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Molecular approaches identify known species, reveal cryptic species and verify host specificity of Chinese *Philotrypesis* (Hymenoptera: Pteromalidae)

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Abstract

***Philotrypesis*, a major component of the fig wasp community (Hymenoptera: Pteromalidae), is a model taxon for studying male fighting and mating behaviour. Its extreme sexual dimorphism and male polymorphism render species identification uncertain and in-depth research on its ecology, behaviour and other evolutionary topics challenging. The fig wasps' enclosed habitat within the syconia makes their mating behaviour inaccessible, to the extent of matching conspecific females and males. In this study, we combine morphological and molecular analyses to identify species of *Philotrypesis* sampled from south China and to associate their extraordinarily dimorphic genders and labile male morphologies. Morphological evaluations of females identify 22 species and 28 male morphs. The mitochondrial cytochrome c oxidase I and nuclear internal transcribed spacer 2 data detect 21 species using females, and 15 species among the males. Most of the males match the species as delimited by females. Both markers reveal cryptic species in *P. quadrisetosa* on *Ficus vasculosa*. Most species of wasps live on one species of fig but three species co-occur in two hosts (*F. microcarpa* and *F. benjamina*), which indicates host switching.**

Keywords: DNA barcoding, fig wasp, gender association, male polymorphism, sexual dimorphism

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Introduction

Philotrypesis (Sycoryctinae, Pteromalidae; Rasplus *et al.* 1998) is a major component of fig wasp communities (Chen *et al.* 1999; Zhen *et al.* 2004; Peng *et al.* 2005; Zhai *et al.* 2008; Lu *et al.* 2009). As a nonpollinator, it exploits either monoecious fig trees such as *Ficus microcarpa* (Chen *et al.* 1999) and *F. benjamina*, or diecious species, including *F. hispida* and *F. auriculata* (Jiang *et al.* 2006a), as parasitoid of pollinators or inquiline (Chen *et al.* 1999). The approximately 50 species of *Philotrypesis*, including undescribed ones, are widely distributed from southern Europe throughout Africa and southern Asia to Australia (Bouček 1988).

Fig wasps show morphological adaptations to living in the syconium of figs (Weiblen 2002; Cook & Rasplus 2003), and *Philotrypesis* is no exception. Extreme sexual dimorphism and male polymorphism are adaptations. Females have functional wings and eyes that greatly facilitate their ability to colonize new hosts. In contrast, most of males are apterous with vestigial eyes, antennae and tarsi, which are also closely correlated with their existence solely within the syconium (Weiblen 2002). Species of *Philotrypesis* are one of the most variable with respect to male morphological divergence and polymorphism. They are ideal species for investigating fighting behaviours of conspecific males and the evolution of mating strategies (Jousselin *et al.* 2004; Cook & Bean 2006; Moore *et al.* 2009) because different intraspecific morphs adopt unique mating strategies (Hamilton 1979; Murray 1987; Herre *et al.* 1997). For example, small males have reduced fighting adaptations, and they move between the tightly packed seeds and galls to find receptive females. Large males possess morphologies that promote aggressive fighting to defend enclosed females. And winged males can disperse from the figs to mate with

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females outside of the syconium (Greeff & Ferguson 1999; Greeff *et al*. 2003; Jousselin *et al*. 2004).

The prevalence of *Philotrypesis* in fig syconia, their important ecological role and their intriguing biological and morphological characteristics increasingly provoke research interests. However, the identification of species is difficult, and this blocks further study. Fewer than five species are identified and described in China (Chen *et al*. 1999; Jiang *et al*. 2006a). Traditional morphologically identification for *Philotrypesis* is often difficult and error prone when matching conspecific genders. On one hand, some figs host two or more species of *Philotrypesis*, and mating evidence is inaccessible because most of the mating behaviours are completed within the compact and dark syconia; on the other hand, species of *Philotrypesis* show extreme morphological sexual dimorphism and male polymorphism, which makes species identification difficult. Three female and nine male morphological forms of *Philotrypesis* occur in *F. benjamina* (Xiao *et al*. 2010). Identification is sometimes confused. The number of long seta on the hind tarsi I and II can serve to identify males of *Philotrypesis* (Baker 1913; Chen *et al*. 1999). *Ficus fistulosa* hosts three morphological forms of female *Philotrypesis* and a variety of males characterized by different numbers of seta in first two hind tarsi. Further, seta numbers on the left and right hind tarsi vary within individuals. In those cases, the combination of molecular and morphological data is essential.

As a common and widely distributed nonpollinating fig wasp, host specificity of *Philotrypesis* attracts much attention. *Philotrypesis* associated with African host figs of the section Galoglychia indicate that the wasp's phylogeny is congruent with host speciation at the level of subsection (Jousselin *et al*. 2004). However, a study on *Philotrypesis* from seven sections of Chinese figs shows that host-switch is rampant, even between sections (Jiang *et al*. 2006a). However, owing to the limited number of individuals sequenced and the few sampled localities, there is no report of a single species using multiple hosts in China.

The primary goals of this study are to (i) use molecular and morphological data to identify the species of *Philotrypesis* in China, including conspecific genders determination and the identification of polymorphic males and (ii) explore the extent of host specialization in *Philotrypesis* collected from 13 species of *Ficus* in southern China (Table 1). Besides, we employ molecular evidence to test whether or not the number of seta on the hind tarsi can be used to diagnose species.

Materials and methods

Taxon sampling and morphological study

All fig wasps were collected from 13 fig species of eight sections of wild figs in Yunnan, Hainan and Fujian provinces, China, from 2002 to 2010 (Table 1). Some individuals were stored in 75% ethanol for the preparation of pinned specimens, and other individuals of the same morphotypes were placed in 95% ethanol for DNA extraction. Specimens were identified by comparing the wasps with the images taken by Dr. Jean-Yves Rasplus (Professor from INRA-UMR Centre de Biologie et de Gestion des Populations, Campus International de Baillarguet in France) in Yunnan, China (unpublished images) mainly according to the following female morphological characters: length ratio of extended 7th tergite (T7) and T8, length ratio of ovipositor sheath and T7 plus T8, length ratio of extended tergites plus ovipositor and body (Chen *et al*. 1999), body colour pattern, mandible form, antennal formula, the ratio of pronotum width and length, and whether the axilla groove is distinct and the fore wing is ciliated (Jiang *et al*. 2006a). Unidentified females were assigned sequential numbers as *Philotrypesis* sp. Because gender association was virtually impossible without molecular analysis, male morphs on each fig species were named as M plus either a number or letter that was independent of names of the female. For example, M7JaBen means the seventh male collected on *F. benjamina*. Images of the wasps were captured by using Nikon AZ100 microscope system. Species from the closely related genera *Sycoscapter* and *Walkrella* were chosen as outgroup taxa.

DNA extraction, PCR and sequencing

Total genomic DNA was extracted nondestructively from adult wasps preserved in 95% ethanol using Easypure Genomic DNA Extraction kit (*TransGen Biotech*, Beijing, China). After nondestructive DNA extraction, some specimens, especially the males, were stored in 75% ethanol for further morphological study.

Mitochondrial cytochrome c oxidase I (COI) and nuclear ribosomal DNA internal transcribed spacer 2 (ITS2) were sequenced to clarify species boundaries and to match genders. Both COI and ITS2 have been shown to effectively identify species of hymenopteran insects (Dowton *et al*. 2001; Alvarez & Hoy 2002; Pinto *et al*. 2002; Scheffer & Grissell 2003; Jousselin *et al*. 2004; Jiang *et al*. 2006a,b; Lotfalizadeh *et al*. 2008; Li *et al*. 2010). One to 11 individuals (depending on availability) of each species as determined by females and each of the distinguishable male morph were sequenced. ITS2 was amplified and sequenced with primers ITS2F (5′-ATT CCC GGA CCA CGC CTG GCT GA) and ITS2R (5′-TCC TCC GCT TAT TGA TAT GC; White *et al*. 1990) with the following cycling conditions: 5 min at 94 °C; 30 s at 94 °C, 30 s at 50 °C, 40 s at 68 °C (35 cycles); 10 min at 72 °C. COI was amplified using primers FWCOIF (5′-CCT GGT TCT TTR ATT GGT

Table 1 *Philotrypesis* species used in this study and the individual numbers with cytochrome c oxidase I (COI) or internal transcribed spacer 2 (ITS2) sequence obtained (the accession numbers of the sequences were provided)

				ITS		COI	
Ficus host	Wasp species	Province	Code	*N*	Accession Number	*N*	Accession Number
F. microcarpa	*P. emeryi*	Hainan	EmeMicHN	3	JN545190–JN545192	3	YLCFX035-08–YLCFX037-08
F. microcarpa	*P. okinavensis*	Hainan	OkiMicHN	2	JN545193–JN545194	3	YLCFX029-08–YLCFX031-08
F. microcarpa	*P. taiwanensis*	Hainan	TaiMicHN	2	JN545195–JN545196	3	YLCFX023-08–YLCFX025-08
F. microcarpa	M3G2	Hainan	M3G2MicHN	3	JN545177–JN545179	–	–
F. microcarpa	M6	Hainan	M6MicHN	3	JN545183–JN545185	3	JN545265–JN545267
F. microcarpa	M8	Hainan	M8MicHN	1	JN545186	1	JN545268
F. microcarpa	M10	Hainan	M10MicHN	3	JN545187–JN545189	1	YLCFX194-08
F. microcarpa	M3	Hainan	M3MicHN	3	JN545180–JN545182	3	JN545262–JN545264
F. microcarpa	M3G1	Hainan	M3G1MicHN	2	JN545175–JN545176	2	JN545260–JN545261
F. benjamina	*P. tridentata*	Hainan	TriBenHN	3	JN545118–JN545120	3	YLCFW242-08, YLCFW244-08, YLCFW245-08
F. benjamina	*P.* sp4	Hainan	Sp4BenHN	2	JN545121–JN545122	3	YLCFW235-08–YLCFW237-08
F. benjamina	*P. distillatoria*	Hainan	DisBenHN	3	JN545123–JN545125	3	YLCFW248-08, YLCFW250-08, YLCFW251-08
F. benjamina	M7a	Hainan	M7aBenHN	3	JN545106–JN545108	2	JN545236–JN545237
F. benjamina	M7b	Hainan	M7bBenHN	–	–	1	JN545238
F. benjamina	M7Ja	Hainan	M7JaBenHN	3	JN545109–JN545111	3	JN545239–JN545241
F. benjamina	M7Jb	Hainan	M7JbBenHN	1	JN545112	3	JN545242–JN545244
F. benjamina	M7Jc	Hainan	M7JcBenHN	2	JN545113–JN545114	3	JN545245–JN545247
F. benjamina	M7G1	Hainan	M7G1BenHN	3	JN545103–JN545105	3	JN545233–JN545235
F. benjamina	M5	Hainan	M5BenHN	–	–	3	YLCFW367-08–YLCFW369-08
F. benjamina	M6	Hainan	M6BenHN	2	JN545173–JN545174	–	–
F. benjamina	M9	Hainan	M9BenHN	3	JN545115–JN545117	2	YLCFW389-08–YLCFW390-08
F. drupacea v. pubescens	*P.* sp1	Yunnan	Sp1DruYN	3	JN545126–JN545128	3	YLCFW047-08, YLCFW048-08, YLCFW050-08
	P. longispinosa	Yunnan	LonDruYN	3	JN545129–JN545131	1	YLCFW052-08
F. drupacea v. pubescens	M2	Yunnan	M2DruYN	–	–	2	YLCFW043-08, YLCFW046-08
F. religiosa	*P. anguliceps*	Yunnan	AngRelYN	3	JN545211–JN545213	2	JN545274–JN545275
F. religiosa	M2	Yunnan	M2RelYN	3	JN545208–JN545210	2	JQ408678, JN545273
F. nervosa	*P. marginalis*	Hainan	MarNerHN	3	JN545199–JN545201	3	YLCFX031-08–YLCFX033-08
F. nervosa	M3	Hainan	M3NerHN	2	JN545197–JN545198	–	–
F. vasculosa	*P. quadrisetosa*	Hainan	QuaVasHN	5	JN545228–JN545232	6	JQ408680–JQ408685
F. vasculosa	M2	Hainan	M2VasHN	6	JN545222–JN545227	4	JQ408686–JQ408689
F. semicordata	*P. dunia*	Yunnan	DunSemYN	3	JN545216–JN545218	2	JN545276–JN545277
F. semicordata	M2	Yunnan	M2SemYN	2	JN545214–JN545215	1	JQ408679
F. tinctoria	*P. jacbosoni*	Hainan	JacTinHN	3	JN545219–JN545221	3	YLCFX487-08–YLCFX489-08
F. tinctoria	M1	Hainan	M1TinHN	–	–	2	YLCFX497-08, YLCFX504-08
F. hispida	*P. pilosa*	Hainan	PilHisHN	3	JN545170–JN545172	2	YLCFX090-08, YLCFX091-08
F. hispida	*P.* sp	Hainan	SpHisHN	3	JN545167–JN545169	3	YLCFX078-08–YLCFX080-08
F. hispida	M1	Hainan	M1HisHN	3	JN545162–JN545164	1	JN545258
F. hispida	M1G1	Hainan	M1G1HisHN	1	JN545161	–	–
F. hispida	Mx	Hainan	MxHisHN	2	JN545165–JN545166	1	JN545259
F. fistulosa	*P. spinipes*	Hainan	SpiFisHN	3	JN545143–JN545145	3	YLCFW147-08–YLCFW149-08
F. fistulosa	*P. longiventris*	Hainan	LonFisHN	3	JN545146–JN545148	3	YLCFW153-08, YLCFW155-08, YLCFW156-08
F. fistulosa	*P. collaris*	Hainan	ColFisHN	3	JN545149–JN545151	3	YLCFW159-08, YLCFW160-08, YLCFW162-08
F. fistulosa	M2	Hainan	M2FisHN	11	JN545132–JN545142	10	JQ408673, JN545248–JN545251, JQ408674–JQ408676, JN545252, JQ408677
F. oligodon	*P.* sp1	Yunnan	Sp1OliYN	3	JN545205–JN545207	2	JN545271–JN545272
F. oligodon	M1	Hainan	M1OliHN	3	JN545202–JN545204	2	JN545269–JN545270
F. auriculata	*P. longicaudata*	Hainan	LonAurHN	3	JN545100–JN545102	3	YLCFX211-08–YLCFX213-08
F. auriculata	M2	Hainan	M2AurHN	3	JN545097–JN545099	3	YLCFX230-08–YLCFX232-08

Table 1 *(Continued)*

				ITS		COI	
Ficus host	Wasp species	Province	Code	*N*	Accession Number	*N*	Accession Number
F. hirta	*P. josephi*	Fujian	JosHirHN	3	JN545155–JN545157	3	YLCFX601-08, YLCFX602-08, YLCFX604-08
F. hirta	*P.* sp2	Fujian	Sp2HirFJ	3	JN545158–JN545160	2	JN545256–JN545257
F. hirta	M1	Fujian	M1HirFJ	3	JN545152–JN545154	3	JN545253–JN545255

No sequence was obtained owing to the lack of specimens or experimental failure.

AAT GATC) and COI2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA; Simon *et al.* 1994), with 35 cycles of 30 s at 94 °C, 40 s at 50 °C, 58 s at 68 °C.

The amplified DNA products were either directly sequenced (for most of the ITS2) or cloned into Peasy-T1 vector according to the manufacture's protocols (*TransGen Biotech*, Beijing, China). Sequencing was carried by the BioSune Sequencing Centre (Beijing, China). Genomic DNA and specimen vouchers were kept in the Institute of Zoology, Chinese Academy of Sciences. COI sequences were deposited in GenBank under accession numbers JN545233–JN545277, JQ408673–JQ408689 and for ITS2 JN545097–JN545232.

Sequence alignment and molecular analyses

We used ClustalX in MEGA version 4.0 (Tamura *et al.* 2007) to align the sequences. Sequences were initially aligned using the default multiple alignment parameters (gap opening penalty = 15, gap extension penalty = 6.66, delay divergent sequences = 30%) for COI. We tried different parameter settings for ITS2, because this noncoding region had many indels (Xiao *et al.* 2010); ultimately, the default parameters were chosen. The sequences were uploaded to TREEBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S12299).

A neighbour-joining (NJ) tree was built for COI and ITS2 using the Kimura 2-parameter (K2P) model and pairwise deletion by using MEGA. Bootstrap analyses were performed with 1000 replicates. Both trees were rooted with *Walkerella* sp. (Hymenoptera: Pteromalidae) and *Sycoscapter* sp. (Hymenoptera: Pteromalidae) from *F. microcarpa*.

Results

Morphological studies

We identified 22 species based on the females with five unnamed species (Table 1; Fig. S1, Supporting information). Almost half of the figs (6/13) hosted more than one species of *Philotrypesis*. In total, *Ficus microcarpa*, *F. benjamina* and *F. fistulosa* had three species of *Philotrypesis*, respectively.

Distinct male polymorphism occurred in the two monoecious fig trees from the subsection Conosycea, *F. benjamina* and *F. microcarpa*. Six male morphs were recognized in the latter fig, and they differed in fore wing, body colour, head shape and hair alignment on head (Table 1; Figs S1 and S2, Supporting information). Morph M3G1 was similar to M3 except for the alignment of hair beside the eyes (not shown because of specimen quality). Owing to the lack of direct mating evidence, we could not associate so many male morphs with the three females observed in *F. microcarpa* (Table 1; Fig. S1, Supporting information). What's more, the two fig trees *F. benjamina* and *F. microcarpa* hosted six species of *Philotrypesis* altogether (three species for each fig tree), and the species were striking similar morphologically, which makes identification of the males further confused. This discovery suggested that some species of *Philotrypesis* occurred on more than one species of fig tree.

In *F. fistulosa*, three species based on females, *P. spinipes*, *P. longiventris* and *P. collaris*, were identified. Although the males had different patterns of setae on the hind tarsi, we could not a priori morphologically determine whether this variation indicated intraspecific or interspecific differences.

Molecular analysis, species delimitation, gender association and cryptic taxa identification

A total of 125 and 138 sequences from COI and ITS2, respectively, were aligned and used for NJ tree construction. The final alignment length for COI was 522 bp, and for ITS2, 466 bp. Both NJ trees showed similar topologies (Figs 1 and 2), although the topology indicated larger intraspecific divergence in COI sequences than in ITS2. According to some studies in other insects and also in fig wasps in our lab, we here presume that if the branch length difference between various species in COI NJ tree is <0.02, we take them as the same species. Branch

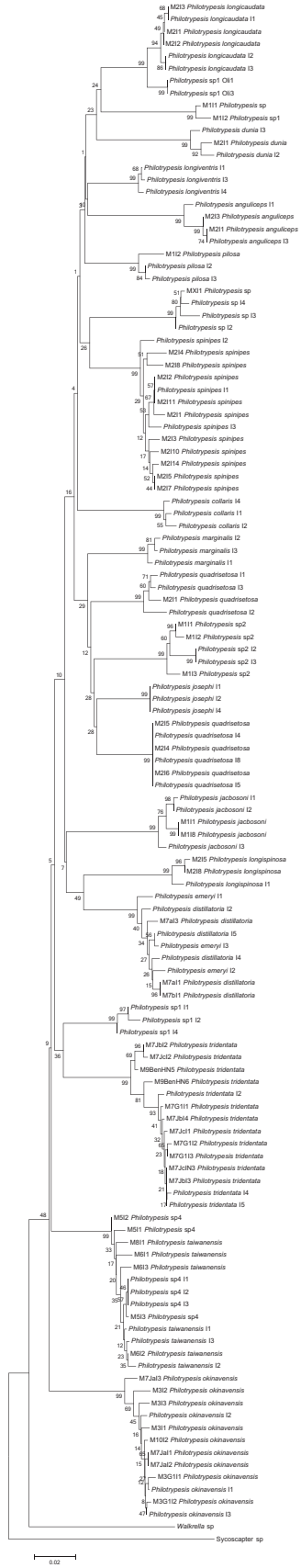

Fig. 1 The NJ tree for parasitic wasps of the genus *Philotrypesis* constructed with cytochrome c oxidase I sequences. Values on the nodes are Bootstrap supports. The code for each specimen is denoted in Table 1.

support for each node was high (>99%) in the COI tree (Fig. 1), and similarly in the ITS2 tree except for two nodes (52% for MarNerHN and 66% for SpHisHN, respectively). The weakly supported nodes did not influence species delimitations. The 21 clusters of females suggested the existence of 21 species, rather than 22 as determined a priori, and 15 male lineages were detected. Most female and male *Philotrypesis* from the same fig clustered into the same lineage except for species in *F. nervosa* and *F. oligodon*. *Philotrypesis marginalis* and M3 in *F. nervosa* formed two distinct, divergent groups and females of *P.* sp1 and M1 in *F. oligodon* clustered with *P. longicaudata* from *F. auriculata*, although M1 formed a distinct group.

The NJ trees unambiguously associated the genders and delimitated the species with complicated male polymorphisms, such as the species of *Philotrypesis* in *F. benjamina* and *F. microcarpa*. For example, *P. tridentata* had four male morphs: M9, M7G1, M7Jb and M7Jc. Whereas M9 had complete wings, the other three were wingless, which was consistent with previous research (Xiao *et al.* 2010). In both trees, morphologically similar morphs of fig wasps associated with *F. benjamina* and *F. microcarpa* clustered together with high support. For example, *P. emeryi* from *F. microcarpa* and *P. distillatoria* and their related male morphs M3G2MicHN, M7aBenHN and M7bBenHN from *F. benjamina* formed a highly supported group. *Philotrypesis taiwanensis* from *F. microcarpa* clustered with *P.* sp4 from *F. benjamina*, and they shared four male morphs: M6MicHN, M8MicHN, M5BenHN and M6BenHN. One male morph, M7Ja, which was unidentified in a previous study on fig wasps associated with *F. benjamina* (Xiao *et al.* 2010), clustered with *P. okinavensis* in *F. microcarpa*.

The existence of three species in *F. fistulosa* was confirmed in both trees. However, the males with different setae on the enlarged hind tarsi (10 and 11 individuals on the COI and ITS2 trees, respectively) clustered together into a single clade as *P. spinipes*. The other two species occurred in a different group without associated males. Surprisingly, both trees split *P. quadrisetosa* in *F. vasculosa* with identical morphology into two divergent groups; this discovery suggested the existence of cryptic species.

Discussion

We combined morphological and molecular data to identify 22 female morphological species of *Philotrypesis*

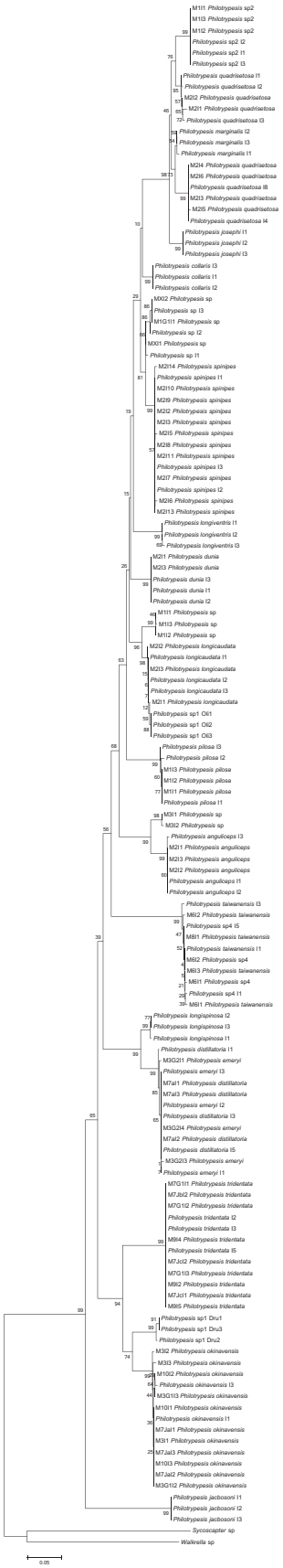

Fig. 2 The NJ tree for parasitic wasps of the genus *Philotrypesis* constructed with internal transcribed spacer 2 sequences. Values on the nodes are Bootstrap supports. The code for each specimen is denoted in Table 1.

collected from southern China. The corresponding males were identified using molecular data.

Pattern of host specificity: host switching is common

Direct observations on *Philotrypesis* suggest that it feeds on plant rather than insect tissue, although they oviposit in flowers galled by pollinators (Joseph 1959; Cook & Rasplus 2003). When identifying species of *Philotrypesis*, taxonomists usually relied on the species of host fig, because of the lack of knowledge on its host specificity characters. Two lines of evidence indicate that host switching is common among nonpollinating fig wasps. First, in one genus, at least one species occurs on more than one fig host, and second, multiple species of wasps commonly occur in one species of fig (Marussich & Machado 2007). One study reports strong host specificity in African host figs in the subsections of section Galoglychia (Jousselin *et al*. 2004). However, given that the different species in the same fig are not sister species, host switching appears to be rampant in *Philotrypesis* (Jiang *et al*. 2006a).

No study on Chinese *Philotrypesis* indicates one species lives in multiple hosts (Jiang *et al*. 2006a). Herein, most species of *Philotrypesis* from the same fig cluster together. However, our studies show that some species of *Philotrypesis* colonize more than one fig host. For example, *P. emeryi* and *P. distillatoria*, which occur in *F. benjamina* and *F. microcarpa*, respectively, are actually conspecific. In a taxonomic context, *P. distillatoria* is now suggested as a new synonym of *P. emeryi*. *Philotrypesis taiwanensis* in *F. microcarpa* is identical to *P.* sp4 in *F. benjamina*. Further, *F. auriculata* and *F. oligodon* both host *P. longicaudata*. Interestingly, *F. benjamina* and *F. microcarpa* belong to Section *Conosycea*; *F. auriculata* and *F. oligodon* are in Section *Neomorphe*. This observation suggests that closely related figs may have the same *Philotrypesis* species.

The clustering pattern also suggests that sympatric species are not closely related taxa, and this has important implications for host switching. Three species of *Philotrypesis* (*P. emeryi*, *P. taiwanensis* and *P. longicuadata*) co-occur in two fig species. Conversely, *F. auriculata* and *F. oligodon* host *P. longicaudata*. Indeed, 6 of 13 species of fig trees host two or three species of *Philotrypesis* that are unlikely to be closely related. Based on the abovementioned findings, we can say that host switching in *Philotrypesis* may be more common than not in South China.

Implications of the molecular methods: useful support for the identification of Philotrypesis

Morphological identification of *Philotrypesis* is often problematic. The three most prominent challenges are (i) extreme sexual dimorphism and male polymorphism combined with unobservable mating, (ii) intra-individual morphological variation and (iii) putative cryptic species. Our molecular studies clearly confirm morphological species. The approach also identifies sympatric species in the same fig, which have morphologically complicated male polymorphism. The molecular data also identify complicated species assemblages that colonize more than one species of fig tree, as occurs in *F. benjamina* and *F. microcarpa*.

The number of long seta on the hind tarsi I and II serves as a diagnostic characteristic in the key to male *Philotrypesis* (Baker 1913; Chen *et al.* 1999). However, M2 in *F. fistulosa* has a varying number of seta on hind tarsi yet the individuals cluster together, indicating intraspecific variation. This situation also occurs in male *Philotrypesis* sp. in *F. hispida*. For example, MxHisHN1 has two and one setae on left first two enlarged hind tarsi, respectively, but one and one setae on the right tarsi. Similarly, MxHisHN2 possesses different numbers of seta on left and right legs. Seta numbers on enlarged tarsi do not seem to be a good diagnostic character.

The molecular analyses unveil morphologically cryptic species. For example, both trees indicate that *P. quadrisetosa* consists of two species. In this case, as in others (Hebert *et al.* 2003), the DNA barcoding gene COI identifies known species and discovers new ones. However, because mitochondrial genes can be integrated into the nuclear genomes (nuclear mitochondrial DNA or Numts; Lopez *et al.* 1994; Bensasson *et al.* 2001), or be influenced by *Wolbachia* infections (Ballard 2000; Shoemaker *et al.* 2004) and heteroplasmy (Magnacca & Brown 2010), which in some cases makes the results based on sole COI sequences inaccurate, nuclear genes are required to confirm the identifications. Nuclear ITS2 appears to be an effective DNA marker in other fig wasps (Li *et al.* 2010; Xiao *et al.* 2010). The discovery of cryptic species in our case indicates that morphology might not be essential for the reproductive isolation of organisms living in enclosed habitats (Li *et al.* 2010).

The utility of gene markers is, in part, a function of levels of divergence. Herein, COI divergence between any two lineages is large enough (5.8–6.0%) to indicate different species. Levels of ITS2 sequence divergence within each lineage are nearly identical, while the divergence between any lineages is in the region of 5.8–6.6%. Similar divergences are reported in pollinating (Molbo *et al.* 2003; Haine *et al.* 2006; Sun *et al.* 2011) and nonpollinating fig wasps of the genus *Sycophila* (Li *et al.* 2010).

Wolbachia might play a vital role in the formation of the cryptic species of pollinators, as exemplified by *Eupristina verticillata* in *F. microcarpa* (Sun *et al.* 2011). The same mechanism may act on *P. quadrisetosa* as suggested by the high incidence of *Wolbachia* infection in fig wasps (Shoemaker *et al.* 2002; Haine & Cook 2005; Chen *et al.* 2010). Further research on the speciation mechanism of this nonpollinator species is highly desirable.

Extensive overlaps on the Philotrypesis *species in* F. microcarpa *and* F. benjamina

One unresolved question remains from our previous case study on parasitic wasps in *Ficus benjamina*: no females were found to cluster with male morph M7Ja (Xiao *et al.* 2010). Herein, the NJ trees for COI and ITS2 still do not match this male to any female individuals from *F. benjamina*. However, the analyses associate the male with *P. okinavensis* in *F. microcarpa*. The two independent studies further indicate the occurrence of one species in the two similar fig species, *F. microcarpa* and *F. benjamina*.

Present observations can tell us that the three species of wasps in *F. microcarpa* (*P. emeryi*, *P. taiwanensis* and *P. okinavensis*) can freely oviposit in *F. benjamina*, although no mature females of *P. okinavensis* are known from *F. benjamina*. Further sampling on *F. benjamina* may help detect the existence of females of *P. okinavensis* on this fig tree. However, it is possible that *P. okinavensis* only oviposit very few unfertilized eggs that thus produce M7Ja on *F. benjamina*. Whereas most species of *Philotrypesis* occur on both *F. microcarpa* and *F. benjamina*, *P. tridentate* is only detected on the latter only. It remains unrecorded from *F. microcarpa*.

Remaining cases of unresolved identifications

Philotrypesis marginalis, which is found in *F. nervosa*, is not associated with M3, the only male *Philotrypesis* in our specimens on this fig. The same is true for *Philotrypesis* sp.1 and M1 in *F. oligodon*. These observations indicate the need for extended sampling. Alternatively, we cannot associate genders precipitately even though there was only one female and one male morph sampled from the same fig tree.

In conclusion, the combined analyses of COI and ITS2 gene sequences provide a valid and useful approach to identifying 21 'molecular' species of *Philotrypesis* from southern China. The molecular analysis methods not only easily and unambiguously identify morphological species but also associate the genders of species that exhibit extreme sexual dimorphism and male polymorphism. The approach also reveals cryptic species. Because half of the fig species host two or more species of *Philotrypesis*, and three species of *Philotrypesis*

occur sympatrically on two fig species, host switching appears to be common in *Philotrypesis*.

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Conceived and designed the project: JHX, DWH; performed the experiments: MJZ, SNB, YWL, LMN, HYH, WSW; analyzed the data: MJZ, JHX, DWH; wrote the paper: MJZ, JHX, DWH, RM.

Data Accessibility

Taxa information: Table 1.

DNA sequences: Genbank accessions JN545233–JN545277, JQ408673–JQ408689 and JN545097–JN545232.

Phylogenetic data: TreeBASE Study accession no. S12299.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Morphological images of all female and male morphs of *Philotrypesis*.

Fig. S2 The images of the male morphs of *Philotrypesis* sampled in *Ficus microcarpa*.

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