The Lack of Pollinator Specificity in a Dioecious Fig Tree: Sympatric Fig-pollinating Wasps of *Ficus septica* in Southern Taiwan

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ABSTRACT

Although species specificity between fig trees and their pollinators has been considered a classic example of obligate mutualism, increasing exceptions to the one-to-one relationship suggest that multiple pollinator species per fig host species may be a pervasive phenomenon. Based on both mitochondrial DNA and microsatellite analyses, we found three putative pollinator species (*Ceratosolen* spp.) associated with *Ficus spetica* in southern Taiwan, two of which, that differ in color, routinely coexist within single figs. These three pollinators are substantially divergent from the pollinator of *F. spetica* in New Guinea, implying that wasp diversity may be highly underestimated when the one-to-one rule is assumed. Our findings not only provide the first case of coexistence of pollinators within single figs on a shared dioecious *Ficus* host, but also an ideal system to investigate interspecific competition and sex allocation, especially when coexisting pollinators are visually distinguishable by their colors.

Abstract in Chinese is available at http://www.blackwell-synergy.com/loi/btp.

Key words: *Ceratosolen*; coexistence; fig wasp; pollination.

THE OBLIGATE MUTUALISM between fig trees (Moraceae, *Ficus*) and their pollinating wasps (Hymenoptera, Chalcidoidea, Agaonidae) has generally been regarded as a classic system for studying coevolution (*e.g.*, Marussich & Machado 2007, Jousselin *et al.* 2008). The genus *Ficus* is characterized by the enclosed inflorescence (syconium) that renders its pollination solely dependent on wasps; the fig-pollinating wasps, in turn, rely on the host flowers for reproduction. Traditionally, each *Ficus* species is assumed to associate with a single specific pollinating wasp. The validity of the 'one-to-one rule', however, has recently been challenged as exceptions are continually being documented (Ware & Compton 1992, Michaloud *et al.* 1996, Kerdelhue *et al.* 1997, Molbo *et al.* 2003, Machado *et al.* 2005, Haine *et al.* 2006, Jackson *et al.* 2008, Peng *et al.* 2008, Su *et al.* 2008).

The departure from species specificity has most commonly been found where multiple pollinator (sub-)species exist in geographical isolation from one another across the host's distribution range (Weiblen 2002). However, molecular diagnosis has revealed many morphologically undetected yet genetically divergent pollinators (or 'cryptic species') that exist in sympatry and even within the same fig (Molbo *et al.* 2003, Haine *et al.* 2006, Su *et al.* 2008; see also this study), indicating that the partnership of fig trees and their pollinators is more complicated than previously thought. The coexistence of multiple pollinator species thus raises questions concerning the evolutionary relationships among these wasps, their potential for hybridization within the same fig, interspecific competition for resources within figs, and sex-ratio adjustment un-

der the local mate competition theory (LMC; Hamilton 1967, Herre 1985, Raja *et al.* 2008). For example, if multiple pollinators coexist within single figs, one may be interested to know whether heterospecific foundresses act as foundresses of the same pollinator species in reducing the intensity of LMC (*i.e.*, competition between related males).

Here we report another exception to the one-to-one rule in *Ficus septica* Burm. f. in subtropical Taiwan. *Ficus septica* is a dioecious fig tree that is widely distributed throughout lowland Taiwan (< 1000 m asl), neighboring Green and Lanyu Islands (Tzeng 2004), as well as in SE Asia and in Queensland, Australia (Liao 1996). Across its distributional range, *F. septica* is thought to be associated with two allopatric pollinator subspecies *Ceratosolen bisulcatus bisulcatus* Mayr and *Ceratosolen bisulcatus jucundus* Grandi (Rasplus 1994, Weiblen 2002). Although *C. b. jucundus* is considered the sole pollinator of *F. septica* in Taiwan (Chen & Chou 1997), our genetic analyses—based on both mitochondrial and nuclear marker systems—revealed three putative pollinator species that exist sympatrically and two of which routinely coexist within single figs in southern Taiwan. Potential scenarios for the origin of multiple pollinator species of *F. septica* in southern Taiwan are discussed.

METHODS

SAMPLE COLLECTION AND DNA PREPARATION.—Pollinating wasps (*Ceratosolen* spp.) of *F. septica* were collected from 2004 through 2006 in Taiwan (25°00′ N, 121°31′ E) and Lanyu Islands (22°04′ N, 121°31′ E; Fig. 1). We collected 1–12 mature male figs (D phase; Galil & Eisikowtich 1968) from each tree, and placed them
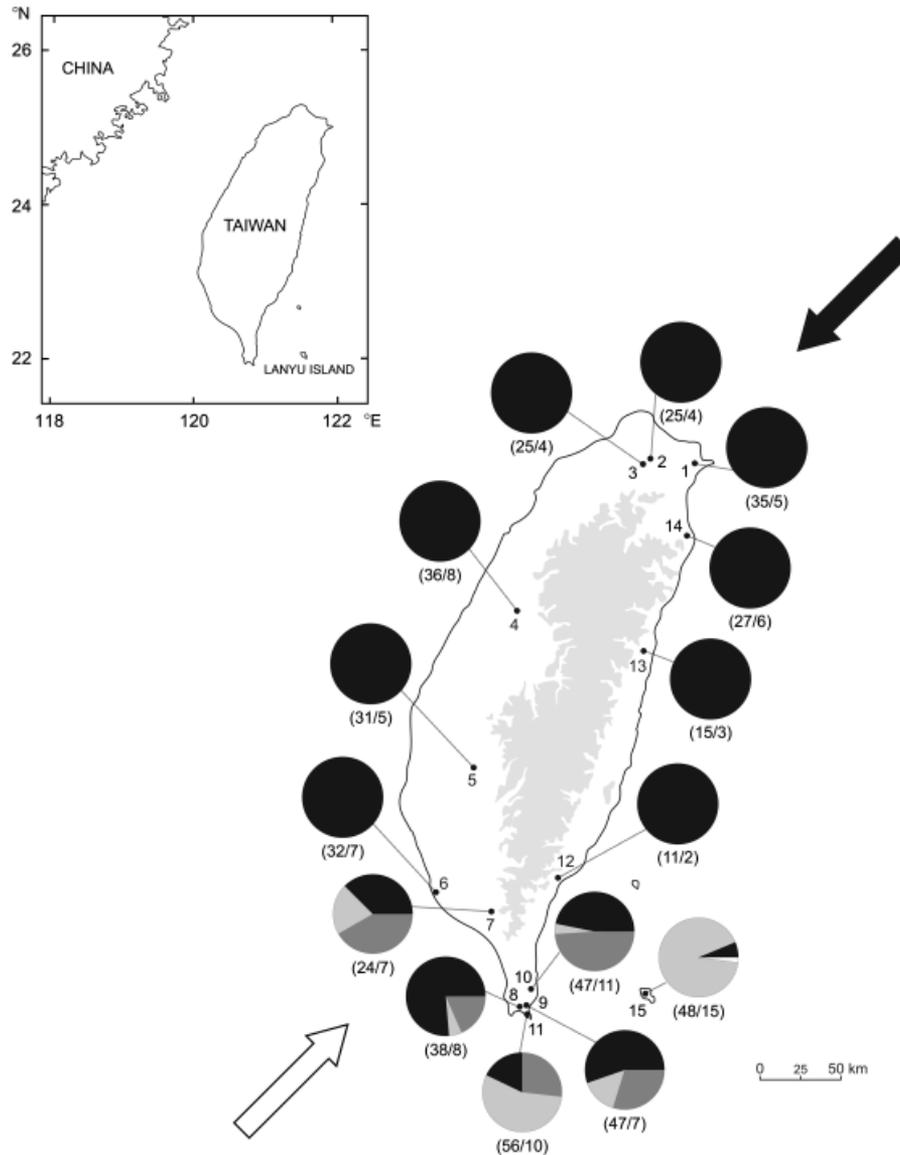
FIGURE 1. The proportion of figs that are used by different pollinators in Taiwan: *Ceratosolen* sp. 1 (black), tentative *C. bisulcatus jucundus* (light gray), *C. b. jucundus*+ *C.* sp. 1 (dark gray) and *C.* sp. 2 (white). The solid arrow indicates the northeasterly monsoon during winter, while the open arrow indicates the southwesterly monsoon during summer. Locality codes correspond to numbers in Table S1. Sample sizes (the number of figs/the number of trees) in each location are indicated within parentheses beneath the pie diagram.

in separate mesh-covered containers. Adult female pollinating wasps were then allowed to emerge naturally. Because we observed two color morphs (black and yellow) of female pollinating wasps in southern Taiwan, the color of individuals emerging from each fig was recorded. Of all the wasps that emerged from each fig, one specimen of each color morph was used for molecular analysis. Genomic DNA was extracted individually using the modified LiCl method (Gemmell & Akiyama 1996). The other *Ceratosolen* sp. in Taiwan (*i.e.*, *Ceratosolen wui*, the pollinator of *Ficus bengutense*; Chen & Chou 1997, Tzeng 2004) was also sampled and served as outgroup for phylogenetic analysis. Our wasp collections were deposited at the Insect Specimens Museum of Taiwan Forestry Re-

search Institute (TAIF, Taipei, Taiwan) and National Museum of Natural Science (NMNS, Taichung, Taiwan).

PCR AMPLIFICATION.—A 1052 bp region of the mitochondrial cytochrome oxidase *c* subunit I (*COI*) gene was amplified and sequenced for 280 wasps following the protocols described in Lin *et al.* (2008) (Table S1). Sequences proofread by eye were aligned with the aid of the software SEQUENCHER 4.2 (Gene Codes, Ann Arbor, Michigan, U.S.A.) and deposited in GenBank (Accession Number: EF439899–EF440119 and EF440121–EF440181). Wasps were also genotyped at seven unlinked microsatellite loci: WCAG8, WCAG67, WGATA5, WGATA29, WGATA39, WGATA42 (Lin

et al. 2007), and CB3-61 (Zavodna *et al.* 2005a), following the amplification conditions described in Lin *et al.* (2007). Alleles were scored manually with the aid of the software GENETIC PROFILER 2.2 (Amersham Biosciences, Sunnyvale, California, U.S.A.).

DATA ANALYSES.—To understand the seasonal effect on sampling of the two coexisting morphs, we divided the time period during which the wasps were collected into six sampling seasons: fall 2004 (September–November 2004), summer 2005 (June–August 2005), fall 2005 (September–November 2005), winter 2005 (December 2005–February 2006), spring 2006 (March–May 2006), and summer 2006 (June–August 2006; Table S1). For wasps sampled in southern Taiwan, where coexistence of pollinators within single figs was observed, a chi-square test, as implemented in the software JMP 7.0 (SAS Institute, Cary, North Carolina, U.S.A.), was performed to check for seasonal differences in the number of sampled coexisting pollinators.

The phylogenetic relationships among mitochondrial haplotypes of these wasps were reconstructed by a maximum likelihood (ML) algorithm using PHYML-approximate likelihood-ratio tests (aLRT) 1.1 (Guindon & Gascuel 2003, Anisimova & Gascuel 2006) under the K81uf+I+G model as suggested by MODELTEST 3.06 (Posada & Crandall 1998), where the proportion of invariable sites (I) was 0.6201 and the γ shape parameter (*a*) was 0.5719. Estimated base frequencies were $A = 0.3498$; $C = 0.0674$; $G = 0.1326$; $T = 0.4502$. Statistical support for clades was assessed by aLRT based on a Shimodaira–Hasegawa-like procedure (Anisimova & Gascuel 2006) and by bootstrap analysis (500 replicates), as implemented in PHYML-aLRT. To examine whether pollinating wasps of *F. septica* in Taiwan were monophyletic, one published sequence of *C. bisulcatus* (the pollinator of *F. septica* in New Guinea) (GenBank accession no. AF200375, Weiblen 2001) was included in the phylogenetic analysis. Two published sequences (*C. corneri*, AF200386 and *C. dentifer*, AF200387, Weiblen 2001) and the sequence of *C. wui* were used as outgroups to root the phylogenetic tree. For microsatellite data, to visualize the pattern of genetic differentiation, a factorial correspondence analysis (FCA) of microsatellite genotypes was performed using GENETIX 4.05 (Belkhir *et al.* 2004). In addition, F_{ST} value between coexisting wasps was estimated with 10,000 permutations using ARLEQIN 3.01 (Excoffier *et al.* 2005).

Genetic polymorphisms between the two pollinator morphs in southern Taiwan were compared. Parameters of genetic diversity, including the number of haplotypes (K), haplotype diversity (*h*), nucleotide diversity (π; the average number of nucleotide differences per site between two sequences; Li 1997), and the number of polymorphic sites, were calculated using the software DNASP 5.0 (Librado & Rozas 2009). For microsatellite data, the level of polymorphism in each coexisting wasp was measured as the number of alleles per locus (N_A), observed heterozygosity (H_O), and heterozygosity expected from Hardy–Weinberg assumptions (H_E) using the software CERVUS 2.0 (Marshall *et al.* 1998). As the number of detected alleles is highly dependent on sample size, we further calculated allelic richness (A_R) by using the software FSTAT 2.9.3.2 (Goudet 2001). Tests for departures

from Hardy–Weinberg expectations were examined using GENE-POP 3.4 (Raymond & Rousset 1995) and a Bonferroni correction was applied to calibrate bias arising from multiple comparisons (Rice 1989). Differences in genetic polymorphisms between these two morphs were assessed using nonparametric Mann–Whitney *U*-tests.

RESULTS

COEXISTENCE OF TWO POLLINATING WASPS OF *F. SEPTICA* IN SOUTHERN TAIWAN.—We conducted a thorough sampling across Taiwan and Lanyu Islands: a total of 497 fig fruits were collected from 102 male fig trees, representing 11–70 pollinating wasps (*Ceratosolen* spp.) for each of 15 locations. We observed two color morphs of pollinating wasps associated with *F. septica* in Taiwan: black morphs were found throughout Taiwan, while yellow morphs were only found at six locations in southern Taiwan: Laiyi, Kenting Forest Recreation Area, Sheding Park, Nanranshan, Eluanbi, and Lanyu Island (locality codes 7, 8, 9, 10, 11, and 15; Fig. 1). Among 260 figs collected from southern Taiwan, 27 percent were used by both morphs, which usually emerged within the same day (R.-C. Lin, pers. obs.), 38 percent were used by black morphs and the remaining 35 percent were used by yellow morphs, both exclusively (Fig. 1). These two color morphs were also morphologically distinguishable in terms of the shapes of their heads and antennae (J.-Y. Rasplus, pers. comm.). We tentatively named the yellow morph *C. b. jucundus* because their morphological characteristics matched those originally described for *C. b. jucundus* (Grandi 1927).

The black morphs were sampled mostly in fall 2005, while *C. b. jucundus* (the yellow morphs) were sampled mostly in spring 2006 (Table S1). Few wasps were sampled in winter ($N = 5$ and 0 for black morphs and *C. b. jucundus*, respectively); none of the pollinators was found in southern Taiwan during winter. Considering the pollinators in southern Taiwan only, sampling of the two coexisting morphs was significantly different among sampling seasons ($\chi^2 = 54.95$, $P < 0.001$), indicating that the ratio of the number of *C. b. jucundus* to black morphs varied with the seasons.

MOLECULAR PHYLOGENETIC RELATIONSHIP AMONG POLLINATORS.— The ML tree (Fig. 2) revealed that the fig-pollinating wasps of *F. septica* in Taiwan formed a well-supported clade (aLRT = 0.96/ bootstrap = 90). These wasps were divided into three deep lineages, however, one consisting of all except one black morph, the second consisting of all *C. b. jucundus* individuals, and the last lineage composed of a single black wasp sampled from Lanyu Island. This suggests that the black wasps may represent two undescribed species: *Ceratosolen* sp. 1 (*C.* sp. 1) which is commonly distributed around Taiwan and Lanyu Islands, and *C.* sp. 2 that is only found on Lanyu Island.

GENETIC DIFFERENTIATION AMONG POLLINATORS AT MICROSATELLITE LOCI.—F_{ST} between two coexisting pollinating wasps, *C. b. jucundus* and *C.* sp. 1, was significantly greater than zero ($F_{ST} = 0.56$, $P < 0.001$), indicating significant genetic differentiation. Similarly, the results of FCA revealed three well-defined groups of individuals
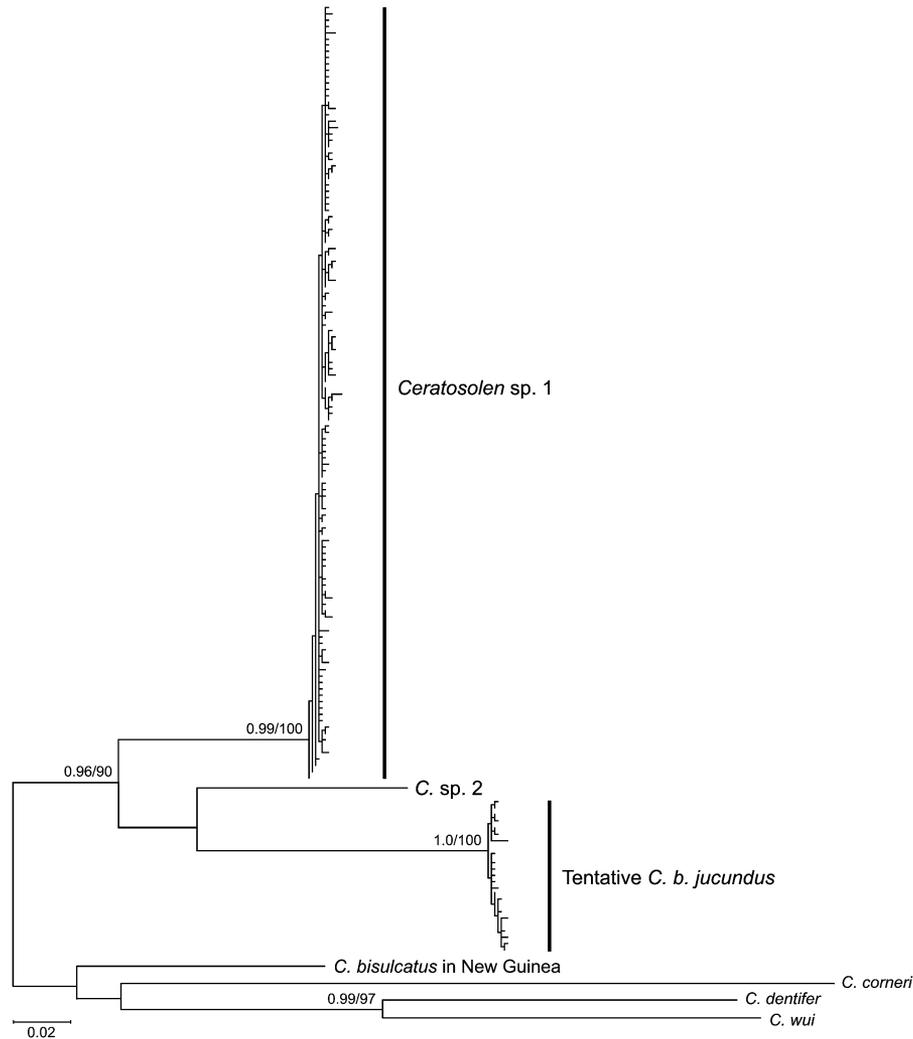
FIGURE 2. Maximum Likelihood tree reconstructed based on mtDNA *COI* gene of pollinating wasps of *Ficus septica* in Taiwan and their outgroups using a K81uf+I+G distance model. Branch support values (aRLT statistics/500 bootstrap replicates) are shown above the nodes.

in good agreement with the mitochondrial grouping into *C. b. jucundus*, *C.* sp. 1 and *C.* sp. 2 (Fig. S1). The first two axes of the FCA explained 15.6 percent of the total genetic variance: 10.2 percent and 5.44 percent for the first and second axes, respectively.

GENETIC POLYMORPHISMS WITHIN POLLINATORS.—*Ceratosolen* sp. 2 was not subjected to genetic polymorphism analysis due to a limited sample size ($N = 1$). The *COI* alignment (1052 bp) from 59 *C. b. jucundus* individuals revealed 24 polymorphic sites, which defined 26 haplotypes (Table 1); 10 haplotypes were shared by more than 1 (up to 10) individual, while 16 were unique to single individuals. Based on the sequences of 90 *C.* sp. 1 individuals sampled in southern Taiwan, 42 polymorphic sites and 53 haplotypes were identified (Table 1); 9 haplotypes were shared by multiple (up to 12) individuals, while 44 were unique to single individuals. Haplotype diversity was significantly lower for *C. b. jucundus* than for *C.* sp. 1 (Mann–Whitney *U*-test, $P < 0.05$), but not so for nucleotide diversity. Microsatellite characteristics for six locations in southern Taiwan of *C. b. jucundus*

and *C.* sp. 1 are shown in Table S2. In total, 93 alleles were amplified at seven microsatellite loci—33 and 63 alleles for *C. b. jucundus* ($N = 158$) and *C.* sp. 1 ($N = 164$), respectively; only three alleles were shared between the coexisting pollinators. The number of alleles at each locus ranged from two to nine and from four to 16 for *C. b. jucundus* and *C.* sp. 1, respectively. Levels of allelic richness calculated based on the minimum sample size ($N = 9$ and 3 for *C. b. jucundus* and *C.* sp. 1, respectively) ranged from 1.0 to 5.7 for *C. b. jucundus* and from 1.0 to 5.0 for *C.* sp. 1. Twenty-one of 84 locus-location comparisons (9 and 12 comparisons for *C. b. jucundus* and *C.* sp. 1, respectively) showed significant deviations from Hardy–Weinberg expectations ($P < 0.05$); moreover, four comparisons (two comparisons each for *C. b. jucundus* and *C.* sp. 1) were still significant after Bonferroni's correction ($P < 0.0012$). For these two pollinators, the mean observed heterozygosities were lower than the expected from Hardy–Weinberg assumptions, which may be due to the sib-mating system in fig-pollinating wasps (Molbo *et al.* 2004; Zavodna *et al.* 2005b, 2007; Lin *et al.* 2008). The mean expected heterozygosities,

TABLE 1. *Nucleotide polymorphism of a 1052 bp region of mtDNA* COI *gene for six locations in southern Taiwan of* Ceratosolen bisulcatus jucundus *and* C. *sp. 1. Sample size (*N*), number of haplotypes (*K*), haplotype diversity (*h*), nucleotide diversity (*π*) and number of polymorphic sites are shown.*

	C. b. jucundus					*Ceratosolen* sp. 1				
Sampling site	*N*	*K*	*h*	*π*	Number of polymorphic sites	*N*	*K*	*h*	*π*	Number of polymorphic sites
7. Laiyi	9	5	0.722	0.00127	4	13	12	0.987	0.00246	11
8. Kenting Forest Recreation Area	8	6	0.929	0.00329	10	16	15	0.992	0.00325	17
9. Sheding Park	7	6	0.952	0.00362	12	16	15	0.992	0.00299	16
10. Nanrenshan	12	4	0.742	0.00181	5	26	17	0.957	0.00283	18
11. Eluanbi	12	7	0.879	0.00305	10	16	10	0.900	0.00211	11
15. Lanyu Island	11	9	0.964	0.00259	9	3	3	1.000	0.00507	8
Overall	59	26	0.935	0.00310	24	90	53	0.967	0.00285	42

however, were significantly lower in *C. b. jucundus* (0.15–0.38) than in *C.* sp. 1 (0.42–0.51) (Mann–Whitney *U*-test, $P < 0.05$).

DISCUSSION

With a detailed survey across Taiwan and Lanyu Islands, we found three genetically distinctive taxa in two color morphs of fig-pollinating wasps associated with *F. septica*. Coexisting species, *C. b. jucundus* and *C.* sp. 1, showed substantial mitochondrial divergence (uncorrected *p*-distance = 7.8%) and high genetic differentiation at microsatellite loci ($F_{ST} = 0.56$), indicating the presence of reproductive isolation despite their frequent coexistence within the same fig. The emergence times of *C. b. jucundus* and *C.* sp. 1, however, seem to overlap—when coexisting within the same fig, both pollinators usually emerged within the same day (R.-C. Lin, pers. obs.). Moreover, both *C. b. jucundus* and *C.* sp. 1 were sampled in each of six sampling seasons (except for fall 2004), implying that their coexistence occurs across seasons.

In general, *C. b. jucundus* showed less genetic diversity than *C.* sp. 1, which may be related to the restricted distribution of *C. b. jucundus* to southern Taiwan. Many terrestrial organisms in Taiwan show an east–west split, due to the mountain ranges that run north-south across the island (*e.g.*, Toda *et al.* 1998, Huang *et al.* 2002, Lin 2003, Yeh *et al.* 2004, Oshida *et al.* 2006); however, there is no apparent east-west barrier in Taiwan to preclude *C. b. jucundus* from dispersing northward. Additionally, long-distance dispersal of fig-pollinating wasps has been well documented (Nason *et al.* 1998, Harrison 2003, Zavodna *et al.* 2005a, Harrison & Rasplus 2006, Ahmed *et al.* 2009). Specifically, the distance of 40–60 km across open sea might not limit dispersal of the pollinators of *F. septica* (Zavodna *et al.* 2005a, Lin *et al.* 2008). Such long-distance dispersal is believed to be achieved passively by wind (Ware & Compton 1994a, b; Ahmed *et al.* 2009) and may be facilitated by strong monsoons or irregular typhoons, such as the northeasterly monsoon during the cold season (September–April) and the southwesterly monsoon during the warm season (May–August) in Taiwan (Jan *et al.* 2002, Chen & Chen 2003; Fig. 1). Based on the wind patterns, the northeasterly monsoon winds would prevent *C. b.*

jucundus from dispersing northward, but the southwesterly monsoon winds would facilitate northward expansion. If long-distance dispersal is mediated predominantly by the southwesterly monsoon, however, *C. b. jucundus* would be distributed throughout Taiwan Island, instead of being restricted to southern Taiwan. Therefore, the restricted distribution of *C. b. jucundus* to the southern part of this island does not seem to be due to the presence of geographic barriers or to its dispersal ability, but rather some other limiting factors.

We suggest that the clear north–south difference in pollinator species diversity across Taiwan may be partially attributed to the thermal adaptation of the two pollinator species. The average annual temperature (2003–2007) in northern Taiwan is approximately 1–2°C lower than that in southern Taiwan (Taipei: 25°02′23″ N, 121°30′24″ E, 23.4°C; Taichung: 24°08′51″ N, 120°40′ 33″ E, 23.6°C; Hengchun: 22°00′20″ N, 120°44′17″ E, 25.4°C; data retrieved from Central Weather Bureau, Taipei, Taiwan). Interestingly, the north–south distribution pattern found in *C. b. jucundus* and *C.* sp. 1 is also found in other animal taxa (*e.g.*, skinks and butterflies) which are thought to be temperature sensitive (S.-M. Lin & Y.-F. Hsu, pers. comm.). For example, although the South China forest skink (*Sphenomorphus incognitus*) and the Indian forest skink (*Sphenomorphus indicus*) both utilize similar habitats, *S. incognitus* is restricted to southern Taiwan, while *S. indicus* is widely distributed throughout lowland Taiwan and is sympatric with *S. incognitus* in southern Taiwan (Zhu 2002). Also, for two butterflies (*Copera nadina* and *Copera nerissa*) feeding on the same host plants (*Capparis* spp.), *C. nerissa* is restricted to southern Taiwan, while *C. nadina* is sympatric in southern Taiwan and extends its range to the north (Shirozu 1960). Furthermore, genetically based differences such as the 2–3°C of difference in thermal preference have been documented to explain the ecologically isolated distribution between two *Drosophila* species (Matute *et al.* 2009). Consequently, the restricted distribution of *C. b. jucundus* is likely due to the genetically based physiological constraints, rather than extrinsic factors such as the environmental wind direction (but see Ahmed *et al.* 2009).

Even more intriguing, *C.* sp. 2 found in Lanyu Island, which is difficult to be discriminated from *C.* sp. 1 morphologically, differed from *C.* sp. 1 by an uncorrected nucleotide difference of 7.2 percent. This finding is similar to other studies on cryptic fig-pollinating wasp species (Molbo *et al.* 2003, Haine *et al.* 2006, Su *et al.* 2008). Compared with the fact that all previously published cryptic fig-pollinating wasps are the pollinators of monoecious figs, however, our study provides the first case revealing coexistence of pollinators within single figs on a shared dioecious *Ficus* host. Therefore, such coexistence may be independent of the fig breeding system and more pervasive than imagined previously. Additionally, *C. bisulcatus* in New Guinea differs from fig-pollinating wasps of *F. septica* in Taiwan by 8.4–8.8 percent sequence divergence, implying that *C. bisulcatus* in New Guinea should be an evolutionary independent taxon. Taken together, the diversity of fig-pollinating wasps is highly underestimated when an exclusive one-to-one host-pollinator relationship is assumed.

We here propose three scenarios that may account for the lack of species-specific association between *F. septica* and its pollinating wasps in Taiwan: colonization by other pollinators, host shift, and *in situ* speciation. First, coexistence of multiple divergent taxa may be due to colonization by other *F. septica*-associated pollinators from neighboring areas. Colonizer could have dispersed from the Ryukyu Islands of Japan and/or the Philippine Archipelago, as a result of long-distance dispersal facilitated by seasonal monsoons. This scenario also implies speciation in geographically isolated pollinators across the distribution range of *F. septica*. Such a speciation event, consistent with the view that high insularity and natural fragmentation in the Oriental region may favor pollinator differentiation (Rasplus 1994), is supported by our findings of genetic differences between *F. septica*-associated pollinators in Taiwan and *C. bisulcatus* in New Guinea. Second, *F. septica* in Taiwan may have multiple pollinators due to host shifts. Host shifts have been proposed for the lack of specificity between fig trees and their pollinating wasps (*e.g.*, Michaloud *et al.* 1996, Molbo *et al.* 2003, Machado *et al.* 2005, Su *et al.* 2008); however, an extensive phylogenetic study of *F. septica*-associated pollinators from different geographical regions and other *Ficus*-associated pollinators in Taiwan is required to examine monophyly of these pollinators in order to adequately address this hypothesis in an evolutionary framework. The third scenario we propose is that the presence of multiple pollinating wasps of *F. septica* in Taiwan may be due to an *in situ* speciation event. By applying the substitution rate of 1.9×10^{-8} substitutions per site per year assumed for the *COI* gene in fig-pollinating wasps (Machado *et al.* 2001), the uncorrected nucleotide divergences (7.2–7.8%) between the *F. septica*-associated pollinators in Taiwan suggested that these pollinators diverged from each other roughly 1.9–2.0 million years ago (mya). Geological studies revealed that Taiwan was uplifted by the collision of the Eurasian plate and the Philippine Sea plate *ca* 5 mya, followed by the rapid rise of the mountain ranges 1–3 mya (Teng 1990, Huang *et al.* 1997, Liu *et al.* 2000). Vicariance events resulting from orogenic uplift may have prompted the differentiation of *F. septica*-associated pollinators. Also, habitat fragmentation and vegetational changes caused by climatic oscillations during the past 3 million years (Hewitt 2000) may have also facilitated differ-

entiation of fig-pollinating wasps in Taiwan. In addition, divergence among pollinators may be partially due to random genetic drift and/ or natural selection, such as differential selection on temperature tolerance. This scenario seems unlikely, however, given the substantial dispersal distance of pollinating wasps and the relatively small geographical scale of Taiwan Island (< 400 km in length).

In conclusion, we conducted a geographically and genetically comprehensive survey that has seldom been done on fig-pollinating wasps and, accordingly, we found the coexistence of multiple pollinating wasp species associated with *F. septica* in southern Taiwan. Our findings provide not only the first case of coexistence of pollinators within single figs on a dioecious host, but also an ideal system to study the ecological niche differentiation resulting from competition within a confined and resource-limited environment, as well as sex ratio adjustment under LMC, as such studies may be more practicable when coexisting wasps are visually distinguishable by their colors.

ACKNOWLEDGMENTS

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

TABLE S1. *The number of* Ceratosolen bisulcatus jucundus, C. *sp. 1 and* C. *sp. 2 used in mitochondrial* COI *gene sequencing and microsatellite genotyping for each sampling locality and their sampling seasons.*

TABLE S2. *Characteristics of seven microsatellite loci for six locations in southern Taiwan of* Ceratosolen bisulcatus jucundus *and* C. *sp. 1.*

FIGURE S1. FCA of three pollinators of *Ficus septica* in Taiwan based on seven microsatellite loci. A total of 560 wasps are included: 401 individuals of *Ceratosolen* sp. 1, 158 *C. bisulcatus jucundus* and one *C.* sp. 2.

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LITERATURE CITED

AHMED, S., S. G. COMPTON, R. K. BUTLIN, AND P. M. GILMATION. 2009. Wind-borne insects mediate directional pollen transfer between desert fig trees 160 kilometers apart. Proc. Natl. Acad. Sci. 106: 20342–20347.

ANISIMOVA, M., AND O. GASCUEL. 2006. Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. Syst. Biol. 55: 539–552.

BELKHIR, K., P. BORSA, L. CHIKHI, N. RAUFASTE, AND F. BONHOMME. 2004. GENETIX 4.05, logiciel sous Windows pour la genetique des populations. Laboratoire Genome, Populations, Interactions, CNRS UMR 5171, Universite de Montpellier II, Montpellier, France.

CHEN, C.-H., AND L.-Y. CHOU. 1997. The blastophagini of Taiwan (Hymenoptera: Agaonidae: Agaoninae). J. Taiwan Mus. 50: 113–154.

CHEN, C.-S., AND Y.-L. CHEN. 2003. The rainfall characteristics of Taiwan. Mon. Wea. Rev. 131: 1323–1341.

EXCOFFIER, L., G. LAVAL, AND S. SCHNEIDER. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evol. Bioinform. Online 1: 47–50.

GALIL, J., AND D. EISIKOWTICH. 1968. On the pollination of *Ficus sycomorus* in East Africa. Ecology 49: 259–269.

GEMMELL, N. J., AND S. AKIYAMA. 1996. An efficient method for the extraction of DNA from vertebrate tissues. Trends Genet. 12: 338–339.

GOUDET, J. 2001. FSTAT. A program to estimate and test gene diversities and fixation indices (version 2.9.3). Updated from Goudet (1995). Jerome Goudet, Lausanne, Switzerland. Available at http://www.unil.ch/izea/softwares/fstat.html.

GRANDI, G. 1927. Hyménoptères sycophiles récoltés aux iles Philippines par C.F. Baker, i. Agaonini. Phillipp. J. Sci. 33: 309–329.

GUINDON, S., AND O. GASCUEL. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52: 696–704.

HAINE, E. R., J. MARTIN, AND J. M. COOK. 2006. Deep mtDNA divergences indicate cryptic species in a fig-pollinating wasp. BMC Evol. Biol. 6: 83.

HAMILTON, W. D. 1967. Extraordinary sex ratios. Science 156: 477–488.

HARRISON, R. D. 2003. Fig wasp dispersal and the stability of a keystone plant resource in Borneo. Proc. R. Soc. Lond. B 270: S76–S79.

HARRISON, R. D., AND J.-Y. RASPLUS. 2006. Dispersal of fig pollinators in Asian tropical rain forests. J. Trop. Ecol. 22: 631–639.

HERRE, E. A. 1985. Sex ratio adjustment in fig wasps. Science 228: 896–898.

HEWITT, G. M. 2000. The genetic legacy of the Quaternary ice ages. Nature 405: 907–913.

HUANG, C.-Y., W.-Y. WU, C.-P. CHANG, S. TSAO, P. B. YUAN, C.-W. LIN, AND X. KUAN-YUAN. 1997. Tectonic evolution of accretionary prism in the arc-continent collision terrain of Taiwan. Tectonophysics 281: 31–51.

HUANG, S. S. F., S.-Y. HWANG, AND T.-P. LIN. 2002. Spatial pattern of chloroplast DNA variation of *Cyclobalanopsis glauca* in Taiwan and East Asia. Mol. Ecol. 11: 2349–2358.

JACKSON, A. P., C. A. MACHADO, N. ROBBINS, AND E. A. HERRE. 2008. Multilocus phylogenetic analysis of neotropical figs does not support cospeciation with the pollinators: The importance of systematic scale in fig/wasp cophylogenetic studies. Symbiosis 45: 57–72.

JAN, S., J. WANG, C.-S. CHERN, AND S.-Y. CHAO. 2002. Seasonal variation of the circulation in the Taiwan Strait. J. Mar. Syst. 35: 249–268.

JOUSSELIN, E., S. VAN NOORT, V. BERRY, J.-Y. RASPLUS, N. RONSTED, J. C. ERASMUS, AND J. M. GREEFF. 2008. One fig to bind them all: host conservatism in a fig wasp community unraveled by cospeciation analyses among pollinating and nonpollinating fig wasps. Evolution 62: 1777–1797.

KERDELHUE, C., M. E. HOCHBERG, AND J.-Y. RASPLUS. 1997. Active pollination of *Ficus sur* by two sympatric fig wasp species in West Africa. Biotropica 29: 69–75.

LI, W.-H. 1997. Molecular evolution. Sinauer Associates, Sunderland, Massachusetts.

LIAO, J.-C. 1996. Moraceae. *In* Editorial Committee of the Flora of Taiwan. (Ed.). Flora of Taiwan, Vol. 2 (2nd Edition), pp. 177–180. Editorial Committee of the Flora of Taiwan, Taipei, Taiwan.

LIBRADO, P., AND J. ROZAS. 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451–1452.

LIN, R.-C., C.-C. HUANG, AND S.-H. LI. 2007. Development and characterization of microsatellite markers for a fig-pollinating wasp (*Ceratosolen* sp. 1) of *Ficus septica*. Mol. Ecol. Notes 7: 1360–1362.

LIN, R.-C., C. K.-L. YEUNG, AND S.-H. LI. 2008. Drastic post-LGM expansion and lack of historical genetic structure of a subtropical fig-pollinating wasp (*Ceratosolen* sp. 1) of *Ficus septica* in Taiwan. Mol. Ecol 17: 5008–5022.

LIN, S.-M. 2003. Phylogeny and phylogeographic studies of *Takydromus* in Taiwan and adjacent regions (Squamata: Lacertidae). PhD dissertation, National Taiwan Normal University, Taipei, Taiwan.

LIU, T.-K., Y.-G. CHEN, W.-S. CHEN, AND S.-H. JIANG. 2000. Rates of cooling and denudation of the Early Penglai Orogeny, Taiwan, as assessed by fission-track constraints. Tectonophysics 320: 69–82.

MACHADO, C. A., E. JOUSSELIN, F. KJELLBERG, S. G. COMPTON, AND E. A. HERRE. 2001. Phylogenetic relationships, historical biogeography and character evolution of fig-pollinating wasps. Proc. R. Soc. Lond. B 268: 685–694.

MACHADO, C. A., N. ROBBINS, M. T. P. GILBERT, AND E. A. HERRE. 2005. Critical review of host specificity and its coevolutionary implications in the fig/fig-wasp mutualism. Proc. Natl. Acad. Sci. 102: 6558–6565.

MARSHALL, T. C., J. SLATE, L. E. B. KRUUK, AND J. M. PEMBERTON. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. Mol. Ecol. 7: 639–655.

MARUSSICH, W. A., AND C. A. MACHADO. 2007. Host-specificity and coevolution among pollinating and nonpollinating New World fig wasps. Mol. Ecol. 16: 1925–1946.

MATUTE, D. R., C. J. NOVAK, AND J. A. COYNE. 2009. Temperature-based extrinsic reproductive isolation in two species of *Drosophila*. Evolution 63: 595–612.

MICHALOUD, G., S. CARRIÉRE, AND M. KOBBI. 1996. Exceptions to the one:one relationship between African fig trees and their fig wasp pollinators: Possible evolutionary scenarios. J. Biogeogr. 23: 513–520.

MOLBO, D., C. A. MACHADO, E. A. HERRE, AND L. KELLER. 2004. Inbreeding and population structure in two pairs of cryptic wasp species. Mol. Ecol. 13: 1613–1623.

MOLBO, D., C. A. MACHADO, J. G. SEVENSTER, L. KELLER, AND E. A. HERRE. 2003. Cryptic species of fig-pollinating wasps: Implications for the evolution of the fig-wasp mutualism, sex allocation, and precision of adaptation. Proc. Natl. Acad. Sci. 100: 5867–5872.

NASON, J. D., E. A. HERRE, AND J. L. HAMRICK. 1998. The breeding structure of a tropical keystone plant resource. Nature 391: 685–687.

OSHIDA, T., J.-K. LEE, L.-K. LIN, AND Y.-J. CHEN. 2006. Phylogeography of Pallas's squirrel in Taiwan: Geographical isolation in an arboreal small mammal. J. Mammal. 87: 247–254.

PENG, Y.-Q., Z.-B. DUAN, D.-R. YANG, AND J.-Y. RASPLUS. 2008. Co-occurrence of two *Eupristina* species on *Ficus altissima* in Xishuangbanna, SW China. Symbiosis 45: 9–14.

POSADA, D., AND K. A. CRANDALL. 1998. MODELTEST: Testing the model of DNA substitution. Bioinformatics 14: 817–818.

RAJA, S., N. SULEMAN, S. G. COMPTOM, AND J. C. MOORE. 2008. The mechanism of sex ratio adjustment in a pollinating fig wasp. Proc. R. Soc. B 275: 1603–1610.

RASPLUS, J.-Y. 1994. The one-to-one specificity of the *Ficus*-Agaoninae mutualism: How causal? *In* L. J. G. van der Maesen, X. M. van der Burgt, and J. M. van Medenbach de Rooy (Eds.). The biodiversity of African plants, pp. 639–649. Kluwer Academic Publishers, Dordrecht, The Netherlands.

RAYMOND, M., AND F. ROUSSET. 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. J. Hered. 86: 248–249.

RICE, W. R. 1989. Analyzing tables of statistical tests. Evolution 43: 223–225.

SHIROZU, T. 1960. Butterflies of Formosa in colour. Hoikusha, Osaka, Japan (in Japanese).

SU, Z.-H., H. IINO, K. NAKAMURA, A. SERRATO, AND K. OYAMA. 2008. Breakdown of the one-to-one rule in Mexican fig-wasp associations inferred by molecular phylogenetic analysis. Symbiosis 45: 73–81.

TENG, L.-S. 1990. Geotectonic evolution of a late Cenozoic arc-continent collision in Taiwan. Tectonophysics 183: 57–76.

Toda, M., M. Nishida, M. Matsui, K.-Y. Lue, and H. Ota. 1998. Genetic variation in the Indian rice frog, *Rana limnocharis* (Amphibia: Anura), in Taiwan, as revealed by allozyme data. Herpetologica 54: 73–82.

Tzeng, H.-Y. 2004. Taxonomic study of the genus *Ficus* in Taiwan. PhD dissertation, National Chung Hsing University, Taichung, Taiwan (in Chinese).

Ware, A. B., and S. G. Compton. 1992. Breakdown of pollinator specificity in an African fig tree. Biotropica 24: 544–549.

Ware, A. B., and S. G. Compton. 1994a. Dispersal of adult female fig wasps. 1. Arrivals and departures. Entomol. Exp. Appl. 73: 221–229.

Ware, A. B., and S. G. Compton. 1994b. Dispersal of adult female fig wasps. 2. Movements between trees. Entomol. Exp. Appl. 73: 231–238.

Weiblen, G. D. 2001. Phylogenetic relationships of fig wasps pollinating functionally dioecious *Ficus* based on mitochondrial DNA sequences and morphology. Syst. Biol. 50: 243–267.

Weiblen, G. D. 2002. How to be a fig wasp. Annu. Rev. Entomol. 47: 299–230.

Yeh, W.-B., Y.-L. Chang, C.-H. Lin, F.-S. Wu, and J.-T. Yang. 2004. Genetic differentiation of *Loxoblemmus appendicularis* complex (Orthoptera: Gryllidae): Speciation through vicariant and glaciation events. Ann. Entomol. Soc. Am. 97: 613–623.

Zavodna, M., P. Arens, P. J. van Dijk, T. Partomihardjo, B. Vosman, and J. M. M. van Damme. 2005a. Pollinating fig wasps: Genetic consequences of island recolonization. J. Evol. Biol. 18: 1234–1243.

Zavodna, M., S. G. Compton, S. Raja, P. M. Gilmartin, and J. M. M. van Damme. 2005b. Do fig wasps produce mixed paternity clutches? J. Insect Behav. 18: 351–362.

Zavodna, M., S. M. Knapp, S. G. Compton, P. Arens, B. Vosman, P. J. van Dijk, P. M. Gilmartin, and J. M. M. van Damme. 2007. Reconstruction of fig wasp mating structure: How many mothers share a fig? Ecol. Entomol. 32: 485–491.

Zhu, H.-D. 2002. Habitat use of six skinks in southern Taiwan. MSc dissertation, National Sun Yat-Sen University, Kaohsiung, Taiwan (in Chinese).