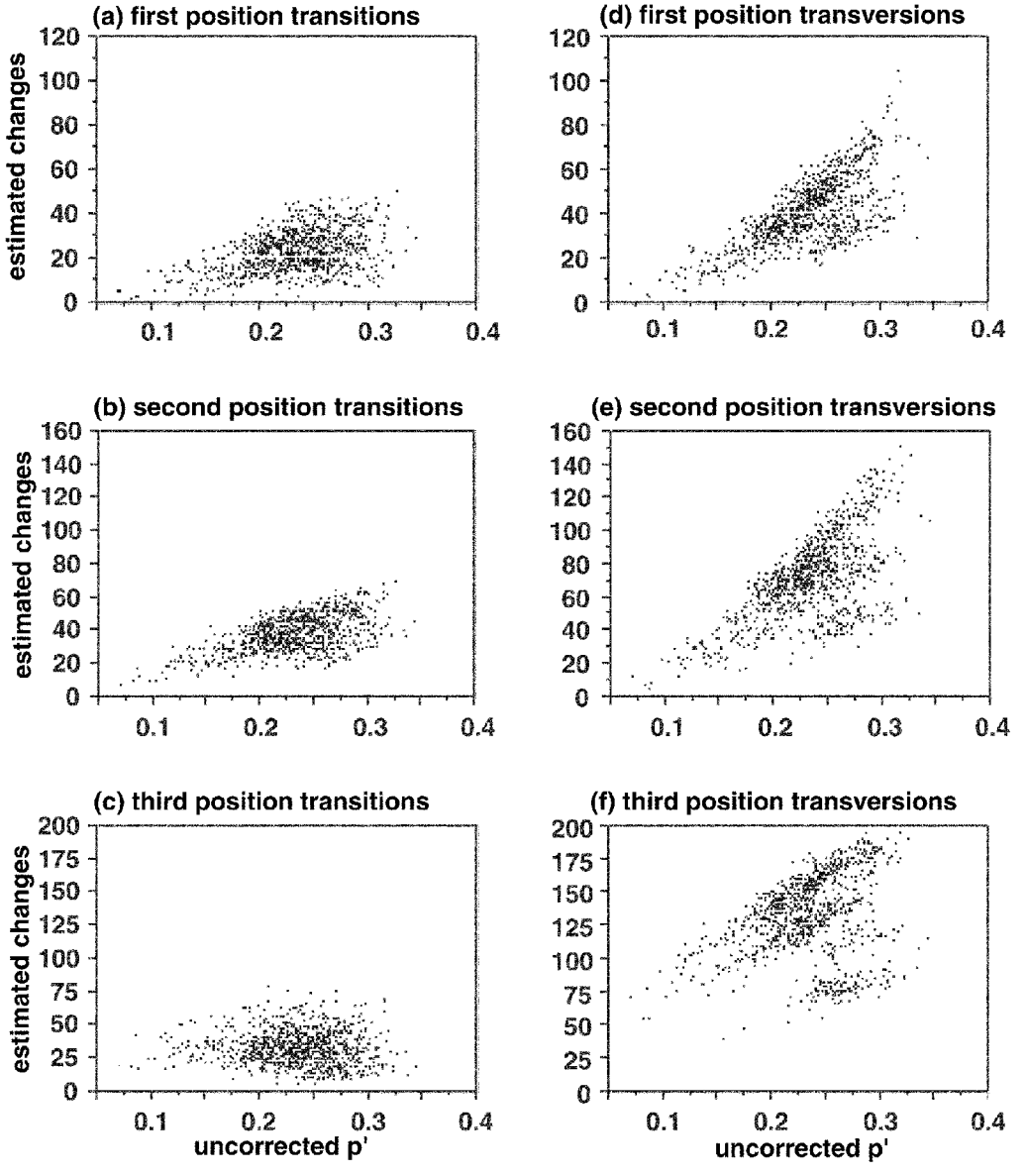

FIGURE 2. Pairwise genetic distance (uncorrected p') versus the absolute number of transitions and transversions for different codon positions in mitochondrial DNA sequences (COI and COII) from Indo-Australian pollinators of figs.

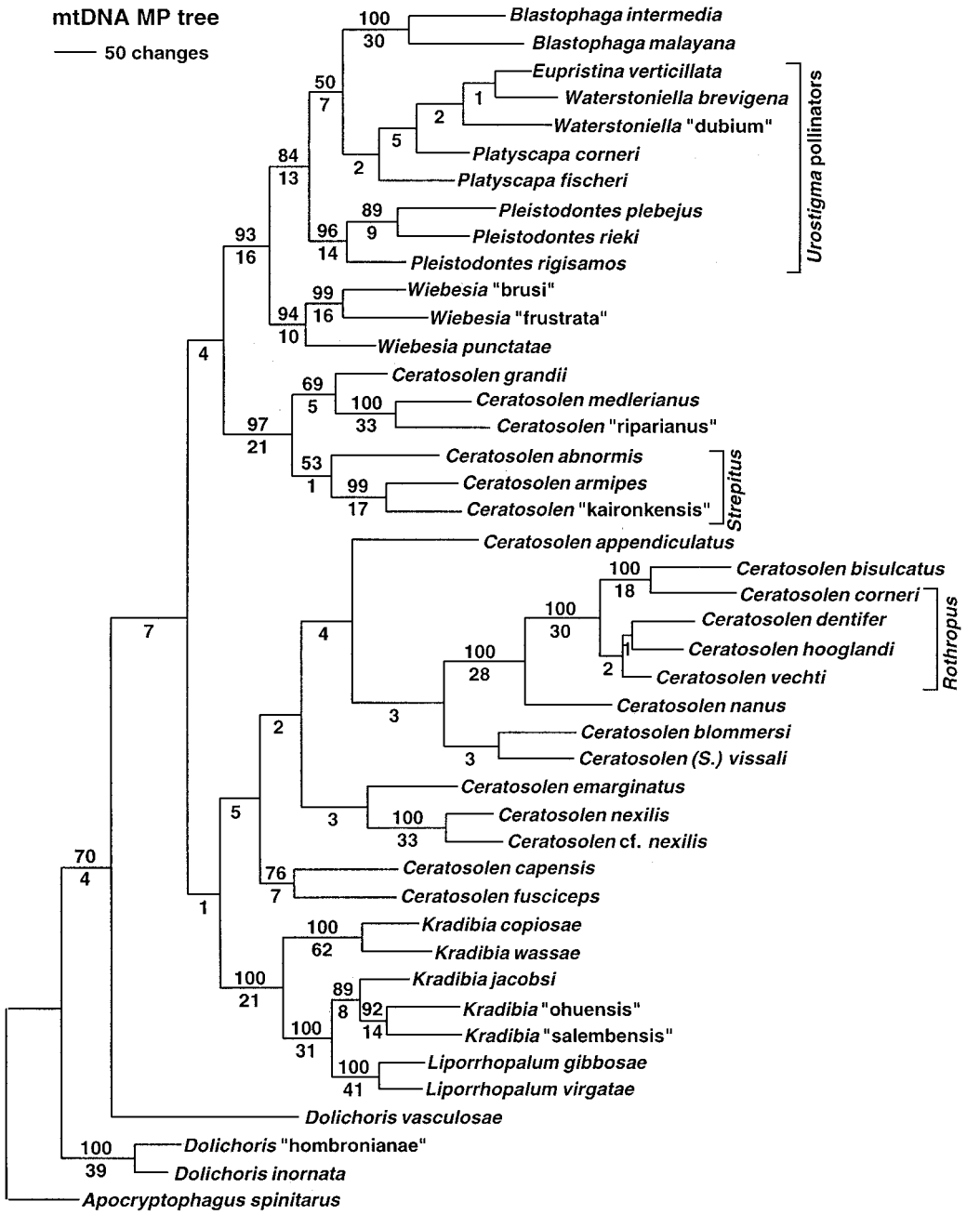

FIGURE 3. The single most-parsimonious tree for mitochondrial DNA sequences (COI and COII) from Indo-Australian pollinators of figs, rooted with nonpollinating *Apocryptophagus spinitarsus* (Sycophaginae). Bootstrap percentages and decay values excluding uninformative characters are listed above and below the branches, respectively. Branch lengths are proportional to the number of unambiguous changes.

vasculosae was 1.6 times longer than the next longest branch in the ML tree; I examined inconsistency in the placement of this species by parametric bootstrapping. Assuming null model parameters, the difference between trees with paraphyletic *Dolichoris* ($-\ln L_{H0} = 31508.66$) and polyphyletic *Dolichoris* (Fig. 4; $-\ln L_{H1} = 31484.76$) was significant ($-\ln L_{H0} + \ln L_{H_1} = 23.90$; $P = 0.02$). Thus, paraphyletic *Dolichoris* rejected the mtDNA data. The source of this inconsistency will be discussed later.

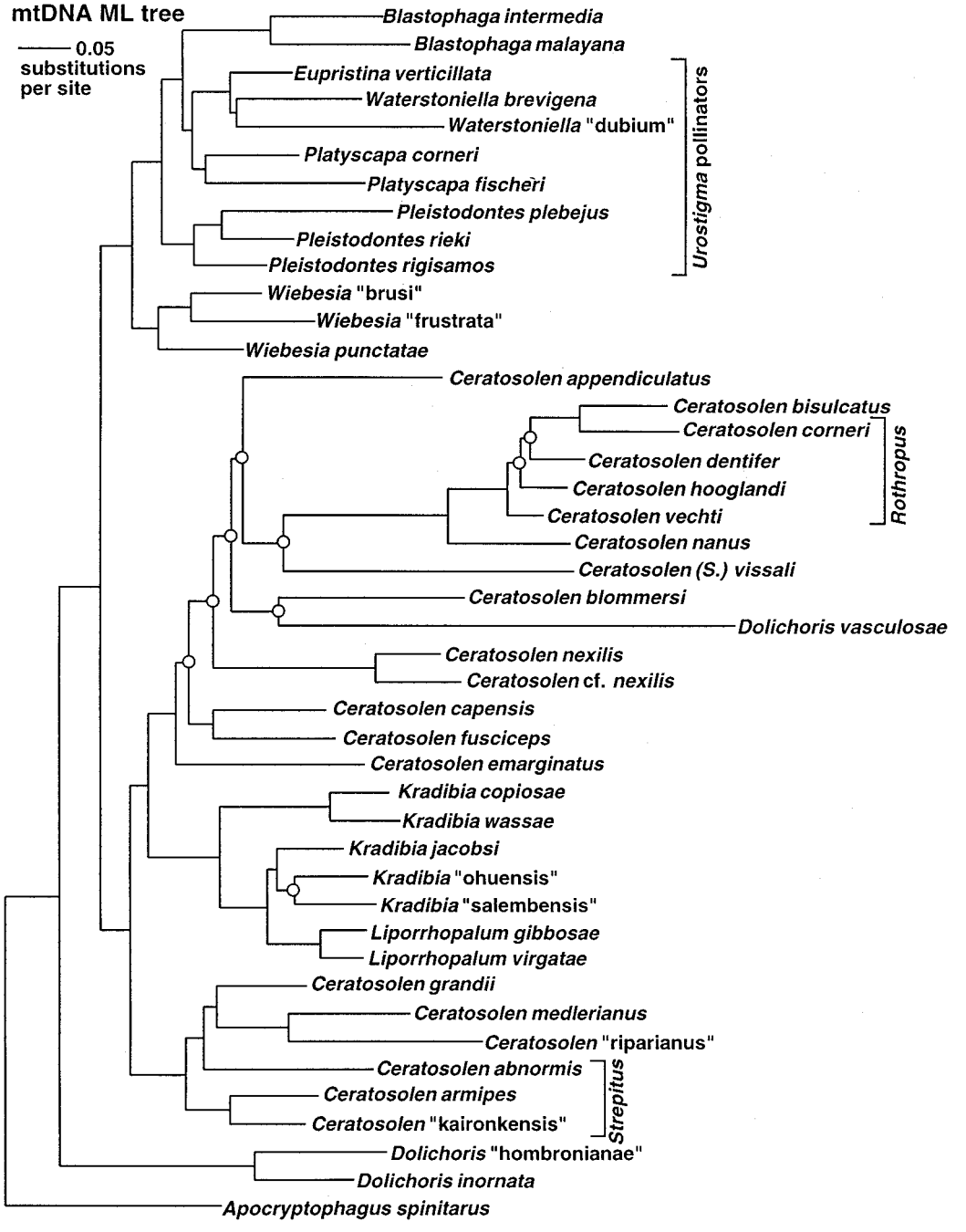

FIGURE 4. ML phylogram for mitochondrial DNA sequences (COI and COII) from Indo-Australian pollinators of figs. The MP tree (Fig. 3) was used to estimate ML parameters for the GTR+Γ model of nucleotide substitution and as a starting tree in a heuristic search. A highly similar ML topology resulted from a heuristic search starting with an NJ tree based on Tamura–Nei genetic distances for transversions only. Open circles mark conflicting nodes in the ML topologies obtained from the two searches. Branch lengths are proportional to the number of substitutions per site. The tree was rooted with nonpollinating *Apocryptophagus spinitarsus* (Sycophaginae).

Morphology

Of 22 male and 35 female features, 54 characters were phylogenetically informative. Morphological analyses yielded 256 most-parsimonious trees of 317 steps (CI = 0.28). Only nine clades were supported by >50% bootstrap values and all were present in the strict consensus tree (circled nodes in Fig. 5). Overall, morphological data supported the

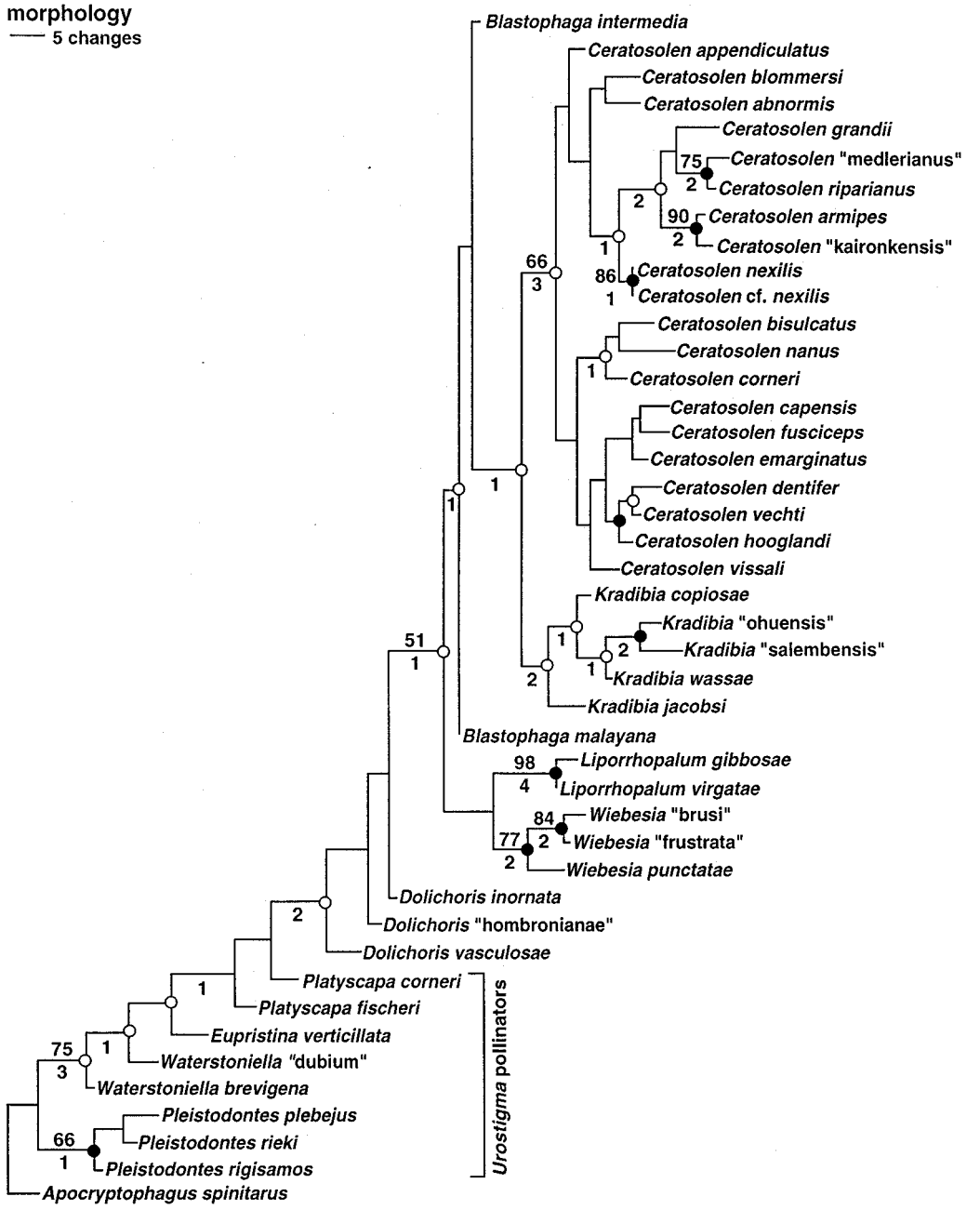

FIGURE 5. One of 256 equally parsimonious trees based on the morphology of Indo-Australian pollinators of figs. Closed circles indicate resolved nodes in the strict consensus that are congruent with the mtDNA MP tree. Open circles indicate conflicting nodes. Bootstrap percentages and decay values excluding uninformative characters are listed above and below the branches, respectively. Branch lengths are proportional to the number of unambiguous changes.

monophyly of *Ceratosolen*, *Kradibia*, *Liporrhopalum*, *Pleistodontes*, and *Wiebesia*. A major difference between the mtDNA and the morphological trees concerned the placement of the root, *Apocryptophagus spinitarus*. In the mtDNA tree (Fig. 3), *A. spinitarus* attached to *Dolichoris*, but morphology placed the outgroup in the middle of the *Urostigma* pollinators (Fig. 5). *Pleistodontes* appeared as a sister group to the rest of the Indo-Australian pollinators with 75% bootstrap support, and other genera associated with subgenus *Urostigma* appeared to be paraphyletic. The possibility of morphological convergence between *Pleistodontes* and the outgroup will be discussed. A clade consisting of all genera associated with functionally dioecious figs had 51% bootstrap support, but the relationship of *Dolichoris* to this clade was unresolved. Nor were the relationships of *Blastophaga*, *Liporrhopalum* and *Wiebesia* to the rest of the functionally dioecious fig pollinators resolved. The *Kradibia–Liporrhopalum* clade detected in mtDNA analyses was contradicted by morphology, which suggested instead a sister relationship between *Kradibia* and a moderately supported *Ceratosolen* (66%).

Conflict and Congruence

Only 9 of 23 resolved nodes in the morphological strict consensus (closed circles in Fig. 5) were in absolute agreement with the mtDNA MP tree (Fig. 3). However, the overwhelming majority of conflicting nodes were weakly supported in one analysis or the other. For example, only 3 of 26 clades with >50% bootstrap support in the mtDNA analysis were contradicted by clades in the morphological bootstrap consensus. In addition, 3 of 10 nodes supported by >50% in the bootstrap consensus were in conflict with the mtDNA MP tree. The 3 conflicting nodes in the 50% morphological bootstrap consensus included (1) the sister relationship of *Pleistodontes* to the rest of the Indo-Australian pollinators with 75% support, (2) a weakly supported clade of pollinators associated with functionally dioecious *Ficus* (51%), and (3) the monophyly of *Ceratosolen* with 66%.

An ILD test found that the sum of the tree lengths from separate analyses of mtDNA and morphological data was significantly less than the sum of tree lengths taken from random partitions of the combined data ($P = 0.01$), pointing to marked conflict between the data sets. Further ILD tests after removal of the outgroup and taxa with highly diverged mtDNA sequences (e.g., *D. vasculosae*) also showed considerable conflict. The ILD does not indicate, however, whether conflict results from different phylogenetic histories, different rates of change, or from systematic error in either data set. Wilcoxon's sum of signed ranks tests indicated that mtDNA rejected the shortest morphological trees and that morphology rejected the mtDNA tree (Table 4). However, taking into account bootstrap support in morphological analyses had a measurable impact on the results. At the $\alpha = 0.05$ significance level, mtDNA did not reject the morphological 70% or 90% bootstrap consensus trees. These findings agree with the observation that those few clades with morphological support are

TABLE 4. Wilcoxon's sum of signed ranks test results for incongruence between mtDNA, morphology, and combined data sets. The length of shortest trees (L) resulting from a heuristic search of each data set constrained by a rival topology was compared with the length of shortest trees resulting from an unconstrained search. *P*-values indicate the probability of obtaining a higher test statistic (*z*) by chance given the null hypothesis that the lengths (L) of rival trees are not different.

Comparison	L	Rank sum	*n*	*z*	*P*
mtDNA data and tree vs.					
Morphology MP tree	7616	−20463.0	612	−17.1837	<0.0001
Morphology 50% bootstrap	6858	−11354.5	238	−2.9969	0.0027
Morphology 70% bootstrap	6839	−23174.0	321	−1.7671	n.s.
Morphology 90% bootstrap	6806	0	0	—	—
Combined strict consensus	6818	−3683.0	127	−1.0525	n.s.
Morphological data and tree vs.					
mtDNA MP tree	378	−57.5	41	−4.9379	<0.0001
mtDNA 50% bootstrap	351	−76.0	31	−3.4483	0.0006
mtDNA 70% bootstrap	341	−154.5	35	−2.7213	0.0065
mtDNA 90% bootstrap	337	−253.0	41	−2.4335	0.0150
Combined strict consensus	356	−88.5	35	−3.8079	<0.0001

generally congruent with clades supported by mtDNA. In contrast, the shortest morphological tree rejected all rival topologies derived from mtDNA, including the 90% bootstrap consensus. The only case of conflict with strong support in separate analyses, that is, the attachment of the root near *Pleistodontes*, will be discussed later.

Combined Analyses

The combined analysis recovered three most-parsimonious trees of 7174 steps (CI = 0.29) that differed in the placement of *Ceratosolen* associated with section *Sycomorus* (Fig. 6). Thirty-one nodes in the combined MP tree were supported by >50% bootstrap

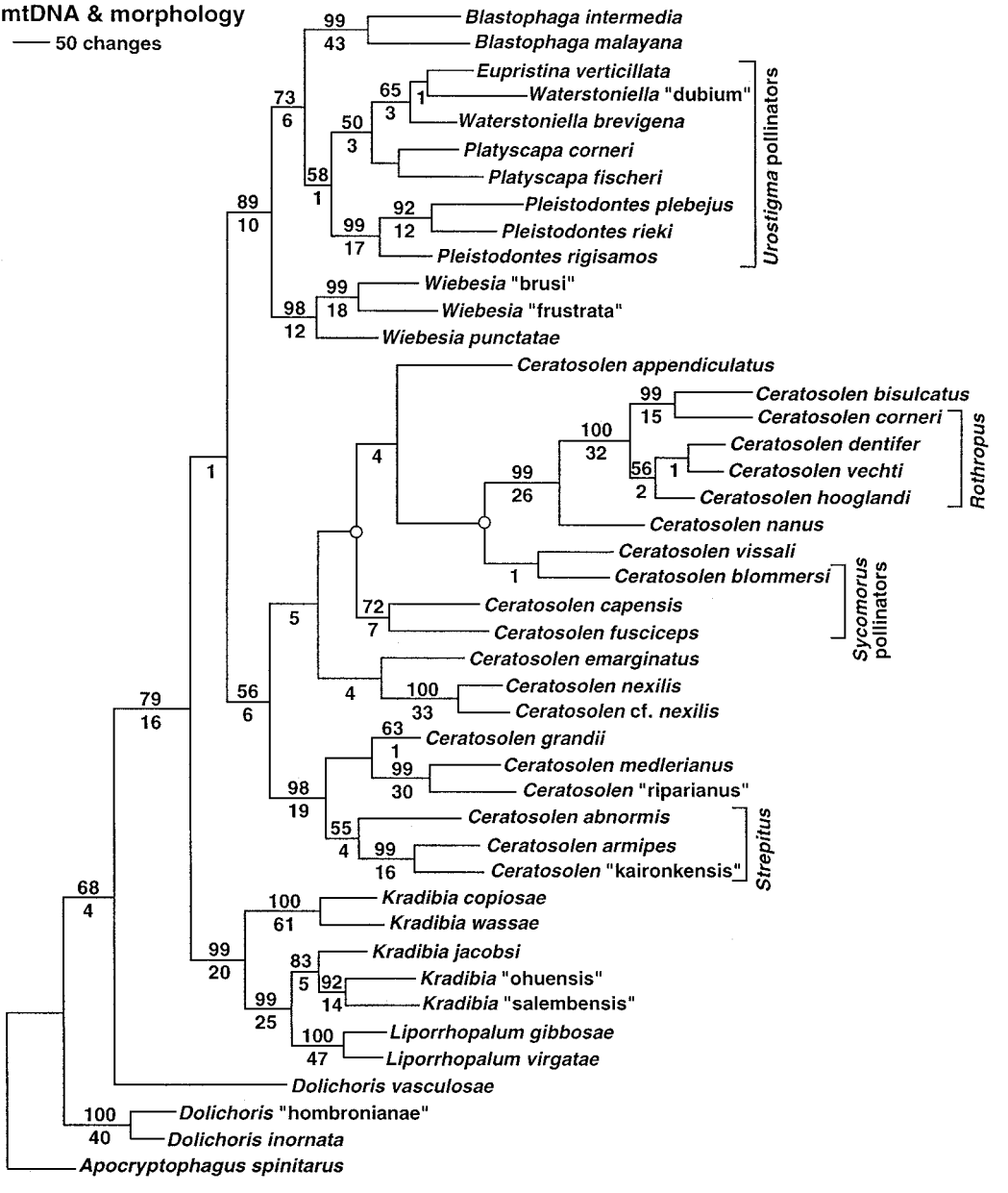

FIGURE 6. One of three equally parsimonious trees from the combined analysis of mtDNA sequences and morphology for Indo-Australian pollinators of figs. Collapsed nodes in the strict consensus are indicated by open circles. Bootstrap percentages and decay values excluding uninformative characters are listed above and below the branches, respectively. Branch lengths are proportional to the number of unambiguous changes.

values, compared with 26 and 10 nodes in the mtDNA and morphology MP trees, respectively. Clades with >50% bootstrap support in the separate mtDNA and combined analyses were generally in agreement, indicating that the addition of morphology to mtDNA data did not have a dramatic impact on clade support. For example, of 26 clades recovered in both separate mtDNA and combined analyses (Figs. 3 and 6), bootstrap support for 8 clades increased, for 10 decreased, and for 8 remained unchanged after morphology was combined with mtDNA in a single analysis.

Morphology strongly rejected a combined MP tree, whereas the mtDNA tree and the combined tree did not show significant conflict (Table 4). Thirty-one nodes in the mtDNA and combined trees (Figs. 3 and 6) were in agreement and 6 nodes were in conflict. Two of the conflicting nodes supported by >60% bootstrap in the combined tree were attributable to morphology, namely, the monophyly of *Ceratosolen* and the monophyly of the *Urostigma* pollinators.

However, two clades in the morphological 50% bootstrap consensus were not supported in the combined analysis, namely, a clade including all genera associated with functionally dioecious figs and the placement of *Pleistodontes*. The attachment of the outgroup forced *Pleistodontes* to be sister to the rest of the pollinators and the issue of rooting is discussed in terms of morphological convergence (see Discussion, Morphological Evolution).

The combined analysis supported placing *Dolichoris* in a paraphyletic relationship with respect to the rest of the Indo-Australian pollinators (Fig. 6). A *Kradibia–Liporrhopalum* clade was weakly supported as sister to the rest of the pollinators excluding *Dolichoris* (<50% bootstrap). Relationships within the *Kradibia–Liporrhopalum* clade were in complete agreement with the separate mtDNA MP tree. A clade that included *Blastophaga, Wiebesia*, and the *Urostigma* pollinators was well supported and sister to *Ceratosolen*. A monophyletic and well-supported *Wiebesia* was the sister group to the other genera in this clade and, in contrast to the mtDNA results, *Blastophaga* was sister to the *Urostigma* pollinators. *Pleistodontes* and *Platyscapa* were each monophyletic, and *Waterstoniella* appeared to be paraphyletic but weakly supported with respect to *Eupristina*. The monophyly of *Ceratosolen* had marginal support in the combined analysis (56%). Within the genus, there was strong support for a clade including *C. grandii, C. medlerianus, C.* sp. "riparianus," and subgenus *Strepitus* excluding *C. vissali*; however, support for deep relationships in *Ceratosolen* was weak. Support was strong for *C. nanus* as sister to a clade including mostly subgenus *Rothropus*, which was paraphyletic as a result of the highly supported relationship of *C. bisulcatus* to *C. corneri*. The relationship between *C. vissali* and *C. blommersi* was unexpected and will be discussed.

DISCUSSION

Issues in mtDNA Analysis

An advantage of mitochondrial DNA is that high interspecific sequence divergence provides a large pool of potentially informative characters (Simon et al., 1994). However, the inference of species phylogenies from mitochondrial gene trees can be problematic because genes and species may not share the same history (Doyle, 1992; Maddison, 1995, 1997). For instance, the potential for lineage sorting is great if the alleles shared a most recent common ancestor before speciation (Hoelzer, 1997). In this regard, Moore (1995) argued that mtDNA haplotypes are less prone to lineage sorting because of their smaller effective population sizes and shorter coalescence times than those for the alleles of nuclear genes. Introgression may also lead to incongruence between gene trees and species trees (McDade, 1995) but hybridization among pollinators of figs seems unlikely. Premating reproductive isolation of pollinator species results from host specificity, and evidence suggests hybridization is not a major force in the evolutionary history of the host plants (Weiblen, 2000).

Other explanations for discrepancies between mtDNA and species trees involve positively misleading estimates of phylogeny because of systematic error or unequal rates of molecular evolution (Cunningham, 1997a; Huelsenbeck, 1997). For example, unequal base composition and differing rates of nucleotide substitution across sites in fig wasp mtDNA are potential sources of systematic error (Simon et al., 1994). In this regard, ML has an advantage over parsimony in evaluating the fit of data to explicit models of nucleotide substitution (Swofford et al., 1996). A model assuming unequal base frequencies,

TABLE 5. Log likelihood scores of mtDNA data under alternative tree topologies for the Indo-Australian pollinators of figs. GTR+Γ model parameters were estimated separately for each codon position and then used to calculate the likelihood of the data under the MP and ML topologies (Figs. 3 and 4).

	$-\ln L_{ML}$	$-\ln L_{MP}$	Diff.	SD diff.	t	P
GTR+Γ	31484.71	31533.61	48.89	22.58	2.16	0.03
1st only	9553.95	9574.70	20.74	14.08	1.47	n.s.
2nd only	6380.79	6393.85	13.05	12.61	1.03	n.s.
3rd only	14284.62	14282.23	−2.39	7.16	0.33	n.s.
1st + 2nd + 3rd	30219.36	20250.78	31.42	22.22	1.41	n.s.

unequal rates of transitions and transversions, and unequal rates of substitution rates across sites was significantly better than simpler models (Table 3), but even the best model may not be robust to violations of its assumptions (Swofford et al., 1996).

It is encouraging, however, that the overall ML and MP results for pollinator mtDNA were very similar (Figs. 3 and 4). Huelsenbeck and Hillis (1996) demonstrated that, under simple conditions, equally weighted parsimony can be as accurate as weighted parsimony and ML for >1000 bp. Although equally weighted parsimony may not be especially sensitive to A-T bias or transition bias at third positions (Fig. 2), given the influence of a large number of potentially informative characters at first and second codon positions, parsimony may converge on the wrong tree if rates of change in different lineages are unequal (i.e., "long-branch attraction"; Felsenstein, 1978).

Particular conflicts between the MP and ML results, especially in lineages with unequal rates of substitution, invite further consideration with respect to accuracy. An interesting example concerns the longest branch, that leading to *Dolichoris vasculosae*. This species fell within *Ceratosolen* in the ML topology (Fig. 4), whereas under parsimony the species was sister to all Indo-Australian pollinators except for Papuasian *Dolichoris* (Fig. 3). Parametric bootstrapping showed that the mtDNA data were significantly more likely supportive for polyphyletic *Dolichoris* (Fig. 4) than for paraphyletic *Dolichoris*. Nor was polyphyletic *Dolichoris* an artifact of the starting tree used in heuristic searches under ML, because searches starting with MP and NJ starting trees that differed in the placement of *D. vasculosae* yielded the same result. However, the derivation of *D. vasculosae* within *Ceratosolen* is very doubtful based on morphology and host associations (see Evolution of host associations). Could it be that likelihood rather than parsimony is inconsistent in this case? Even the best available model of molecular evolution may be unrealistic (B. Chang, pers. comm.), and inadequate models may perform poorly in likelihood ratio tests (Zhang, 1999). For example, GTR + Γ assumes that base composition bias is equal across codon positions and across taxa; however, A-T bias is 14–15% greater at third positions than at first or second positions in pollinator mtDNA, and the species showed considerable heterogeneity in base composition at third positions. To examine the sensitivity of the model to this assumption, parameters were estimated separately for each position and used to compare the likelihood scores of the MP and ML trees (Figs. 3 and 4).

Models based on first and second positions agreed with the overall model in favoring the ML topology (Table 5). However, those based on third codon positions were more likely under the MP topology. This result is surprising, given that the ML topology was used to estimate the model parameters. That some codon positions favor the exclusion of *D. vasculosae* from *Ceratosolen* but others do not suggests that GTR + Γ is oversimplified for pollinator mtDNA. The likelihood of a GTR + Γ model that takes codon position bias into account is obtained by summing the log likelihood values for the three separate models (Table 5). Despite tripling the number of parameters, the new model significantly rejects GTR + Γ ($\chi^2 = 2530.70$, $df = 18$, $P < 0.0001$).

In the case of *D. vasculosae*, weighted parsimony is apparently more robust than ML to deviations from the assumption of equal base composition across codon positions. Future analyses of fig wasp mtDNA should consider new models that allow base frequencies to vary among codon positions. The effect of transition:transversion weighting under parsimony should also be examined in light

of the differing rates of these substitutions across codon positions (Fig. 2; Simon et al., 1994). A-T bias is often most extreme at third positions in insect mtDNA (Brower, 1994; Brown et al., 1994), and the influence of this bias on phylogenetic analyses ought to be explored generally. Additional sampling of taxa and nuclear genes (Brower and DeSalle, 1994) is also needed to corroborate these findings.

Conflict and Congruence in mtDNA and Morphology

Convergence in the functional traits of pollinators and their host plants might lead to inaccurate phylogenetic hypotheses if based on morphology alone (Herre et al., 1996, Machado et al., 1996), and molecular data can provide an independent source of evidence for evaluating the classification and evolution of fig wasps. Nonetheless, as shown here, mtDNA can also fail to provide an accurate estimate of species phylogeny. In the case of the fig pollinators, morphology provided less phylogenetic signal than did mtDNA, as indicated by numbers of characters, clade support, and resolution. Although the mtDNA data set included almost 20 times as many informative characters as morphology, amounts of homoplasy were comparable between the data sets ($CI_{mtDNA} = 0.29; CI_{morph} = 0.28$). The lesser phylogenetic utility of fig wasp morphology than of molecular data is not the result of rampant convergence as supposed by Herre et al. (1996) and Kerdelhue et al. (1999) but rather reflects the greater number of potentially informative characters in DNA sequence data.

The question then becomes whether a combination of mtDNA and morphology provides a more accurate phylogenetic hypothesis than does mtDNA alone (Kluge, 1989). Recent reviews have advocated a conditional approach to combining data sets, based on statistical tests of congruence (Huelsenbeck et al., 1996). In the case of fig pollinators, morphology and mtDNA were significantly incongruent according to an ILD test and some Templeton tests (Table 4). However, global tests of incongruence do not distinguish data sets with different histories from those affected by systematic error (de Querioz et al., 1995; Mason-Gamer and Kellogg, 1996; Cunningham, 1997a, 1997b; Munro and Linder, 1998). With respect to the latter possibility, Templeton's (1993) test has the advantage of considering the extent of clade support. Significant conflict between mtDNA and morphology was attributable to weakly supported clades (i.e., <70% bootstrap support; Table 4), and the unilateral rejection of mtDNA by morphology supports the notion that incongruence reflects systematic error because of homoplasy in the much smaller morphological data set. The only instance of incongruence with strong support from morphology resulted from correlated homoplasy in *Pleistodontes* (see next section). The overall similarity of mtDNA and combined trees also reflects the greater phylogenetic signal in the molecular data set. However, signal hidden in the separate analyses also could have been recovered in the combined analysis (Barrett et al., 1991). For example, *Platyscapa* was monophyletic in the combined analysis (Fig. 6) but the clade did not appear in the mtDNA MP tree (Fig. 3) or in the morphology strict consensus (Fig. 5). Some unique clades in the morphological analysis also appeared in the combined analysis (e.g., *Ceratosolen*), whereas others did not (e.g., pollinators of functionally dioecious figs). In any event, more nodes were supported by bootstrap values in the combined analysis than in either separate analysis, and thus the combined data provided the best-supported estimate of pollinator phylogeny.

Other arguments against the inclusion of morphological characters in phylogenetic analysis involve concerns about circularity (de Queiroz, 1996) and subjectivity in the delimitation of discrete states for continuous characters (Gift and Stevens, 1997). Kerdelhue et al. (1999) argued, for instance, that fig wasp morphology should be excluded from phylogeny reconstruction to avoid biased inferences of morphological evolution. However, de Queiroz (1996) correctly pointed out that inferences of character evolution depend on the assumptions of the method used and that parsimony consistently underestimates homoplasy. Excluding the characters of interest in the case of fig wasps could actually bias the results in favor of the very assumption we wish to evaluate, namely, that morphology is homoplasious and more indicative of functional constraints imposed by host associations than of phylogenetic relationships. The effect of inclusion versus exclusion was evaluated by comparing the number of changes in

putatively convergent characters on the morphological, mtDNA, and combined trees. Traits interacting closely with host morphology, such as head shape (van Noort and Compton, 1996), ovipositor length (Ramirez, 1980), and pollen pockets (Ramirez, 1978), had equal numbers of changes in separate and combined analyses, indicating that their inclusion had no effect on the inference of convergence in these traits (see next section).

Without question, analyses of pollinator morphology would benefit from improved homology assessment, just as molecular analyses benefit from improved sequence alignment and models of substitution. Ordering character states into transformation series could refine future morphological analyses (Liljeblad and Ronquist, 1998). For example, future analyses ought to consider reduction series in female wing venation, male eyes, male tarsi, and mouthparts in both sexes (Ramirez, 1978, 1991; Wiebes, 1982a). In general, further study of conflicts between mtDNA and morphology would benefit from considerations of bias in both morphological and mtDNA evolution through new weighting schemes and models of substitution. In the meantime, the best-supported topology will be used to discuss morphological evolution, classification, and the evolution of host associations (Fig. 6).

Morphological Evolution

A single character, the length of the ovipositor relative to the abdomen, was correlated with the breeding system of the host fig (Fig. 7). As noted by Ramirez (1980), ovipositors longer than the abdomen tend to be associated with monoecious figs, whereas ovipositors shorter than the abdomen tend to be associated with functionally dioecious figs. For example, the ancestral ovipositor of *Ceratosolen* was short but three different lineages within *Ceratosolen* have evolved longer ovipositors in association with monoecious figs. Figure 8 also suggests that a shift from monoecious section *Oreosycea* to functionally dioecious *Ficus* was followed by shifts back to monoecious hosts in subgenus *Urostigma* and section *Sycomorus*. In that scenario, pollinators of functionally dioecious figs gave rise to the pollinators of subgenus *Urostigma*, but functionally dioecious figs did not give rise to subgenus *Urostigma*. This conflict between pollinator and host phylogeny is noteworthy, although the host clade of interest is not strongly supported (Weiblen, 2000). If functional dioecy in *Ficus* is related to the interaction of fig styles and pollinator ovipositors (Fig. 1), then the coadaptation of these traits could play an important role in the stability of the mutualism. This is a promising area for future comparative studies.

Morphological evolution inferred from the combined phylogeny supports the view of Wiebes (1982a) that pollinators of figs show trends toward the reduction and loss of multiple features including mouthparts (character nos. 43 and 44 in Appendix), tarsi (nos. 53 and 54), male eyes (no. 42), and female wing venation (no. 31). The Indo-Australian pollinators are distinguished from paraphyletic *Dolichoris* by at least six unambiguous morphological changes. In females, for example, the maxillary palpus is lost (no. 13), whereas in males the mouthparts are reduced to a maxillolabial complex (no. 42) and the mesonotum is separated from the metanotum (no. 47).

Wiebes (1982b) regarded the similarity of the female head in *Dolichoris*, *Tetrapus*, and *Pleistodontes* as pleisiomorphic. Interestingly, the morphological analysis suggested that *Pleistodontes* is sister to the Indo-Australian pollinators, a position akin to that of *Tetrapus* in the global mtDNA study (Machado, 1998), in which *Pleistodontes* was not near *Tetrapus*. Combined analyses strongly suggest convergence between *Pleistodontes* and the outgroup in six different features of the head (Fig. 7). For example, in females, the facial groove fitting the antennae (no. 4) is closed in both genera, and the scape (no. 6) and pedicel (no. 7) are elongate. Convergence in these correlated characters placed the root near *Pleistodontes* in the morphological analysis, the only strongly supported case of incongruence between morphology and mtDNA (75% bootstrap in Fig. 3). *Tetrapus* and *Pleistodontes* are also convergent in four of these features, as well as the elongation of the female head (no. 1) and in the modification of the mandibular appendage. Convergent head shapes in African pollinating and nonpollinating fig wasps have been related to ostiole morphology (van Noort and Compton, 1996) and, in the case of *Tetrapus* and *Pleistodontes*, the possibility of similar ostioles in sections *Pharmacosycea* and *Malvanthera* ought to be

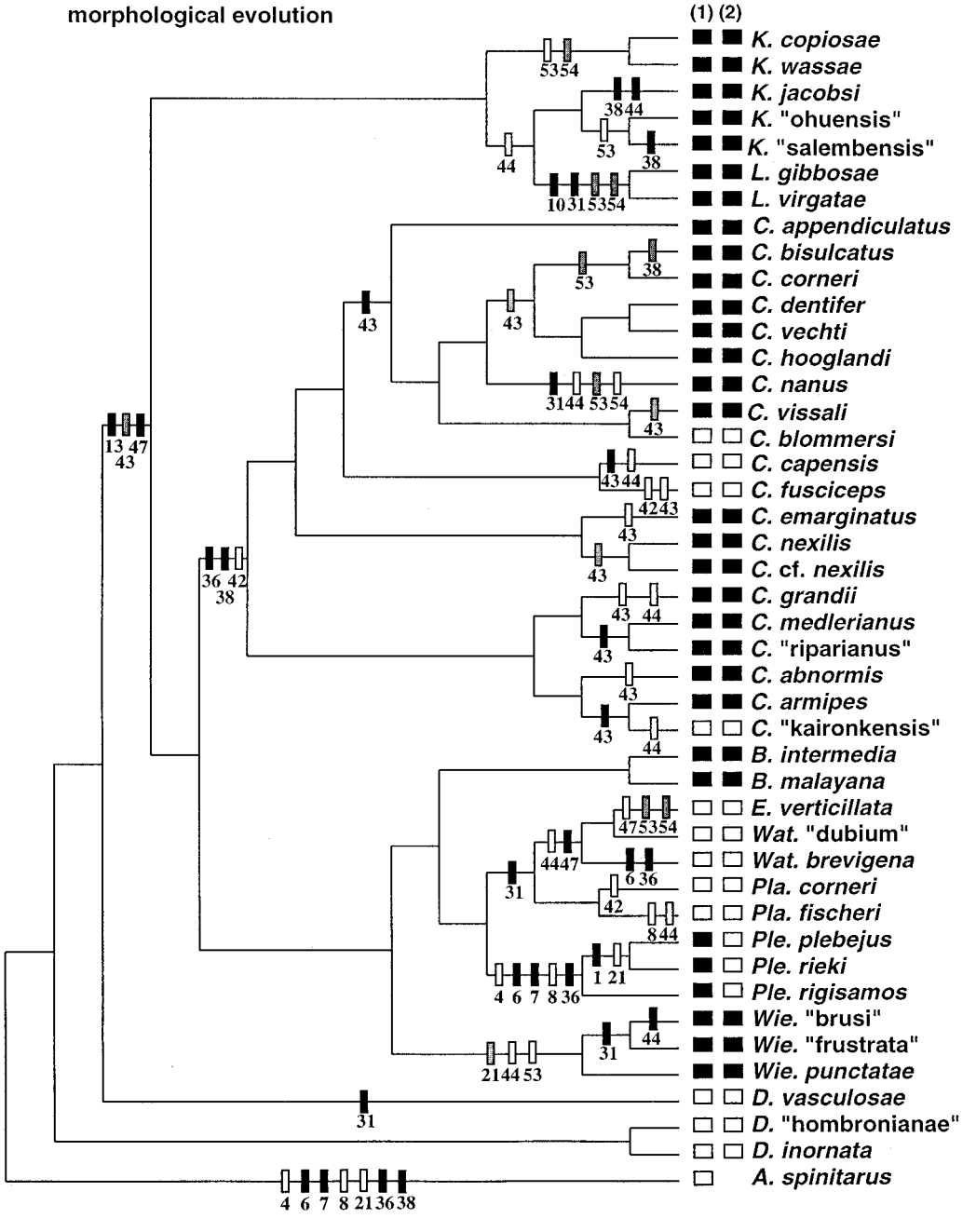

FIGURE 7. Evolution of some morphological features in Indo-Australian pollinators of figs reconstructed on one of the shortest trees from the combined analysis of mtDNA and morphology. Numbered bars on the branches refer to characters in the Appendix; open, closed, and shaded vertical bars indicating shifts to states 0, 1, and 2, respectively. States for ovipositor length (1) and fig breeding system (2) are indicated by bars to the left of the taxon names. The ovipositor is either longer than the abdomen (open bars) or shorter than the abdomen (closed bars). Fig breeding systems are either monoecious (open bars) or functionally dioecious (closed bars).

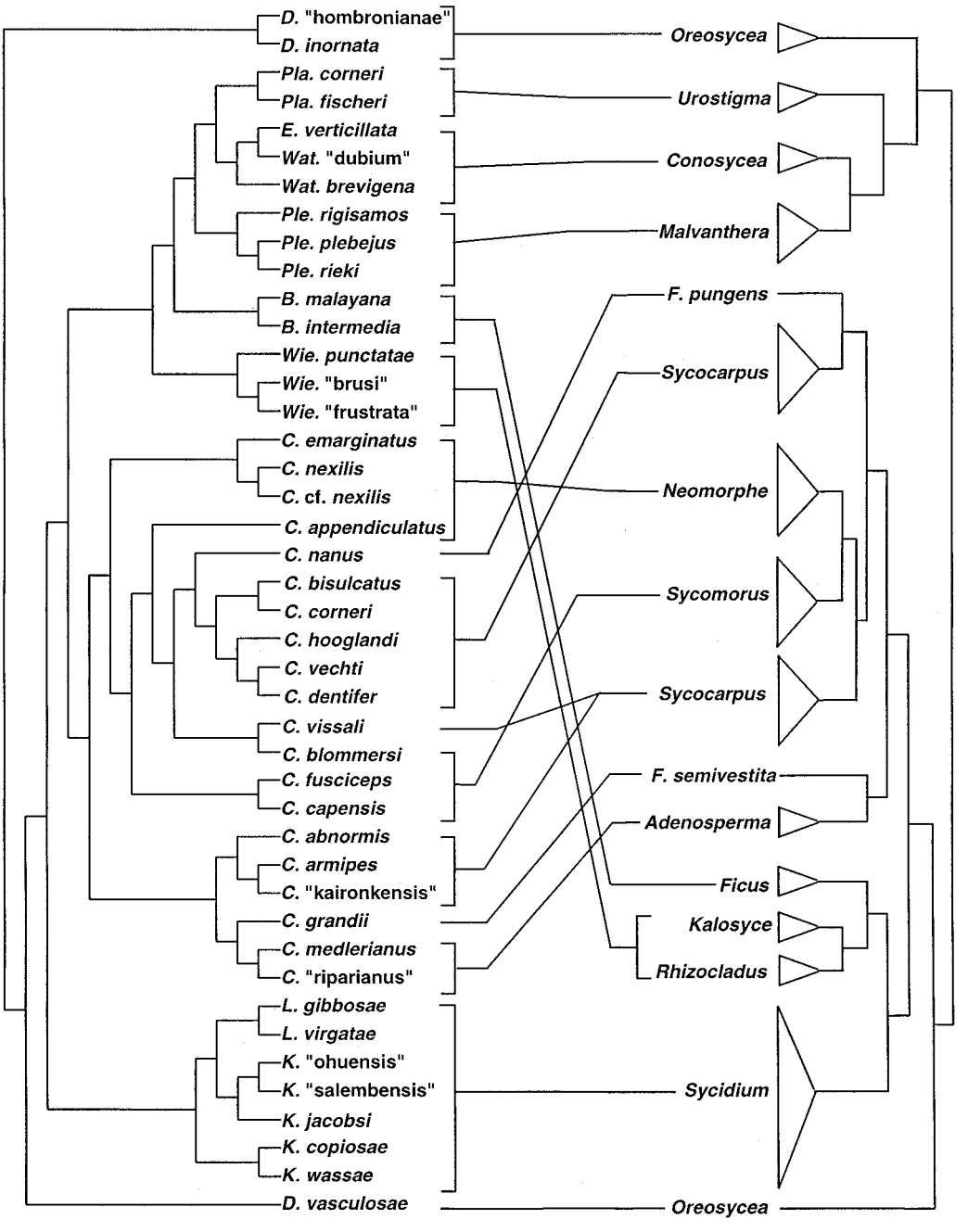

FIGURE 8. Evolution of host associations in Indo-Australian pollinators of figs. One of the shortest trees from the combined analysis of mtDNA and morphology is shown in relation to a phylogeny of *Ficus* inferred from nuclear ribosomal DNA sequences and morphology (Weiblen, 2000). Groups of pollinators marked by brackets are uniquely associated with *Ficus* sections in the classification of Corner (1965). In the majority of cases, pollinator groupings and their host sections are monophyletic.

explored. The elaboration of other features, including the female antennae and mesosternal pockets, have been proposed to reflect adaptation to host figs (Ramirez, 1978, 1991), and similar adaptations to functional constraints imposed by different hosts have indeed occurred independently in different lineages (vanNoort and Compton, 1996). Most morphological features in Figure 7 showed patterns of homoplasy that provide a basis for testing adaptation hypotheses. On the other hand, morphological apomorphies are useful tools for identification. For example, *Liporrhopalum* can be recognized by the elongate funicle in females (no. 10) and *Ceratosolen* by the trilobate clypeus in males (no. 38).

Classification of Fig Wasps Pollinating Functionally Dioecious Ficus

The combination of mtDNA and morphology provides new insights on the classification and proposed phylogenetic relationships of fig wasps pollinating the functionally dioecious figs (Boucek, 1988; Wiebes, 1994a). Contrary to Ramirez' (1991) proposal, the five genera known to pollinate functionally dioecious figs do not belong to a clade. Instead, evidence strongly supports a close relationship between functionally dioecious–pollinating *Blastophaga* and the pollinators of monoecious subgenus *Urostigma* (Wiebes, 1994a). The combined results also indicate that pollinators of functionally dioecious figs are paraphyletic with respect to the pollinators of monoecious *Sycomorus* (Fig. 6). Three clades pollinating only the functionally dioecious figs correspond to *Blastophaga*, *Wiebesia*, and *Kradibia–Liporrhopalum*. The *Kradibia–Liporrhopalum* clade was not detected in earlier morphological analyses (Ramirez, 1978, 1991; Wiebes, 1982a), but mtDNA and combined analyses indicate that *Kradibia* is paraphyletic with respect to *Liporrhopalum*. The combined results also generally agree with the global phylogenetic analysis of fig pollinators (Machado, 1998). For example, the pollinators of subgenus *Urostigma* and most genera were monophyletic in both studies. However, the position of *Dolichoris* requires special consideration. In contrast to findings in the present study, previously this genus appeared to be closely related to the pollinators of subgenus *Urostigma*, but the only sample was taken from a light trap and its host association is unknown (Machado, 1998). Inclusion of pollinators reared from vouchered host plants minimizes problems associated with unknown or misidentified hosts. Despite this discrepancy, the extent of agreement between studies based on different samples of taxa is encouraging for future phylogenetic studies of fig wasps.

Evolution of Host Associations

Studies of phytophagous insects often reconstruct the evolution of host associations in light of phylogeny. Futuyma et al. (1993), for example, used such a reconstruction to demonstrate that genetic constraints have influenced the evolution of host associations in *Ophraella* (Coleoptera: Chrysomelidae). This approach was also used by Brown et al. (1994) to argue that speciation in the yucca moth family Prodoxidae (Lepidoptera: Incurvarioidea) resulted from multiple host shifts. In the case of fig pollination, the specificity of associations has been suggested to be indicative of host conservatism (Ramirez, 1974; Corner, 1985; Wiebes, 1987). Reconstructing ancestral associations bears directly on the question because host shifts result in homoplasy, whereas cospeciation maintains host conservatism. Wiebes (1994b) relied on the classification of *Ficus* as a guide in the placement of wasp species within genera, which raises the possibility that congruence with host associations could be a taxonomic artifact. However, the case for host conservatism is strengthened by evidence of pollinator clades that are associated with host clades. Such evidence is all the more compelling when clades without previous taxonomic designation show such patterns.

The host associations of pollinators showed less homoplasy (CI = 0.80 for host sections) than most mtDNA or morphological characters, and agreement between pollinator and fig classifications (Corner, 1965; Wiebes, 1994a) is also supported. However, a direct comparison of pollinator phylogeny with a phylogeny for *Ficus* based on nuclear ribosomal DNA sequences and morphology is even more informative (Weiblen, 2000). Monophyletic genera of pollinators that are uniquely associated with host sections include *Blastophaga* with section *Ficus*, *Platyscapa* with section *Urostigma*, and *Pleistodontes* with section

Malvanthera. The apparent conservatism of host associations in each of the three clades could be spurious if the taxonomic limits of pollinator genera were set by host classification. The *Eupristina–Waterstoniella* clade pollinating section *Conosycea* and the *Kradibia–Liporrhopalum* clade pollinating section *Sycidium* provide stronger evidence of conservatism in this respect. Also, monophyletic *Wiebesia* is associated with a clade that includes sections *Rhizocladus* and *Kalosyce*, and Papuasian *Wiebesia* is restricted to section *Rhizocladus*.

Two examples from *Ceratosolen* illustrate how botanical classification can confuse the inference of shifts in pollinator associations. *Ceratosolen* and its three subgenera were sampled intensively because of the complexity of their host associations. For example, *C. grandii* pollinates *F. semivestita* in section *Neomorphe*, whereas *F. semivestita* is more closely related to section *Adenosperma* than to section *Neomorphe* (Fig. 8). Wiebes (1963) recognized that the absence of cerci in male genitalia and the fusion of three apical segments of the female antennae into a club were similar features between *C. grandii* and *C. appendiculatus*, another pollinator of section *Neomorphe*. However, separate and combined analyses demonstrate that *C. grandii* is more closely related to the pollinators of section *Adenosperma* than to *C. appendiculatus*; moreover, both morphological characters are very homoplasious (CI = 0.09 and 0.18, respectively) and appear to have converged in the two pollinator species. A revised classification placing *F. semivestita* in section *Adenosperma* would alter the inference of an ancestral host shift between sections *Neomorphe* and *Adenosperma* to yet another case of host conservatism.

Another kind of mistaken identity having a strong effect on inferences of host-switching involves *C. nanus*, the pollinator of *F. pungens* in section *Sycidium*. Wiebes (1963) asserted the pollinator belonged to *Ceratosolen* despite its association with *Kradibia*-pollinated section *Sycidium*. From fig classification alone, one would conclude that the *C. nanus* lineage switched from section *Sycocarpus* to section *Sycidium*. However, host phylogeny indicates that *F. pungens* is more closely related to section *Sycocarpus* than to section *Sycidium* (Fig. 8). Correcting the spurious placement of *F. pungens* in a revised classification of *Ficus* would change the switching scenario for *C. nanus* to one of host conservatism. These examples illustrate the importance of considering the potential effect of host phylogeny on inferences about the conservatism or lability of pollinator associations.

The pollinators of *Sycomorus* present an additional problem with regard to the evolution of host associations. Figure 8 suggests two shifts from functionally dioecious *Ficus* to monoecious *Sycomorus* in *Ceratosolen*. Given that *Sycomorus* is monophyletic (Weiblen, 2001), *C. blommersi* might seem to represent a host switch. However, the sister relationship of *C. blommersi* from Madagascar and *C. vissali* from the Solomon Islands is poorly supported and questionable given the geography (Kerdelhue et al., 1999). Such an implausible relationship could result from the failure of phylogenetic methods or mtDNA to accurately reflect species phylogeny. In addition to having the longest terminal branch in the genus (Fig. 6), these species were sister to a *Rothropus* clade showing an accelerated rate of nucleotide substitution. The possibility of inconsistency in the placement of *C. blommersi* and *C. vissali* could be explored in future studies by using parametric bootstrapping methods with more realistic substitution models (Huelsenbeck and Hillis, 1996) or additional sampling.

In addition to laying the groundwork for an improved classification, the combined phylogenetic results for pollinators and their hosts provide a framework for studying aspects of the coevolutionary process. In particular, the pollinators of functionally dioecious figs are not monophyletic, and evolutionary changes in ovipositor length are correlated with shifts in fig breeding system. The correlated evolution of interacting morphologies sometimes but not always contributes to inaccurate phylogenetic inferences; similarly, complex patterns of nucleotide substitution can pose problems for phylogeny reconstruction from fig wasp mtDNA. Pollinator mtDNA provides more phylogenetic signal than does morphology, although the strength of support in combined analyses is encouraging for further phylogenetic studies in the group based on multiple sources of evidence. This study has also shown how inferences regarding ancestral host associations can address the question of host conservatism. The pollinators of figs showed little evidence of ancestral host switching,

but more precise comparisons of fig and pollinator phylogenies are needed to test the cospeciation hypothesis.

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APPENDIX. MORPHOLOGICAL CHARACTERS FOR A PHYLOGENETIC ANALYSIS OF POLLINATING FIG WASPS

The rationale for delimiting character states is described in Weiblen (1999). The character matrix for 44 species is deposited in TREEbase (http://www.herbaria.edu/treebase.index/html). Characters 1–35 refer to females and 36–57 refer to males.

1. Head (0) less than twice as long as wide or (1) twice or more as long as wide.
2. Ocelli: (0) three or (1) two.
3. Epistomal margin or clypeus (0) rounded, (1) with a pointed median, (2) bilobate, or (3) trilobate.
4. Facial groove (0) closed or (1) open.
5. Antennae with (0) nine segments, (1) ten segments, or (2) eleven or more segments.
6. Scape (0) $< 2\times$ as long as wide or (1) $> 2\times$ as long as wide.
7. Pedicel (0) as long as wide or (1) elongate.
8. Pedicel (0) with < 10 or (1) with >10 recurved axial spines.
9. Third antennal segment (0) without a pointed apex, (1) with a pointed apical process, or (2) with a pointed apical appendage.
10. Funicular segments (0) $< 3\times$ as long as wide or (1) $> 3\times$ as long as wide.
11. Funicular segments (0) with sensilla linearia or (1) with sensilla chaetica.
12. Sensilla (0) in one row, (1) in two rows, or (2) in three rows.
13. Maxilla (0) with a palpus, (1) with subapical setae, or (2) atrophied.
14. Labium (0) with two or more subapical setae, (1) with one subapical seta, or (2) without setae.
15. Mandibular appendage (0) horizontal in orientation and appended to the mandible or (1) subvertical in orientation and fused to the mandible.
16. Mandible with (0) one or (1) two apical teeth.
17. Mandible with (0) one or (1) two glands.
18. Number of ridges on mandible (0) four or less, (1) five, (2) six, (3) seven, or (4) eight or more.
19. Number of ventral lamellae on mandibular appendage (0) four, (1) five, (2) six, (3) seven, (4) eight, (5) nine, (6) ten, (7) eleven, or (8) twelve or more.
20. Ventral lamellae (0) not produced into teeth or (1) produced into teeth.
21. Mesosternal pollen pockets (0) absent, (1) present, or (2) present but evidently closed.
22. Mesoscutum (0) entire or (1) with a longitudinal groove along the median.
23. Front coxae (0) smooth, (1) with combs, or (2) with corbiculae.
24. Sternal corbiculae (0) absent or (1) present.
25. Fore tibia with (0) two, (1) three, (2) four, or (3) more than four dorso-apical teeth.
26. Midleg with (0) five or (1) four tarsal segments.
27. Ventral spines on the first tarsomere (0) absent or (1) present.
28. Hind coxae (0) without or (1) with a groove for the reception of the tibia.
29. Antaxial tooth in the hind tibia (0) bicuspidate or (1) tricuspidate.
30. Axial tooth in the hind tibia (0) simple or (1) bicuspidate.
31. Forewing venation (0) complete or (1) obsolete beyond the submarginal vein.
32. Forewing with (0) two, (1) three, (2) four, (3) five or more pustules scattered along the stigmal vein, or (4) without pustules.
33. Spiracular peritremata (0) small and subcircular or (1) large and ovoid.
34. Hypopygium (0) without or (1) with a row of hyaline setae.

35. Ovipositor (0) shorter than the abdomen or (1) longer than the abdomen.

36. Head (0) less long than wide or (1) more wide than long.

37. Head (0) without or (1) with dorsal spines.

38. Epistomal margin or clypeus (0) without lateral lobes, (1) bilobate, or (2) trilobate with a distinct medial prominence.

39. Antennae (0) inserted in separate grooves on either side of prominent scrobes or (1) in a common medial groove toward the front of the head.

40. Antennae with (0) four, (1) five, or (2) seven segments.

41. Antennae with (0) slender or (1) clavate (club-shaped) apical segments.

42. Eyes (0) shorter than the cheek or (1) as long as or longer than the cheek.

43. Mouthparts with (0) a distinct labium and maxillae, (1) with a reduced maxillolabial complex, or (2) without a labium and maxillae.

44. Maxillolabial complex (0) without or (1) with setae.

45. Mandibular glands (0) one or (1) two.

46. Pronotum (0) less long than wide or (1) more wide than long anteriorly.

47. Mesonotum (0) entire or (1) fused to the metanotum (i.e., the dorsal part of the metathorax).

48. Metanotum or dorsal part of the metathorax (0) entire or (1) fused to the propodeum.

49. Propodeal peritremata (0) less than half as long as the propodeum or (1) enlarged (as long as the propodeum).

50. Fore tibia with (0) two, (1) three, (2) four, or (3) five dorso-apical teeth.

51. Fore tibia with (0) one, (1) two, or (2) three ventro-apical teeth.

52. Fore tarsi (0) one, (1) two, (2) three, (3) four, or (4) five.

53. Midleg (0) atrophied, (1) with oligomerous tarsi, or (2) complete with five tarsi.

54. Hind leg with (0) three, (1) four, or (2) five tarsi.

55. Armature of the hind tibia (0) with two bicuspidate teeth, (1) with a bicuspidate axial tooth and an antaxial tooth, (2) without teeth, or (3) with a single tooth.

56. Tarsi (0) without or (1) with plantar protuberances.

57. Genitalia (0) without or (1) with clawed claspers.