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Parasitoid–host associations of the genus *Coccophagus* (Hymenoptera: Aphelinidae) in China

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Host relationships among many Aphelinidae are complex due to their heteronomous reproductive behaviour, where males have different host relationships from females. Heteronomous parasitoids present a fascinating problem in host selection and sex ratio decision making. Accurate identification of insect parasitoids is a prerequisite in the determination of parasitoid–host relationships. With the goal of disentangling the parasitoid–host associations in the genus *Coccophagus*, we compared the performances of the *COI* barcode and of the *28S*-D2 rRNA region for identification and delimitation of 17 morphospecies of *Coccophagus* parasitoid wasps collected during a 9-year rearing programme of scale insects in China. Molecular data were analysed by two different methods of species delimitation, the automatic barcode gap discovery and the general mixed yule coalescent. Both methods were effective in discriminating all previously recognized morphospecies. The congruence of morphospecies delimitation with that obtained by DNA barcode and nuclear gene data greatly enhanced our ability to unravel the parasitoid–host associations of genus *Coccophagus*. Most *Coccophagus* species (11 out of 17) can use host species belonging to different genera, with different levels of host specificity, particularly in host selection to produce female offspring. Sex-related differences in host relationships have also been detected and discussed.

ADDITIONAL KEYWORDS: Aphelinidae – generalist parasitoid – mitochondrial gene –nuclear gene – species delimitation.

INTRODUCTION

The parasitoid–host relationship has long been a focus of behavioural ecology, evolutionary biology and community ecology (Price, 1980; Futuyma & Moreno, 1988; Kawecki, 1998; Derocles *et al.*, 2014). Limited parasitoid–host data, particularly host range and host-use patterns, are often an obstacle to understanding true relationships (Hawkins, 1994; Shaw, 1994; Quicke, 1997). As previously noted (Whitfield & Wagner, 1988; Jervis, Kidd & Walton, 1992), the practical reasons mainly include (1) the need for an exhaustive survey of potential hosts for a given parasitoid; (2) the immense effort required for identification of parasitoids, hosts and host plants and (3) interspecies/intraspecies variation, a long-standing problem in species delimitation. Among them, the fundamental issue is the accurate identification of the interacting partners, particularly the identification of sexually dimorphic parasitoid wasps. Because descriptions for many parasitoids are based on the female sex only, and descriptions of males are often uninformative due

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to a general lack of useful characters, biological data associated with the male sex in species inventories are scarce or unreliable. DNA barcoding, as an identification tool, has greatly facilitated the discrimination of both parasitoids (Zhang *et al.*, 2011; Chesters *et al.*, 2012) and host species (Deng *et al.*, 2012, 2016; Wang *et al.*, 2015, 2016). Recent efforts to estimate parasitoid–host relationships show that without DNA-based discrimination of parasitoid and host species, erroneous parasitoid–host relationships may be diagnosed due to inadvertent labelling of morphologically similar, but genetically isolated, lineages as a single species (Smith *et al.*, 2006, 2008; Chesters *et al.*, 2012). The combination of DNA-based discrimination of parasitoids with morphological data and host records has greatly improved our knowledge of the ecological relationships of Diptera and Hymenoptera parasitoids (Smith *et al.*, 2006, 2008; Zhang *et al.*, 2011; Chesters *et al.*, 2012).

The Aphelinidae (Hymenoptera: Chalcidoidea) is an important group with remarkable economic importance as biological control agents against whitefly and scale insect pests (Clausen, 1978; Greathead & Waage, 1986). The subfamily Coccophaginae has host relationships that are different for males and females, and therefore are defined as heteronomous (Walter, 1983; Williams & Polaszek, 1996). These unusual host relationships were described by Flanders (1936, 1937), with subsequent contributions by Zinna (1961), Yasnosh (1976) and Viggiani (1981). Walter (1983) gave a simplified but comprehensive classification, proposing the term 'heteronomous hyperparasitoids' for those species in which males develop as hyperparasitoids at the expense of the preimaginal stages of conspecific and/or heterospecific parasitoid larvae, either on the same or different primary hosts as conspecific females. He also proposed two further categories: (1) diphagous parasitoids, in which sexes are both primary parasitoids, but females develop as endoparasitoids and males as ectoparasitoids and (2) heterotrophic parasitoids, in which conspecific females and males develop on completely different hosts (e.g. whiteflies and Lepidoptera eggs, respectively), both as primary parasitoids. Outside Coccophaginae, heteronomous parasitoids are known only in the family of Myrmecolacidae in the 'twisted winged flies' Strepsiptera (Kathirithamby, 2009), in this case as heterotrophic parasitoids.

Most hosts of Coccophaginae belong to the Hemiptera Sternorrhyncha (Aleyrodoidea, Coccoidea, and rarely Aphidoidea and Psylloidea) (Evans, Polaszek & Bennett, 1995; Polaszek, Evans & Bennett, 1992). In addition, systematically unrelated hosts such as eggs of Heteroptera, Auchenorrhyncha and Lepidoptera may be used (Yasnosh, 1979; Viggiani, 1981; Polaszek, 1991; Polaszek & Luft Albarracin, 2011). Females are usually primary endoparasitoids of scale insects or whiteflies, whereas males may develop as ectoparasitoids of the same (or closely related) host species or as hyperparasitoids of conspecific females or other endoparasitoid species (Williams & Polaszek, 1996; Hunter & Woolley, 2001). While there is increasing information on host-use patterns for some parasitoid groups (Smith *et al.*, 2006, 2008; Zhang *et al.*, 2011; Chesters *et al.*, 2012), few such studies have been conducted on Aphelinidae. Their actual host range remains to be investigated for many species because of very scant knowledge of the systematics of both hosts and parasitoids (Viggiani, 1984; Hayat, 1998; Kim & Heraty, 2012). Consequently, sex-specific host usage, which may follow independent evolutionary trajectories in the two sexes (Williams & Polaszek, 1996; Hayward, McMahon & Kathirithamby, 2011), has not yet been exhaustively investigated.

In China, an intensive survey of numerous scale insects, and rearing of insect parasitoids, has been conducted (Zhang *et al.*, 2011; Chesters *et al.*, 2012). From 2007 to 2015, we processed more than 2000 collections of scale insects, gathering data on host plants and parasitoids belonging to the genus *Coccophagus*. We selected species of *Coccophagus* because, in addition to their economic importance as natural enemies and their heteronomous lifestyle, many host records are available from the literature (Compere, 1931; Annecke & Insley, 1974; Huang, 1994; Hayat, 1998). In this study, we sought to ascertain whether patterns of sequence variation in *COI* and *28S*-D2 rRNA are congruent with each other, and with morphology, in revealing species boundaries. In particular, we employed molecular evidence to test whether morphologically similar males from different hosts represented a single species and consistently matched the putative corresponding females. On the basis of solid taxonomic work, we investigated the host range and host-use pattern of *Coccophagus* species occurring in China, to gain more data on biased sex ratios and differential host usage related to their heteronomous reproductive behaviour.

MATERIAL AND METHODS

SAMPLING, REARING AND MORPHOLOGICAL IDENTIFICATION

All samples of *Coccophagus* species (Supporting Information, Table S1) were reared from wild-collected plant material infested with scale insects across China from 50 sites in 22 provinces (Supporting Information, Fig. S3). Collection of hosts and rearing of parasitoids followed methods described in Zhang *et al.* (2011). Parasitoids and representatives of their scale insect hosts were stored in 95% ethanol for subsequent study. Preliminary morphological identification of

Coccophagus was made by author YZZ and then confirmed by Prof. Huang Jian (Fujian Agriculture and Forestry University) and Prof. Cheng-De Li (Northeast Forestry University). Scale insect species were morphologically identified by author S-AW and then verified by molecular markers (Deng *et al.*, 2012, 2016; Wang *et al.*, 2015, 2016). Voucher specimens of parasitoids and scale insects were deposited in the Institute of Zoology, Chinese Academy of Sciences (IZCAS) and the Insect Collection of Beijing Forestry University, respectively.

DNA extraction, polymerase chain reaction amplification and sequencing

Individuals were removed from ethanol and dried in open Eppendorf tubes prior to extraction. Head, abdomen, legs and wings of specimens were removed and mounted on slides following the method by Noyes (1982) for further morphological examination. Total genomic DNA was extracted from the thorax of each individual using DNeasy TM Tissue Kits (QIAGEN), following the manufacturer's protocols. After DNA extraction, the thorax was also mounted on the same slide along with the other parts for further morphological study.

Primer sequences and polymerase chain reaction (PCR) protocols of *28S* and *COI* partial genes followed Zhang *et al.* (2011) and Yu *et al.* (2014). Each PCR product was subjected to electrophoresis on 1% agarose gel, and positive products were sequenced directly in both directions using BigDye v3.1 on an ABI 3730xl DNA Analyser (Applied Biosystems). Generated sequences were deposited in GenBank (accession numbers: KY605407–KY605787 for *28S* sequences, KY605788–KY606167 for *COI* sequences).

Molecular data analyses and species delimitation

Forward and reverse sequences were assembled and edited with Bioedit v7.1.3.0 (Hall, 1999). The aligned *COI* data set was then translated into amino acids using MEGA 6 (Tamura *et al.*, 2013) to check for stop codons, nonsense and frameshift, such as numts (Song *et al.*, 2008). The *28S* data set was aligned with MAFFT (Katoh *et al.*, 2002) using the Q-INS-i algorithm (Katoh & Toh, 2008). In order to assess the intraspecific and interspecific genetic variability, Kimura 2-parameter distances (Kimura, 1980) for *COI, 28S* (D2 region) and the combined data set were obtained using MEGA 6 (Tamura *et al.*, 2013).

We analysed sequence data using quantitative methods of species delimitation: automatic barcode gap discovery (ABGD) and general mixed yule coalescent (GMYC). Each analysis uses different terminologies to refer to the delimited taxa (ABGD = 'entities'; GMYC = 'putative species'), thus acknowledging that they may not represent biologically meaningful species.

ABGD infers entities by recursively partitioning the data using a range of prior intraspecific sequence divergences and by calculating a model-based confidence limit for intraspecific divergence at each iteration (Puillandre *et al.*, 2012a, b). We calculated the number of entities using the ABGD online tool (Puillandre *et al.*, 2012a; available at http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html). Default parameters were used, except for the number of steps (20 steps from P_{min} to P_{max}) and relative gap width (X) set to 0.25 to take into account small gaps (especially for very closely related species).

GMYC is a maximum likelihood method for delimiting species by fitting within- and between-species branching models to reconstruct gene trees (Pons *et al.*, 2006). GMYC analysis was executed in R (R Development Core Team, 2014) using four different packages (ape, MASS, paran and splits) (from http://r-forge.r-project.org/projects/splits) with the single threshold optimization (Monaghan *et al.*, 2009). Both neighbor-joining trees, obtained with PAUP 4.0b10 (Swofford, 2002), and Bayesian trees, obtained with MrBayes v3.2 (Ronquist *et al.*, 2012), based on single *COI* gene and combined data *COI* + *28S*, were used for GMYC analyses. The best-fit model for Bayesian analyses was estimated using jModelTest v2.1.3 (Darriba *et al.*, 2012) and selected based on the Akaike information criterion (Posada & Buckley, 2004). For each data matrix, two independent Markov Chain Monte Carlo runs of four Metropolis-coupled chains were performed with the gamma shape parameter, the proportion of invariant sites, base frequencies and substitution rates unlinked across all partitions and with default priors.

RESULTS

Delimitation of *Coccophagus* species

Morphological process

At least one *Coccophagus* individual was reared from > 100 rearings of scale insect species (among > 2000 rearings of scale insects). A total of 3129 specimens of *Coccophagus* belonging to 17 morphospecies (Supporting Information, Figs S1 and S2, figures of *Coccophagus longipedicellus* were not presented) were reared from 30 scale insect species collected in the field (Supporting Information, Table S1 and Fig. S3). *Coccophagus* species that could not be identified with certainty were treated with a numeric code (*Coccophagus* sp1, *Coccophagus* sp2). The name originally applied to a morphospecies is retained as a

reference point but is not meant as a firm scientific identification.

Molecular delimitation of species

At least one representative specimen of each successful rearing was subjected to molecular analysis. PCR products were generated from 435 (345 females, 90 males) of 470 specimens. We obtained partial *COI* gene sequences from 380 specimens and *28S* gene sequences from 381 specimens (Supporting Information, Table S1). The final alignment of *COI* includes 501 bp with 220 variable sites (43.9%). The final alignment of *28S* (including gaps) consisted of 609 bp with 224 variable sites (36.8%). The number of haplotypes was 118 for *COI* and 27 for *28S*, respectively. Both *COI* and *28S*, as single genes, and combined data (*COI* + *28S*) were used in our analyses.

In ABGD analysis, the first major barcode gap was evident at the estimated distance *P* value of 0.048 and 0.056 supporting the presence of 17 entities (Fig. 1, Supporting Information, Figs S4 and S5). The best-fit model for Bayesian analyses of both single gene and combined data was GTR + G for first and third codon of *COI* sequences and GTR + I + G for second codon of *COI* and *28S* sequence. In GMYC analyses, the number of putative species ranged between 18 and 22, depending on the phylogram (Supporting Information, Fig. S5, Table S2). Most morphospecies have been assigned to a single delimited taxon, except morphospecies *Coccophagus japonicus* (2–3 delimited taxa) and *Coccophagus ceroplastae* (1–4 delimited taxa), which were split into multi-entities using the other methods under GMYC (Supporting Information, Fig. S3). There were overlaps of sequence divergences between intraspecies and interspecies delimited by GMYC analyses (Table 1), while there is an evident gap in ABGD results. All morphospecies were strongly supported by the nuclear *28S* gene, as individuals of each species have identical *28S* sequences (Supporting Information, Fig. S5). For all the sequenced males (with a single exception) in our analysis, we detected the corresponding female (Supporting Information, Figs S6, S7). Both *COI* and 28S data revealed 17 major genetic clades corresponding to the morphological taxonomic hypothesis of 17 recognized species (Fig. 1).

Host range investigation

Our integrative process of combining morphological, biological and DNA barcoding data has resulted in a better understanding of parasitoid diversity and host range of *Coccophagus*. Most species of *Coccophagus* can utilize several host species, which usually belong to one, two or more genera of Coccidae, but rarely species of other families, such as Eriococcidae and Kermesidae (Fig. 1, Supporting Information, Table S3). *Coccophagus ceroplastae*, *C. japonicus* and *Coccophagus ishiii* were found to be more general in host selection than the others. *Coccophagus ceroplastae* was reared from 13 species in 5 coccid genera (Coccidae). *Coccophagus japonicus* was found to parasitize 12 species in 7 coccid genera (Coccidae) and 1 species in the family Eriococcidae (*Eriococcus lagerostroemiae*). *Coccophagus ishiii*, as well as exploiting seven species of Coccidae, parasitized *Kermes nawae* (Kermesidae). A slightly narrower host range was found for *Coccophagus lycimnia*, *Coccophagus yoshidae* and *Coccophagus* sp4, each using three or four host species. For the remaining species, only one or two hosts were confirmed, indicating a narrower host range than other species.

Sexual differences in host utilization

The genus *Coccophagus* is a typical representative of heteronomous aphelinids, where males and females may develop on different hosts. In our investigation, almost all *Coccophagus* species show a skewed sex ratio in producing offspring on a given host (Fig. 2, see Supporting Information, Table S1). For example, among the 39 rearings of *C. japonicus* (Supporting Information, Table S4), 37 were female biased (30 rearings produced exclusively female offspring and in 6 rearings female offspring outnumbered males), 1 exhibited an even sex ratio, 1 was male biased, while only males were produced on the unique non-coccid host, *Eriococcus lagerostroemiae*. This was also the case for *C.* sp2, which produced only males when reared from the non-coccid host, *Phenacoccus* nr *aceris* sp1. Among the rearings of *C. ishiii*, progenies obtained from *Takahashia japonica*, *Eulecanium kuwanai*, *Parthenolecanium corni* and the non-coccid *K. nawae* were exclusively males. Finally, for *Coccophagus* sp6 only males were found.

DISCUSSION

Congruence of morphological and molecular delimited species of *Coccophagus*

The *COI* barcode was found to accurately distinguish all the provisional species that had been identified through morphological analysis. Two species, *C. japonicus* and *C. ceroplastae*, are worthy of special attention due to their relatively broader barcode divergence. However, this divergence can be observed among individuals of a single rearing (population), while individuals from different locations can share the same haplotypes. Although GMYC analysis separated each entity into two or even three potential species, some studies have

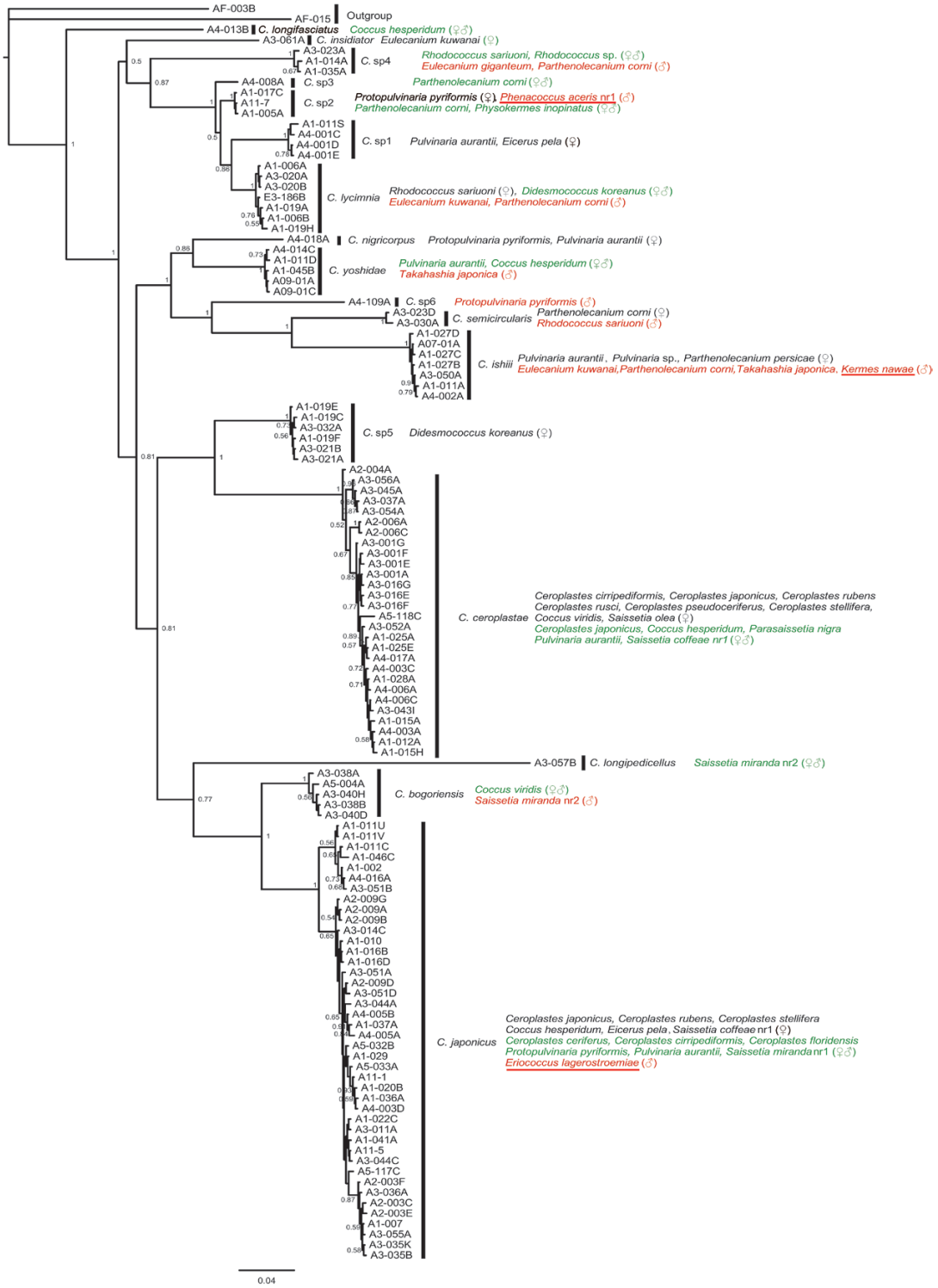

Figure 1. Bayesian inference phylogram of the 17 *Coccophagus* species using 115 haplotypes of combined data (*COI* + *28S*). Numbers above branches indicate Bayesian posterior support for individual clades with two *Coccophagus* species from Africa used as outgroup. Host names were listed right after each *Coccophagus* species: names marked with black and red indicating hosts producing only female and male wasps, respectively; names marked with green indicating hosts producing both female and male wasps. Hosts belonging to Eriococcidae (*Eriococcus lagerostroemiae*), Kermesidae (*Kermes nawae*) or Pseudococcidae (*Phenacoccus aceris* nr1) are underlined.

Table 1. Maximum intraspecific and minimum interspecific divergences of species delimited by ABGD and GMYC analyses based on *COI* and *COI* + *28S* combined data (calculated using MEGA 6 under Kimura 2-parameter model) (Kimura, 1980)

Genetic divergence		ABGD	GMYC			
		COI	NJ (*COI*)	BI (*COI*)	NJ (*COI* + *28S*)	BI (*COI* + *28S*)
COI	Maximum intraspecific	0.0481	0.0203	0.035	0.0328	0.0351
	Minimum interspecific	0.0561	0.0141	0.0141	0.0141	0.0266
COI + *28S*	Maximum intraspecific	0.0226	0.0144	0.0156	0.0156	0.0212
	Minimum interspecific	0.0285	0.0068	0.0077	0.0096	0.0127

BI, Bayesian inference; NJ, neighbor-joining.

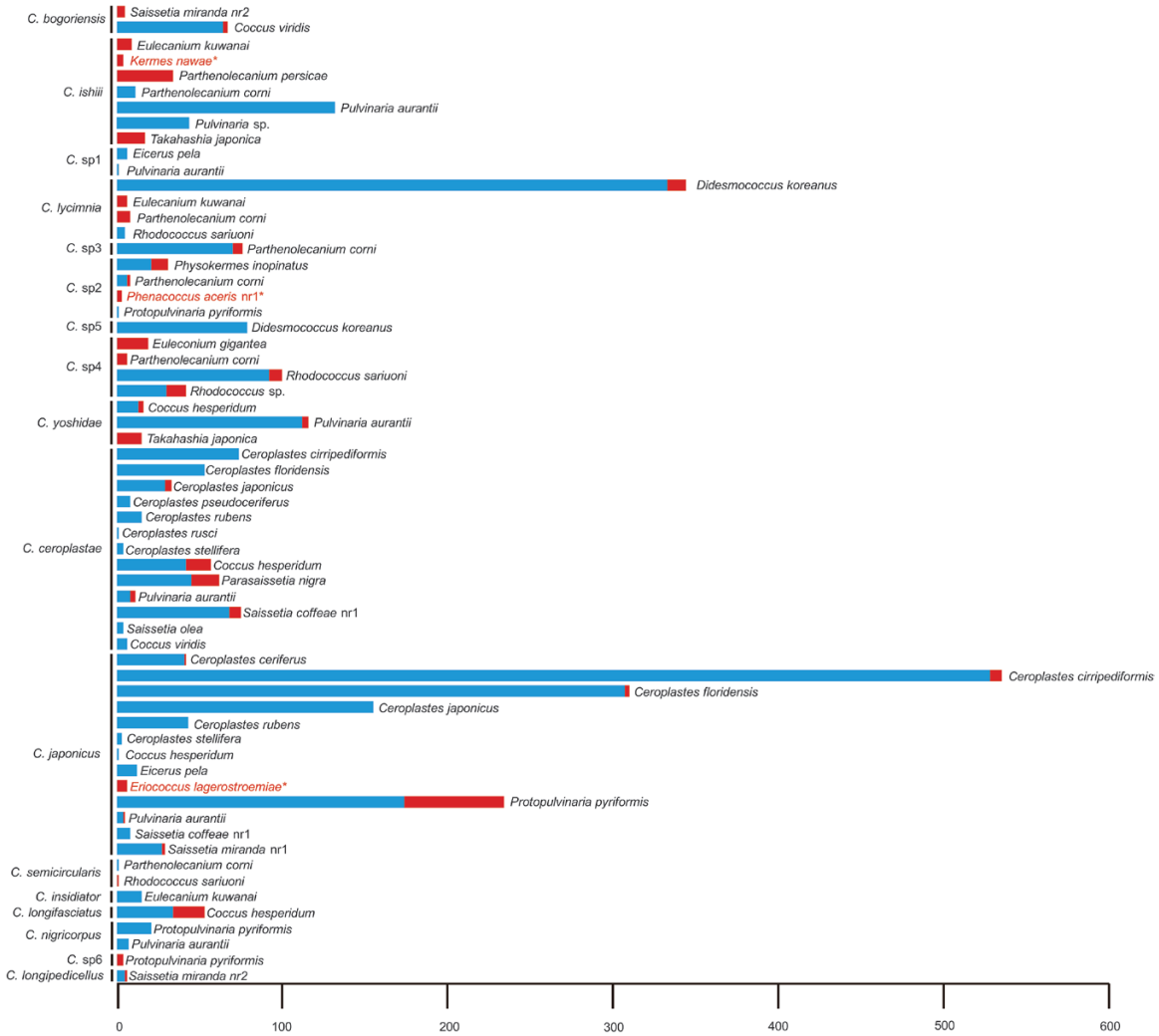

Figure 2. Female (blue) and male (red) composition on each hosts for the 17 *Coccophagus* species. Host species were labelled on the right of histograms. Host species belonging to Eriococcidae (*Eriococcus lagerostroemiae*), Kermesidae (*Kermes nawae*) and Pseudococcidae (*Phenacoccus aceris* nr1) are marked with '*'.

indicated that this method overestimates the number of potential species (Lang *et al.*, 2015; Schwarzfeld & Sperling, 2015). Moreover, all the morphological species were corroborated by the analysis of the unlinked *28S* gene, which appears to be a very useful marker for identification of species in Chalcidoidea (Babcock & Heraty,

2000; De Barro *et al.*, 2000; Babcock *et al.*, 2001; Manzari *et al.*, 2002; Pedata & Polaszek, 2003; Polaszek, Manzari & Quicke, 2004). However, this region in several other cases has been shown to be from highly conserved to invariant, among closely related species, otherwise well differentiated for the *COI* sequence and biological traits (Heraty *et al.*, 2007; Gebiola, Bernardo & Burks, 2010; Gebiola *et al.*, 2016).

The congruence of unlinked gene markers confirms the existence of independent gene pools (Monaghan *et al.*, 2005). In DNA barcoding of very young species, or species with deep diversification, a secondary independent molecular marker to support or confirm identification is of great need (Smith *et al.*, 2007). Taking into account all the evidence provided by molecular and morphological analysis, both *C. japonicus* and *C. ceroplastae* could be considered taking a conservative perspective as single species with relative broader *COI* genetic divergence. Further studies are needed to examine whether this genetic divergence is related to host use or geographic distribution (Baer *et al.*, 2004; Stireman, Nason & Heard, 2005; Stireman *et al.*, 2006). A comparison of *COI* with the *28S*-D2 for most *Coccophagus* species revealed that it was a more sensitive marker in revealing intraspecies diversity. The *COI* gene has the potential to be used for DNA barcoding of *Coccophagus* although a good taxonomic foundation coupled with extensive sampling of taxa is essential for the development of an effective identification system.

An obvious application of DNA barcoding is matching males and females of highly dimorphic species, especially for those groups whose males exhibit reduced interspecific variation (e.g. Hulcr *et al.*, 2007). The effectiveness of DNA barcoding to discriminate both females and males of a species has been tested only in a few groups of insects (e.g. Janzen *et al.*, 2009; Ekrem, Stur & Hebert, 2010; Li *et al.*, 2010). DNA barcoding enabled the association of females with males from different localities and hosts, proving to be an efficient tool for identification of sexually dimorphic male and female parasitoid wasps. This is particularly useful for studies of species with gender-biased sex ratios, which are usually difficult to resolve by traditional methods. In our study, many rearings of scale insect hosts produced exclusively male offspring, whose identification, without the help of highly specialized taxonomy experts, is difficult based on the available literature. DNA barcoding can successfully provide identification at species level for these males based on a solid genetic clustering with the corresponding females.

Host range

Due to our insufficient knowledge of the systematics of both hosts and parasitoids (Viggiani, 1984), the remarkable morphological intraspecific variability of *Coccophagus* species (Compere, 1931; Graham, 1976; Hayat, 1993; Viggiani, 1999), and more importantly the heteronomous reproductive biology (Flanders, 1936, 1937), the actual host range of *Coccophagus* species could be very difficult to define. Our long-term investigation of *Coccophagus* species and particularly the use of molecular markers for their identification and that of their corresponding hosts, resulted in a better understanding of species diversity and host range than any single type of information could provide. The predominant hosts are Coccidae (27 species), although Eriococcidae (1 species), Kermesidae (1 species) and Pseudococcidae (1 species) can be rarely hosts of males. With a few exceptions, most *Coccophagus* species exploit more than one host and show a variable degree of host specificity in producing female offspring. For instance, *C. japonicus* is able to utilize a broad range of species of several coccid genera for female and/or male production, using Eriococcidae for male production only. Moreover, in non-heteronomous parasitoid groups, most species exhibit relatively high levels of host specificity in Encyrtidae (Zhang *et al.*, 2011; Chesters *et al.*, 2012), Microgastrine (Smith *et al.*, 2008) and Tachinidae (Smith *et al.*, 2006, 2007). These studies show that host specificity may vary in different parasitoid groups. The heteronomous reproductive strategy may enable Coccophaginae to use many hosts via other parasitoids. The present study has provided a solid background for unveiling relationships of many Chinese *Coccophagus* species. If this host-use pattern holds for other *Coccophagus* faunas, these results can partially explain the reason for so many successful establishments of *Coccophagus* species in biological control programmes (Greathead & Waage, 1986), in contrast to the host-specific natural enemy groups, such as Encyrtidae (Zhang *et al.*, 2011; Chesters *et al.*, 2012) and Braconidae (Smith *et al.*, 2008).

Heteronomous parasitism and sex ratio

In heteronomous hyperparasitoids, the inability of females to develop in secondary hosts (parasitoid larvae developing inside primary hosts) is supported by careful observation of many species reared for the purposes of biological control, but is not easily tested (Hunter & Woolley, 2001). According to our investigation, though *Coccophagus* species can use more than one host, the host range for production of female offspring is limited (usually species of several closely related genera in Coccidae). On the other hand, the inability of males to develop in primary hosts was also observed in other heteronomous hyperparasitoid aphelinids, where unmated females oviposit reluctantly, if at all, on these hosts,

and no laid egg develops (Williams, 1972; Hunter, Rose & Polaszek, 1996; Hunter, 1999). Some species investigated in the present study use different host species to produce male offspring (Fig. 2, Supporting Information, Table S1), suggesting they could be alloparasitoids, developing on heterospecific females, possibly belonging to other families (Walter, 1983, 1986; Walter & Abeeluck, 2006). Potential secondary hosts of alloparasitoids include many species of Chalcidoidea (unpublished data) because they emerge simultaneously or a little later than *Coccophagus* males from the same scale insect host (see Supporting Information, Table S5).

Some authors (Kennett *et al.*, 1966; Kuenzel, 1977; Viggiani & Mazzone, 1978; Donaldson & Walter, 1991a; Bernal, Luck & Morse, 1998) have reported skewed sex ratios in Aphelinidae. Our investigations show nearly all field populations of *Coccophagus* species in this study have biased sex ratios (either female biased or male biased), indicating that this phenomenon may be common in *Coccophagus*. Moreover, in our investigation, it seems the sex ratio did not change much in relation to size of different host species (also see Bernal *et al.*, 1998), thus excluding effects linked to host-quality-dependent theory (Charnov *et al.*, 1981). The local mate competition (Hamilton, 1967) that may influence sex-ratio evolution in hymenopteran parasitoids may not suit heteronomous aphelinids (see Donaldson & Walter, 1991a, b; Godfray & Hunter, 1992; Walter & Donaldson, 1994; Williams & Polaszek, 1996; Hunter & Woolley, 2001). Flanders (1937) and Williams (1977) supposed that biased sex ratio may reflect host abundance. When hosts are rare and time spent searching for hosts is limited, population sex ratios of heteronomous aphelinids could be determined by the abundance in the environment of the two types of host (Godfray & Waage, 1990). Unfortunately, there has been no detailed field study of sex ratio and host abundance in this group. Laboratory studies have shown sex ratios in *Coccophagus atratus* Compere were largely influenced by the proportion of secondary hosts (Donaldson & Walter, 1991a), in agreement with the prediction of host-limited populations (Godfray & Hunter, 1992). However, in *Encarsia pergandiella*, the oviposition sex ratios, even though influenced by the proportion of secondary hosts, were less female biased than expected from the proportion of secondary hosts alone (Hunter, 1989, 1993). Finally, in *Encarsia tricolor*, sex ratio may be determined by interaction between host density and the proportion of secondary hosts (Hunter & Godfray, 1995). The gender-biased strategy is undoubtedly related to heteronomous parasitism, which has evolved to keep the balance of populations of heteronomous hyperparasitoids, favouring their survival (Colgan & Taylor, 1981; Nadel & Luck, 1992). Further study is needed to elucidate the balance of egg and host limitation in natural populations.

CONCLUSION

The host range and host use of *Coccophagus* parasitoids is more complicated than the picture emerging from previously available data. Our team-based collaboration between field ecology, standard morphological alpha-taxonomy and molecular biology has resulted in a much more profound understanding of parasitoid–host interactions of *Coccophagus* species from China. Use of DNA barcoding, combined with traditional morphological taxonomy, contributed greatly to accelerating the identification of both *Coccophagus* species and their hosts, even though large-scale host sampling and rearing of parasitoids are required to establish actual host ranges. As noted by Hunter & Woolley (2001), Coccophaginae have been, and will continue to be, important in developing and challenging our fundamental understanding of parasitoid physiology, behaviour and evolution. Heteronomous species may offer systems to study the development of different mechanisms in males and females to acquire nutrients and overcome the immune responses of hosts (Strand & Pech, 1995). Here, we have attempted to make a general conclusion about host range and interpret accumulating data relevant to parasitoid–host interactions. We present this case history in the belief that it will cast light on methods, details and logical approaches that will have applications in other systems. Debate on many details of theory and practice will be needed to develop a consensus and a mature body of techniques for use in estimating host ranges of entomophagous arthropods.

DATA ACCESSIBILITY

Sequence data are available in GenBank (accession numbers: KY605407–KY605787 for *28S* sequences, KY605788–KY606167 for *COI* sequences).

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of aphelinids. We thank all the collectors who helped us to sample and sent the scale insects in the past decade.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Information of the specimens of *Coccophagus* species used in this study.
Table S2. Results of General Mixed Yule-Coalescent (GMYC) analysis.
Table S3. List of scale insect species used as host by each *Coccophagus* species.
Table S4. Rearings of *Coccophagus japonicus* collected in China.
Table S5. List of other parasitoids of hosts which might be hyperparasitized by male *Coccophagus* species.
Figure S1. (A–H) Photos of species of *Coccophagus* (all female except G). A, *Coccophagus ceroplastae*; B, *Coccophagus nigricorpus*; C, *Coccophagus aterrimus*; D, *Coccophagus lycimnia*; E, *Coccophagus yoshidae*; F, *Coccophagus* sp4; G, *Coccophagus* sp4 (Male); H, *Coccophagus ishiii*.
Figure S2. (I–P) Photos of species of *Coccophagus* (all female except P). I, *Coccophagus japonicus*; J, *Coccophagus* sp3; K, *Coccophagus* sp5; L, *Coccophagus bogoriensis*; M, *Coccophagus* sp1; N, *Coccophagus longifasciatus*; O, *Coccophagus* sp2; P, *Coccophagus* sp2 (male).
Figure S3. Sample sites of collection of scale insect hosts of *Coccophagus* in mainland China.
Figure S4. ABGD results using the 118 haplotypes of *COI* gene of genus *Coccophagus*. The prior intra-specific divergences and the numbers of groups partitioned were generated using K80 (TS/TV = 2) model.
Figure S5. Demonstration of putative species delimitation of *Coccophagus* as obtained through ABGD (column A, *COI* data), GMYC (column B, *COI* data with NJ phylograms; column C, *COI* data with Bayesian phylograms; column D, combined *28S* + *COI* data with NJ phylogram; column E, combined *28S* + *COI* data with Bayesian phylogram) methods. The phylogenetic tree is the Neighbor-Joining tree of *COI* obtained through PAUP analysis; it is being used for representation, but is not intended to be a definitive phylogenetic tree for the genus.
Figure S6. NJ tree of *COI* genetic distance (K2P) for *Coccophagus* specimens, with representative male information (yellow colored).
Figure S7. NJ tree of *28S* genetic distance (K2P) for *Coccophagus* specimens, with male information (yellow colored).