

## DNA BARCODING

# COI and ITS2 sequences delimit species, reveal cryptic taxa and host specificity of fig-associated *Sycophila* (Hymenoptera, Eurytomidae)

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## Abstract

Although the genus *Sycophila* has broad host preferences, some species are specifically associated with figs as nonpollinator wasps. Because of their sexual dimorphism, morphological plasticity, cryptic mating behaviour and poorly known biology, species identifications are often uncertain. It is particularly difficult to match conspecific females and males. In this study, we employed two molecular markers, mitochondrial COI and nuclear ITS2, to identify *Sycophila* from six Chinese fig species. Morphological studies revealed 25 female and male morphs, while sequence results for both genes were consistent in supporting the presence of 15 species, of which 13 were host specialists and two used dual hosts. A single species of *Sycophila* was respectively found on four fig species, but six species were isolated from *Ficus benjamina* and a same number was reared from *Ficus microcarpa*. Sequence results revealed three male morphs in one species and detected two species that were overlooked by morphological analysis.

**Keywords:** DNA barcoding, host specificity, male polymorphism, sex association, sexual dimorphism, species identification

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## Introduction

Across its global distribution, the genus *Sycophila* (Hymenoptera: Apocrita: Chalcidoidea: Eurytomidae: Eurytominae) includes 117 known species (Noyes 2008). Members of this genus are associated with a wide range of plants (Narendran 1994), but are usually considered as parasitoids or inquiline (Bouček 1988; Zerova & Fulsov 1991; Kerdelhue & Rasplus 1996). They certainly attack the eggs and larvae of some insect groups (e.g. Diptera, Lepidoptera and Hymenoptera) (Compton 1993; Kerdelhue & Rasplus 1996; Gibernau *et al.* 2002) associated with plants such as bamboos (Shibata & Ito 2005), oaks (Eliason & Potter 2001; Stone *et al.* 2002), strawberry guava (Wikler

2000) and figs (Bouček *et al.* 1981). Although they are typically uncommon, species of *Sycophila* are one component of the diverse wasp fauna associated with figs in most subtropical and tropical regions (Kerdelhue & Rasplus 1996). In China, *Sycophila* species have been found in association with *Ficus virens* (Chen *et al.* 2001), *F. microcarpa* (Chen *et al.* 1999), *F. benjamina* (Bai *et al.* 2006) and other monoecious figs. Although some studies have suggested that *Sycophila* are not highly host-specific (Bouček *et al.* 1981), the morphological characters differentiating females are very subtle, making it difficult to be certain that a single species uses multiple hosts. Sexual dimorphism, morphological plasticity and poorly known biology create further challenges for the identification and association of sexes in *Sycophila* (Lotfalizadeh *et al.* 2007). Nevertheless, its high species diversity and broad host use make *Sycophila* an excellent potential model system for the investigation of speciation patterns and host

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**Table 1** Haplogroups and morphospecies of Chinese *Sycophila* and associated host fig species

<i>Sycophila</i> haplogroups			Host						Distribution	COI (N)	ITS2 (N)
			<i>F.</i> <i>benjamina</i>	<i>F.</i> <i>microcarpa</i>	<i>F.</i> <i>glaberrima</i>	<i>F.</i> <i>altissima</i>	<i>F.</i> <i>concinna</i>	<i>F.</i> <i>superba</i>			
S. HP1	S. sp.1-BEN	F	X						Hainan	10	7
	S. M11-BEN	M	X						Hainan	21	20
	S. M11-1-BEN	M	X						Hainan	16	13
	S. M11-2-BEN	M	X						Hainan	8	7
S. HP2	S. sp.5-MIC	F		X					Hainan	3	1
	S. M13-MIC	M		X					Hainan	3	1
S. HP3	S. sp.2-GLA	F			X				Guangdong	4	4
	S. M2-GLA	M			X				Guangdong	5	5
S. HP4	S. sp.1-SUP	F						X	Hainan	6	6
	S. M1-SUP	M						X	Hainan	6	4
S. HP5	S. sp.4-BEN	F	X						Hainan	1	1
	S. M11-MIC	M		X					Hainan	3	3
S. HP6	S. sp.2-CON	F				X			Hainan	2	2
S. HP7	S. sp.3.1-BEN	F	X						Hainan	1	0
	S. M11-MIC	M		X					Hainan	1	1
S. HP8	S. sp.3.1-BEN	F	X						Hainan	2	2
	S. sp.4-BEN	F	X						Hainan	1	1
	S. M10-1-BEN	M	X						Hainan	8	7
S. HP9	S. sp.2-MIC	F		X					Hainan	6	6
S. HP10	S. sp.1-ALT	F				X			Hainan	3	3
	S. M1-ALT	M				X			Hainan	1	1
S. HP11	S. M11-MIC	M		X					Hainan	2	2
S. HP12	S. sp.1-CON	F					X		Hainan	3	3
	S. M1-CON	M					X		Hainan	2	2
S. HP13	S. sp.3-BEN	F	X						Hainan	6	6
S. HP14	S. sp.1-MIC	F		X					Hainan	5	4
	S. M12-MIC	M		X					Hainan	5	1
S. HP15	S. sp.2-BEN	F	X						Hainan	12	11
	S. M15-BEN	M	X						Hainan	8	6

specificity. Despite this fact, careful analyses of the relationships between *Sycophila* and their various hosts have been scarce.

The primary goal of this study was to clarify the diversity of *Sycophila* species on single fig species and the extent of host specialization in the community found in association with six monoecious *Ficus* species in southern China (Table 1). Specimens were first examined morphologically to gain a sense of species diversity and host use. Subsequently, two genes, mitochondrial cytochrome *c* oxidase I (COI) gene and internal transcribed spacer 2 (ITS2) region of the nuclear ribosomal DNA, were employed to clarify species boundaries and to associate sexes. COI is the core gene employed by DNA barcoding initiatives and has the potential to facilitate both the identification of known species and the discovery of new ones (Hebert *et al.* 2003). Many studies have demonstrated that DNA barcodes are effective in diagnosing species (Hebert *et al.* 2004a,b; Barrett & Hebert 2005; Ward *et al.* 2005;

Clare *et al.* 2006; Smith *et al.* 2006, 2007; Seifert *et al.* 2007), although debates remain (Hickerson *et al.* 2006; Meier *et al.* 2006; Whitworth *et al.* 2007; Wiemers & Fiedler 2007; Hunter *et al.* 2008; Moura *et al.* 2008). The ITS2 fragment also usually possesses nucleotide differences between close species pairs (Coleman 2003) and is often an effective tool for species diagnosis (Hackett *et al.* 2000; Hung *et al.* 2004; Ben-David *et al.* 2007). Both COI and ITS2 have been shown to be effective in tracing species boundaries in varied hymenopteran groups (Dowton & Austin 2001; Alvarez & Hoy 2002; Pinto *et al.* 2002; Scheffer & Grissell 2003; Joussetin *et al.* 2004; Jiang *et al.* 2006a,b; Lotfalizadeh *et al.* 2008).

Through a combined morphological and molecular approach, we sought to ascertain if patterns of sequence variation at COI and ITS2 are congruent with one another and with morphology in revealing species boundaries. In addition, we employ molecular evidence to test if morphologically similar *Sycophila* from different fig hosts represent a single species, providing a test of host specificity.

## Materials and methods

### Specimen collection and deposition

*Sycophila* specimens were collected from fruits (syconia) of *F. benjamina*, *F. microcarpa*, *F. glaberrima*, *F. altissima*, *F. concinna* and *F. superba* in Hainan and Guangdong provinces from 2004 to 2008. The dietary choice of the *Sycophila* in this study is unknown, but figs are considered as hosts although some or all wasps may simply be parasitoids of other insects associated with figs (Lotfalizadeh *et al.* 2007). All wasps used for DNA analysis were preserved in 95% ethanol. We sequenced 154 individuals representing 25 *Sycophila* morphs (13 female morphs and 12 male morphs, see Table 1). As past taxonomic study of Chinese *Sycophila* has been very limited, these morphospecies cannot be assigned a species epithet. As a result, we assigned an interim name to each of the 25 morphs. Female morphs gained a morph number, followed by an acronym (see Appendix S1 in Supporting information) representing its host fig, e.g. *Sycophila* sp.1-BEN (*Sycophila* female morph 1 collected on *F. benjamina*). Male morphs were categorized in numeric order across all male morphospecies, independently of female names, because sex association is impossible without genetic analysis. Male morphs were denoted by a letter 'M', followed by a morph number and host fig. For example, *Sycophila* M11-BEN is fig wasp male morph 11 collected on *F. benjamina*.

We gathered sequence records from all representatives of the three close male morphs from *F. benjamina* (i.e. S. M11-BEN, S. M11-1-BEN and S. M11-2-BEN) to ascertain if they were conspecific or not. In fact, because of their rarity, most male morphs were sequenced. By contrast, only a subset of females within a morph was sequenced.

Voucher specimens are deposited in the collections at Shandong Agricultural University and at the Institute of Zoology (IOZ), Chinese Academy of Sciences. All voucher specimens were photographed using a Nikon AZ100M microscope. Detailed specimen information (taxonomy, photograph and collection information) is available in the project 'Chinese *Sycophila*' (project code CNSYC) on the Barcode of Life Data Systems (BOLD, <http://www.barcodinglife.org>). The project will become publicly available upon the publication of this work.

### DNA extraction and PCR amplification

Specimens preserved in 95% ethanol were transferred to a 96-well Eppendorf plate after photography. DNA was extracted nondestructively from whole specimens using AcroPrep™ 96 1 mL filter plate with 3.0 µm Glass fibre

following the manufacturer's protocols. The specimens were restored in 95% ethanol after nondestructive DNA extraction to allow for further morphological study.

Molecular analyses were conducted at the Canadian Centre for DNA Barcoding (CCDB). Full-length DNA barcodes (652 bp) were obtained using standard insect primer sets (Lep-F1, 5'-ATTCAACCAATCATAAAGA TAT-3'; and Lep-R1, 5'-TAAACTTCTGGATGTCCAA AAA-3') (Hebert *et al.* 2004a). As sequencing of the COI amplicon is difficult in hymenopteran because of a poly-T region near the 5' terminus of the barcode region (Smith *et al.* 2008), we designed a new forward primer (FWPTF1, 5'-CCTGGTTCTTTRATTGGTAATGATC-3') that was paired with Lep-R1 to generate a 545-bp amplicon that avoided the poly-T run. The volume of all PCR mixes was 12.5 µL: 2.5 mM MgCl<sub>2</sub>, 5 pmol of each primer, 20 mM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 10–20 ng (1–2 mL) of genomic DNA and 1 unit of Taq DNA polymerase (Platinum® Taq DNA Polymerase; Invitrogen). The PCR regime consisted of one cycle of 1 min at 94 °C, six cycles of 1 min at 94 °C, 90 s at 45 °C, and 75 s at 72 °C, followed by 36 cycles of 1 min at 94 °C, 90 s at 51 °C, and 75 s at 72 °C, with a final step of 5 min at 72 °C.

The primers ITS2F (5'-ATTCCCGGACCACGCCTGG CTGA-3') and ITS2R (5'-TCCTCCGCTTATTGATATGC -3') (White *et al.* 1990) were used to amplify ITS2. ITS2 amplifications were carried out at Shandong Agricultural University using Easy Taq Polymerase (TransGen Biotech Company) with the following thermal regime: one cycle of 5 min at 94 °C; 35 cycles of 30 s at 94 °C, 40 s at 55 °C and 40 s at 72 °C; 10 min at 72 °C. All ITS2 amplifications were performed in a 12.5-µL reaction volume.

All PCR products were visualized on a 2% agarose E-Gel® (Invitrogen). Positive products were then bidirectionally sequenced on an ABI 3730xl DNA Analyzer (Applied Biosystems).

### Sequence analyses

Contigs were assembled from forward and reverse reads using Sequencher version 4.5 (Gene Codes) and aligned by CLUSTALX in MEGA version 4.0 (Kumar *et al.* 2008) and by eye. Separate analyses were performed on COI and ITS2. For COI, genetic distances were calculated using the Barcode of Life Data System (BOLD, <http://www.boldsystems.org>). Bootstrap analysis was performed with 1000 replicates in MEGA version 4.0. COI sequence reads and trace files are deposited in the project 'Chinese *Sycophila*' (project code CNSYC) in BOLD. ITS2 sequences were aligned using CLUSTALX, with default settings (open gap penalty = 10.0; extend gap penalty = 5.0)

and examined by eye with gaps treated as missing data. A neighbour-joining distance tree was built for each gene region using the Kimura 2-parameter (K2P) distance model and pairwise deletion in MEGA4.0. Genetic distances between all species pairs were also calculated using the K2P model. All COI sequences have been deposited in GenBank under accession numbers FJ499663 to FJ499816 and ITS2 accession numbers from FJ513192 to FJ513321.

## Results

### Morphological studies

The 25 *Sycophila* morphs (13 females, 12 males) showed marked variation in abundance (Table 1 and Appendix S1). Morphological examination indicated the presence of several morphs on a single *Ficus* species, and sometimes even in a single fruit. Among the six *Ficus* species, *F. benjamina* appeared to be inhabited by the most *Sycophila* species as 10 morphs (including both sexes) were represented in collections from this tree. However, delineation of these morphs was sometimes uncertain. For example, three male morphs (*S.* M10-1-BEN, *S.* M11-BEN and *S.* M15-BEN) were initially recognized on *F. benjamina*. A fourth male morph, *S.* M11-1-BEN, was separated from the otherwise morphologically similar *S.* M11-BEN by its lack of a triangular tooth on the anterior surface of the fore femur. However, individuals with intermediate morphological characteristics (a blunt tooth at the corresponding position) were subsequently found and were named as *S.* M11-2-BEN. Because males are relatively rare in *Sycophila*, we were not able to determine if this allometric variation in tooth shape represented differences among species or simply phenotypic plasticity. Similarly, three female morphs (*S.* sp.1-BEN, *S.* sp.2-BEN and *S.* sp.4-BEN) from *F. benjamina* could be relatively easily distinguished by variation in body colour (from yellow to black), shape of the antennae and fore wings. However, two other female morphs (*S.* sp.3-BEN and *S.* sp.3.1-BEN) were only separated by a minor difference in body colour, suggesting that they might be the same species.

Similar morphological complexities were observed in *Sycophila* from the other *Ficus* species, particularly *F. microcarpa*. While various *Sycophila* morphs were recognized (Table 1), the morphological characteristics used to

delimit them were sometimes ambiguous because of the subtle variation and the limited number of specimens. The situation was further complicated when members of *Sycophila* species complexes from the two different hosts were compared.

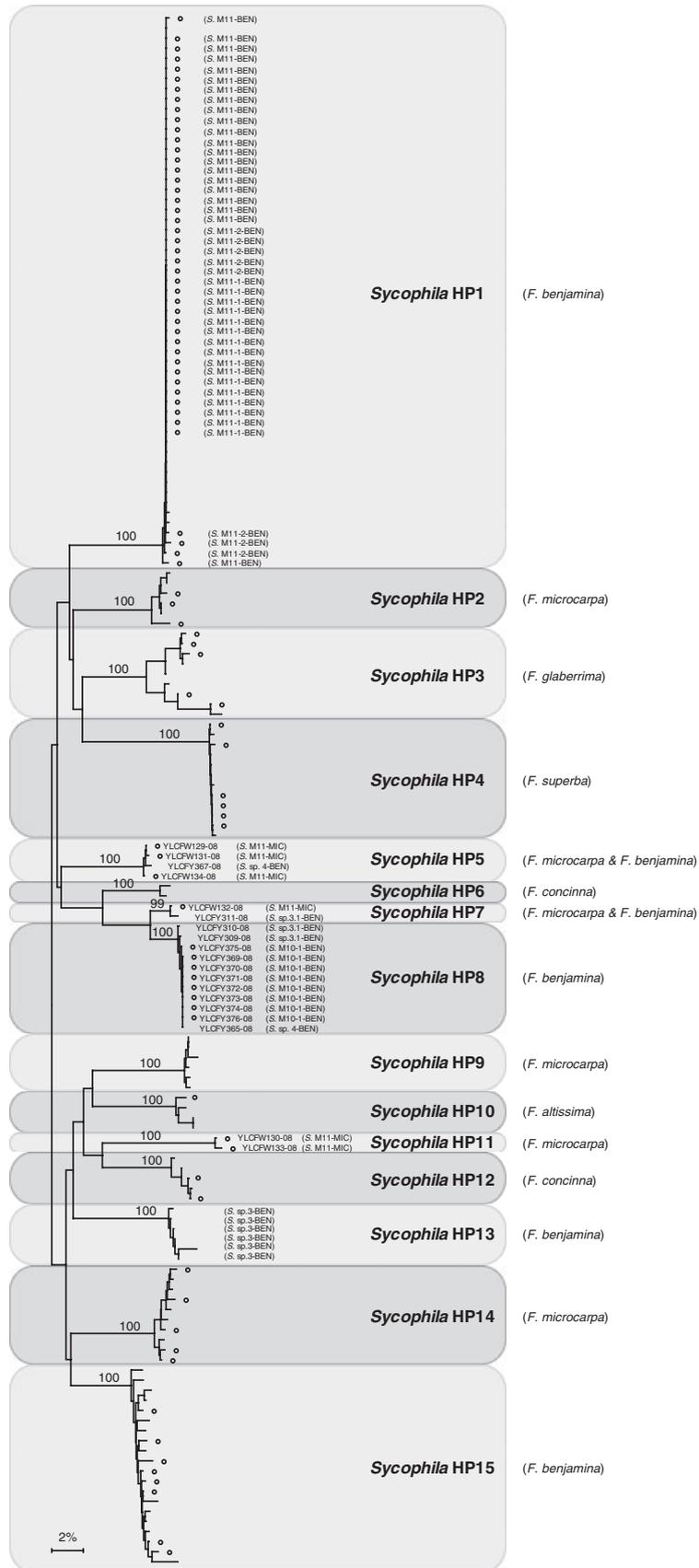
### Nucleotide characteristics

A COI sequence was obtained from all 154 specimens, but only 30 were full-length (652 bp). This low yield reflected sequencing problems caused by poly-T runs. Three COI sequences were just 401 bp, but the remainder (121) were 545 bp in length. The frequency of adenine (A) and thymine (T) was high in COI (A = 29.7%, C = 12.5%, G = 14.0%, T = 43.8%), a typical characteristic in the mitochondrial genomes of Hymenoptera (Crozier *et al.* 1989; Simon *et al.* 1994). An ITS2 sequence was recovered from 130 of the 154 *Sycophila* specimens, and these varied in length (351–459 bp) as a result of indels, and showed a more even nucleotide composition (A = 28.4%, C = 20.6%, G = 22.6%, T = 28.3%). No intra-individual variation of ITS2 was observed in *Sycophila* in this study.

### Species delimitation and sex association in *Sycophila*

Sequencing results for COI and for ITS2 revealed that the 25 *Sycophila* morphs derived from 15 'haplogroups' (HP in Table 1, Figs 1 and 2). Because COI and ITS2 haplogroups were congruent, we conclude that 15 species were present (Figs 1 and 2). Branch support for all 15 haplogroups was high (>99%) in the COI tree (Fig. 1) and for all but one haplogroup in the ITS2 tree (*S.* HP2, Fig. 2, but see Discussion). All but three (see following details) of the 25 *Sycophila* morphs were perfectly categorized into one of the HPs. Eleven of 15 HPs were represented by both sexes while three HPs (*S.* HP6, *S.* HP9 and *S.* HP13) were represented solely by females and one by males (*S.* HP11). Male polymorphism was apparent in one species where a single female morph (*S.* sp.1-BEN) was associated with three male morphs (*S.* M11-BEN, *S.* M11-1-BEN and *S.* M11-2-BEN). Interestingly, this case involved variation in femoral shape indicating that it is an allometric intraspecific variation. The femoral structure was correlated to male size, i.e. large males possessed a sharp tooth, while smaller male had a blunt tooth or none at all.

**Fig. 1** Neighbour-joining tree of Chinese *Sycophila* COI sequences, using Kimura-2-parameter distance. The 15 haplogroups are indicated on the right of the tree. Process IDs (a unique code assigned to each individual) and/or morphospecies are not provided if a haplogroup is clearly delimited by both morphology and by COI and ITS2 divergence. Supplementary materials provide detailed COI and ITS2 trees. Haplogroups 5, 7, 8 and 11 are provided with specific process IDs and associated morphospecies (in brackets) identified using morphology. All male individuals are indicated by a circle. The host fig species is noted in parenthesis after each haplogroup. Bootstrap values for each haplogroup were calculated in MEGA4.0 with 1000 replicates and are shown on each branch.



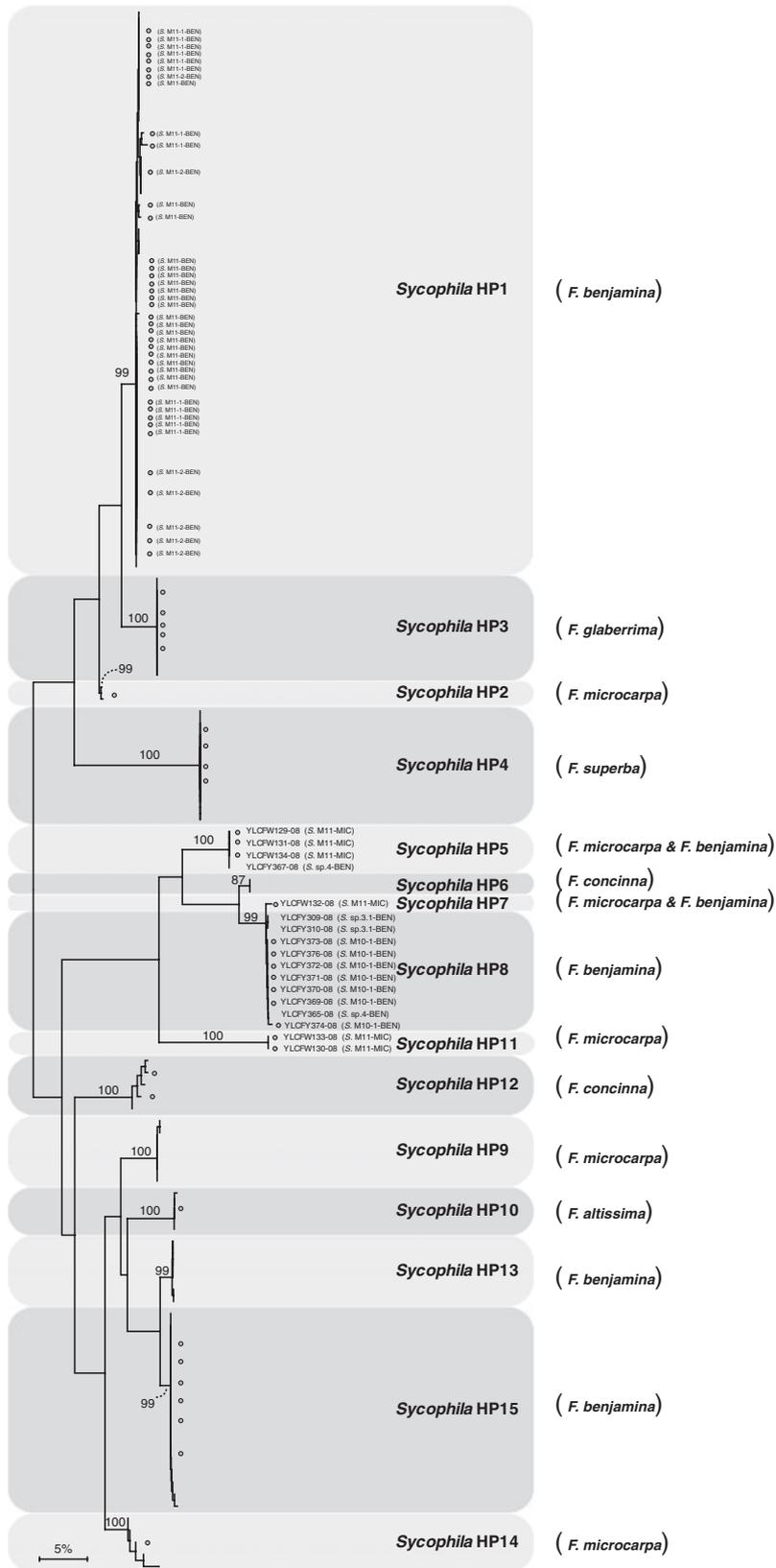


Fig. 2 Neighbour-joining tree of Chinese *Sycophila* ITS2 sequences, using Kimura-2-parameter distance. Figure legends follow those in Fig. 1.

Intra-specific (i.e. within each molecular haplogroup clade) variation at COI ranged from 0% to 1.1%, while inter-specific distances varied from 7.2% to 19.4%. By comparison, intra-specific variation at ITS2 ranged from 0% to 0.6%, while inter-specific variation ranged from 0.6% to 24.6%. Surprisingly, one male morph of *F. microcarpa* (S. M11-MIC) was separated into three very divergent haplogroups (S. HP5, S. HP7 and S. HP11) on both gene trees (Figs 1 and 2). Similarly, one female morph, S. sp.3.1-BEN, was split into two haplogroups (S. HP7 and S. HP8). Two of the three specimens identified as S. sp.3.1-BEN (YLIFY309-08 and YLIFY310-08) are grouped with a different female morph S. sp.4-BEN (YLIFY365-08) in haplogroup S. HP8 although they are morphologically similar to another female morph S. sp.3-BEN, which alone forms a distinct haplogroup (S. HP13) and far from S. HP8. A third female identified as S. sp.3.1-BEN (YLIFY311-08) was associated with one male specimen belonging to male morph S. M11-MIC, forming haplogroup S. HP7. Finally, two female individuals identified as S. sp.4-BEN (YLIFY367-08 and YLIFY365-08) were delimited by molecular analyses as S. HP5 and S. HP8, respectively. The former is associated with three of the six individuals previously recognized as S. M11-MIC; the latter is delimited as S. HP8 and is associated with male morph S. M10-1-BEN and a different female morph S. sp.3.1-BEN mentioned earlier. Interestingly, all these molecular delimitations that appear to be conflicting with morphological examination showed identical clustering patterns on COI and ITS2 gene trees, except one of the two specimens forming S. HP7 (YLIFY311-08) in the COI tree failed to amplify for ITS2.

Most of the morphologically similar morphs found on *F. benjamina* and *F. microcarpa* were clearly separated on COI and ITS2 gene trees. However, two HPs occurred on both fig species suggesting two cases of shared host use.

## Discussion

### *Tracing species boundaries using COI and ITS2*

Taxonomic boundaries for 22 of the 25 *Sycophila* morphs were clearly resolved by COI and ITS2 sequences, but the other three showed conflicts between morphological and molecular data. However, clustering patterns for the independent gene trees of COI and ITS2 were congruent for every case where sequences were available for both genes, including the three cases where DNA results conflicted with morphological evaluation (discussed in details in 'Morphology and DNA analyses'). This observation affirms that COI and ITS2 are both effective DNA markers for species level identification in *Sycophila*. ITS2 data here confirm the pattern for COI thereby refuting

any suspicion that the barcoding data are not indicative of species level boundaries (Smith *et al.* 2006, 2007). Both molecules delimit closely related species that are difficult or impossible to distinguish morphologically. Furthermore, cases of phenotypic plasticity are resolved. For example, the three male morphs of S. HP1 share essentially identical COI and ITS2 sequences, while members of two female morphs in S. HP8 are linked by both DNA markers.

Internal transcribed spacer 2 has been employed in the studies of other hymenopterans (Pinto *et al.* 2002; Joussetin *et al.* 2004; Jiang *et al.* 2006b). This nuclear gene fragment shows species-specific variation in sequence length in the *Sycophila* taxa included in this investigation. Although ITS2 varied from 351 to 459 bp among these species, it was highly conservative within species. Although length variation complicates sequence alignments and phylogenetic analysis, sequence length alone was sufficient to separate all *Sycophila* species examined in this study. It remains uncertain if *Sycophila* from other geographical regions can be separated in a similar fashion. In contrast to ITS2, the barcode region of COI was invariant in length in *Sycophila*, although indels occur in other fig wasps (e.g. *Ceratosolen*, personal observation). The wide acceptance of COI as a DNA marker for species diagnosis in the animal kingdom makes the comparison of various *Sycophila* species collected worldwide feasible.

### *Sex association*

One major advantage of DNA analysis in *Sycophila* as well as in other taxa with sexual dimorphism is the association of males and females. Although morphological differences between male and female *Sycophila* are less extreme than in some other fig wasps, linking sexes is still challenging. This is further complicated by the existence of phenotypic polymorphism within species and vague interspecific morphological variation in both sexes.

Four haplogroups (S. HP6, S. HP9, S. HP11 and S. HP13) were only represented by a single sex, suggesting that our sampling is incomplete. Of course, the likelihood of having extreme sexual dimorphism in these taxa should not be overlooked, in which case individuals of the opposite sex may have been excluded from molecular analyses because they were not identified as *Sycophila*.

### *Host specificity*

Despite their extreme morphological similarity, several *Sycophila* morphs from different hosts proved to be distinct species (e.g. S. sp.1-MIC and S. sp.3-BEN, Figs 1 and 2), forming mutually exclusive haplogroup clusters in both gene trees. Thirteen of the 15 haplogroups were

associated with a single host, while the other two (*S.* HP5 and *S.* HP7) occurred on two host species, *F. benjamina* and *F. microcarpa* (Fig. 1). Consequently, we conclude that all but two of the 15 *Sycophila* species examined in this study are host specialists. It remains uncertain whether these *Sycophila* species parasitize *Ficus* or other insects associated with these trees. In either case, most *Sycophila* show a species-specific association with figs. On the other hand, both *F. benjamina* and *F. microcarpa* supported assemblages of at least six species of *Sycophila*.

The host specificity detected in this study is greater than that revealed in earlier investigations. For example, Bouček *et al.* (1981) found that some African *Sycophila* species appeared to use a range of hosts. As there is no strong reason to believe that Chinese *Sycophila* are exceptional, we suspect that the African investigations may have overlooked cryptic species. A molecular re-evaluation of the African *Sycophila* would clarify host specificity.

We emphasize that molecular insights into species boundaries, such as those gained in this study, enable conclusions concerning ecological and biological attributes of component species before taxonomic issues, such as the description of new species, are completely resolved.

#### *Morphological and DNA analyses*

The separation of three male morphs (*S.* M11-MIC) and lumping of different female morphs (*S.* sp.3.1-BEN and *S.* sp.4-BEN in haplogroup *S.* HP8) revealed by DNA markers are not expected. However, the identical clustering pattern given by independent molecular markers is even more surprising. It is not unusual that a particular DNA marker fails in tracing species boundaries because of the lack of sufficient variation or saturation of nucleotide changes (Whitworth *et al.* 2007; Wiemers & Fiedler 2007). If that were the case, we would have readily accepted the ineffectiveness of COI or ITS2 in diagnosing certain *Sycophila*. This would not have been surprising in such an enclosed natural community (fig syconium) that may enhance rapid speciation, where the retention of ancestral polymorphism and hybridization may have both contributed to the failure of molecular tracing of species boundaries (Mallet & Willmott 2003; Mallet 2005). However, the likelihood of having highly cohesive pattern in mitochondrial and nuclear genes is very small because of the obvious difference in the heritage pathway in these two sets of DNA markers. Thus, we conclude that these genetically distinguishable clusters stated above represent cryptic species. Similar phenomena have been observed in distantly related pollinating fig wasps (Agaonidae) (Molbo *et al.* 2003; Haine *et al.* 2006). The accumulating discovery of cryptic species across various

wasp groups seems to suggest that morphological cues may not always serve as critical restriction in reproductive isolation for organisms living in enclosed habitats such as the fig fruit. This implication may be further supported by the findings where different morphospecies prove to be genetically identical, in which case reproductive isolation remains effective despite the obvious morphological difference.

A comprehensive study of the relevant morphs is needed to clarify the issue, especially because some are only represented in this study by very limited number of individuals. However, we feel that re-evaluation of the significance of some morphological characteristics in *Sycophila* taxonomy (and perhaps in other fig wasps) is necessary. Some characters currently used in differentiating *Sycophila* morphs appear to be unreliable within species, such as the projection on fore femora in the three male morphs associated with *S.* HP1 found on *F. benjamina*. Other characters, such as colour and darkness of body parts, seemed to be correlated with the size of insects (Bouček *et al.* 1981). On the other hand, subtle morphological difference is detected between individuals that were previously assigned to the same morph before DNA analyses. For example, the two individuals of *S.* sp.4-BEN was re-evaluated and found to be different in the length of stigma under the infumated spot and body size. From what we have seen in this work, it is safe to conclude that the extent of morphological difference among members of Chinese *Sycophila* complex is not always even. Several female and male morphs identified in *F. microcarpa* highly resembled those found in *F. benjamina*, with minor morphological differences. As some nonpollinating fig wasps have been demonstrated to have high host specificity (Bouček 1988), it is reasonable to assume that similar morphs associated with different plant species may represent distinct taxa. However, independent lines of evidence such as molecular data are required to verify these hypotheses of species boundaries. While sophisticated morphological work such as dissection of genital structures may help to ultimately clarify the issue, molecular analyses such as the use of DNA barcodes and nuclear genes can certainly assist in the finding of potential cryptic species and draw attention to the taxonomic entities that deserve further investigation.

We can tentatively conclude from our results that both mitochondrial COI and nuclear ribosomal ITS2 sequences proved effective in diagnosing species of Chinese *Sycophila*, and their congruence in results helped to confirm species boundaries. Issues that have long impeded ecological and biological studies on *Sycophila*, such as sex association and host specificity, were readily resolved through DNA analysis. Moreover, new insights into morphological plasticity and cryptic species were obtained.

Some of the conventional morphological characters in species delimitation of *Sycophila* need to be re-evaluated.

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## Supporting Information

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

### Appendix S1 Specimen details and collection data.

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