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ORIGINAL ARTICLE

Application of DNA barcoding to the identification of Hymenoptera parasitoids from the soybean aphid (*Aphis glycines*) in China

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Abstract *Aphis glycines* Matsumura is an important pest of soybean in Asia and North America. Hymenoptera parasitoids play a key role in the control of the soybean aphid. The correct identification of parasitoids is a critical step that precedes the assessment of their potential biological control agents. Accurate identification of the majority of the species attacking the soybean aphid often requires elaborate specimen preparation and expert taxonomic knowledge. In this study, we facilitated the identification of soybean aphid parasitoids by applying a DNA barcoding approach following a preliminary morphological identification. We generated DNA sequence data from the mitochondrial COI gene and the D2 region of 28S rDNA to assess the genetic variation within and between parasitoid species emerging from the soybean aphid in China. Fifteen Hymenoptera parasitoid species belonging to 10 genera of five families were identified with little intra-specific variation (0.09% ± 0.06% for 28S and 0.36% ± 0.18% for COI) and large inter-specific divergence (30.46% ± 3.42% for 28S and 20.4% ± 1.20% for COI).

Key words biological control, COI, 28S-D2, molecular identification, species delimitation

Introduction

The soybean aphid *Aphis glycines* Matsumura (Hemiptera: Aphididae) is currently distributed in China, Japan, Korea, Indonesia, Malaysia, USA and Canada (Wang *et al.*, 1962; Paik, 1965; Iwaki, 1979; Chung *et al.*, 1980; Takahashi *et al.*, 1993; Blackman & Eastop, 2000; Hunt *et al.*, 2003; Venette & Ragsdale, 2004). As a key soybean pest, it can cause great damage by ingesting phloem and/or transmitting viruses (Wang *et al.*, 1962; Wu *et al.*, 2004a). After its invasion to the USA in 2000, *A. glycines* has spread through the vast soybean-growing areas of North America, and frequently resulted in soybean yield loss (DiFonzo & Hines, 2002; Venette & Ragsdale, 2004; Kaiser *et al.*, 2007; Bahlai *et al.*, 2010).

Field investigations in Asia showed Hymenoptera parasitoids contribute a lot to the drastic reduction of soybean aphid populations (Gao, 1992; Liu *et al.*, 2004; Xi *et al.*, 2011; Yan *et al.*, 2011). For example, *Lysiphlebia japonica* (Ashmead) can reach a parasitism rate of 34% on the first generation of soybean aphid populations (Gao, 1994).

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Table 1 Primers used to amplify soybean aphid parasitoid 28S gene.

Primer	Primer sequence	Reference
D2–3549 [F]	5′-AGTCGTGTTGCTTGATAGTGCAG-3′	Campbell *et al.*, 1993
D2–3566 [F]	5′-TGCAGCTCTAAGTTGGTGGT-3′	Gillespie *et al.*, 2005
D2–3665 [F]	5′-AGAGAGAGTTCAAGAGTACGTG-3′	Belshaw and Quicke, 1997
D1–3317 [F]	5′-ACCCGCTGAATTTAAGCATAT-3′	Quicke and Belshaw, 1999
D2–4068 [R]	5′-TTGGTCCGTGTTTCAAGACGGG-3′	Campbell *et al.*, 1993
D3–4283 [R]	5′-TAGTTCACCATCTTTCGGGTC-3′	Belshaw *et al.*, 2001
D2–4057 [R]	5′-TCAAGACGGGTCCTGAAAGT-3′	Heraty *et al.*, 2004

Table 2 Results of molecular analyses by ABGD (Automatic Barcode Gap Discovery), BOLD (Barcode of Life Database) blast and NCBI blast (target species with similarity value less than 98% are not shown).

Morpholo-species	ABGD	BOLD (COI gene)	NCBI		Species delimitated
			COI	28S	
Binodoxys sp.	*Binodoxys* sp.	*B. communis*	*B. communis*	*B. communis*	*Binodoxys communis*
Lysiphlebus sp2	*Lysiphlebus* sp2				*Lysiphlebus orientalis*
Lysiphlebus sp1	*Lysiphlebus* sp1	*L. fabarum*	*L. fabarum*	*L. fabarum*	*Lysiphlebus fabarum*
		L. confusus	*L. confusus*	*L. confusus*	
Aphidius sp.	*Aphidius* sp. H1†	*A. rhopalosiphi*	*A. rhopalosiphi*	*A. rhopalosiphi*	*Aphidius rhopalosiphi*
	Aphidius sp. H2†	*A. uzbekistanicus*	*A. uzbekistanicus*		*Aphidius uzbekistanicus*
		A. avenaphis			
Aphelinus sp.	*Aphelinus* sp1	*A. varipes*	*A. varipes*	*A. varipes*	*Aphelinus albipodus*
			A. abdominalis	*A. albipodus*	
			A. paramali	*A. mali*	
	Aphelinus sp2			*A. varipes*	*Aphelinus* sp2
				A. albipodus	
				A. mali	
Marietta sp.	*Marietta* sp.				*Marietta* sp.
Syrphophagus sp.	*Syrphophagus* sp1			*S. aphidivorus*	*Syrphophagus aphidivorus*
	Syrphophagus sp2				*Syrphophagus* sp2
Pachyneuron sp.	*Pachyneuron* sp.		*P. aphidis*		*Pachyneuron aphidis*
Anisopteromalus sp.	*Anisopteromalus* sp.				*Anisopteromalus* sp.
Asaphes sp.	*Asaphes* sp.	*A. vulgaris*	*A. vulgaris*	*A. suspensus*	*Asaphes* sp.
			A. suspensus		
Alloxysta sp.	*Alloxysta* sp1				*Alloxysta chinensis*
	Alloxysta sp2				*Alloxysta* sp2

†H1 is the haplotype 1 of morphospecies *Aphidius* sp., H2 is the other one.

Correct identification of Hymenoptera parasitoids is crucial for assessing their potential in biological control projects (Bigler *et al.*, 2005; Gariepy *et al.*, 2008). Misidentification of parasitoids used in biocontrol may result in serious economic losses (Compere, 1961; Rosen & DeBach, 1973; Ridgway & Vinson, 1977; Caltagirone, 1981). Due to their small body size (usually 0.5–2.0 mm), high-quality slide and card-mounted specimens are needed in traditional morphological identification. Even the well-known species *Lysiphlebus fabarum* (Marshal) can be confused with *L. confusus* Tremblay and Eady (Rakhshani *et al.*, 2005, 2012).

In the past decade, DNA barcoding has been widely used in identification of Hymenoptera parasitoids (Derocles *et al*., 2012; Smith *et al*., 2013; Novković *et al*., 2011; Babcock & Heraty, 2000; Ratcliffe *et al*., 2002; Jinbo

Table 3 Genetic distances between parasitoid species under K2P model (distances between 28S sequences are shown under the diagonal, distances between COI sequences are above the diagonal; light gray means distance within genus, dark gray means distance within family).

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*Binodoxys communis*		0.1389	0.1300	0.1151	0.1358	0.2227	0.2300	0.2790	0.3011	0.3196	0.2599	0.2853	0.2646	0.2274	0.2282
2	*Lysiphlebus orientalis*	0.1711		0.0895	0.1138	0.1221	0.2443	0.2509	0.2845	0.3021	0.3042	0.2817	0.3129	0.2679	0.2530	0.2630
3	*Lysiphlebus fabarum*	0.1654	0.0301		0.1112	0.1091	0.2462	0.2426	0.3037	0.3111	0.2886	0.2795	0.2915	0.2706	0.2456	0.2640
4	*Aphidius rhopalosiphi*	0.1617	0.0354	0.0362		0.0577	0.2311	0.2409	0.2806	0.3379	0.3091	0.2727	0.2969	0.2770	0.2557	0.2712
5	*Aphidius uzbekistanicus*	0.1812	0.0598	0.0392	0.0391		0.2508	0.2485	0.2968	0.3484	0.3118	0.2705	0.3075	0.2842	0.2682	0.2899
6	*Aphelinus* sp2	0.5561	0.4608	0.4297	0.4746	0.4599		0.0515	0.1388	0.2144	0.2127	0.1422	0.1509	0.1256	0.2205	0.2263
7	*Aphelinus albipodus*	0.5546	0.4563	0.4254	0.4700	0.4502	0.0128		0.1349	0.2111	0.2004	0.1351	0.1544	0.1323	0.2289	0.2439
8	*Marietta* sp.	0.5424	0.4456	0.4153	0.4591	0.4363	0.0905	0.0841		0.2780	0.2646	0.1963	0.2180	0.1765	0.2827	0.2723
9	*Syrphophagus aphidivorus*	0.5078	0.4464	0.4265	0.4671	0.4494	0.1710	0.1747	0.2241		0.1484	0.2238	0.2479	0.2332	0.3003	0.2996
10	*Syrphophagus* sp2	0.4992	0.4369	0.4174	0.4518	0.4400	0.1866	0.1903	0.2298	0.0249		0.2370	0.2476	0.2346	0.2833	0.2985
11	*Pachyneuron aphidis*	0.5482	0.4785	0.4410	0.4869	0.4662	0.0893	0.0865	0.1204	0.1606	0.1761		0.1566	0.1313	0.2457	0.2562
12	*Anisopteromalus* sp.	0.5447	0.4695	0.4323	0.4778	0.4572	0.0920	0.0951	0.1143	0.1735	0.1892	0.0448		0.1500	0.2707	0.2620
13	*Asaphes* sp.	0.5331	0.4510	0.4254	0.4700	0.4502	0.1318	0.1163	0.1451	0.1803	0.1932	0.0722	0.0749		0.2226	0.2402
14	*Alloxysta chinensis*	0.5218	0.4343	0.4098	0.4372	0.4307	0.2763	0.2648	0.2899	0.3131	0.3153	0.2238	0.2454	0.2354		0.1101
15	*Alloxysta* sp2	0.5210	0.4232	0.3991	0.4262	0.4149	0.2714	0.2600	0.2863	0.3038	0.3059	0.2121	0.2408	0.2303	0.0154	

Table 4 Genetic distance within soybean aphid parasitoids under K2P model (mean ± SE).

Species	K2P distance	
	28S	COI
Binodoxys communis	0.00% ± 0.00%	0.16% ± 0.11%
Lysiphlebus orientalis	0.16% ± 0.13%	0.00% ± 0.00%
Lysiphlebus fabarum	0.03% ± 0.03%	0.38% ± 0.17%
Aphidius rhopalosiphi	0.00% ± 0.00%	0.00% ± 0.00%
Aphidius uzbekistanicus	0.00% ± 0.00%	0.00% ± 0.00%
Aphelinus sp2	0.00% ± 0.00%	0.22% ± 0.14%
Aphelinus albipodus	0.00% ± 0.00%	0.00% ± 0.00%
Marietta sp.	0.44% ± 0.21%	1.48% ± 1.20%
Syrphophagus aphidivorus	0.24% ± 0.16%	0.93% ± 0.30%
Syrphophagus sp2	0.15% ± 0.10%	0.74% ± 0.24%
Pachyneuron aphidis	0.00% ± 0.00%	0.19% ± 0.12%
Anisopteromalus sp.	0.00% ± 0.00%	0.00% ± 0.00%
Asaphes sp.	0.00% ± 0.00%	0.58% ± 0.24%
Alloxysta chinensis	0.27% ± 0.20%	0.00% ± 0.00%
Alloxysta sp2	0.00% ± 0.00%	0.71% ± 0.19%

et al., 2011; Zhang *et al*., 2011; Chester *et al*., 2012; Emam *et al.*, 2013). Recent studies of soybean aphid parasitoids (Wyckhuys & Heimpel, 2007; Wyckhuys *et al.*, 2008; Starý *et al.*, 2010; Wu *et al.*, 2004b; Desneux *et al.*, 2009; Petrović *et al.*, 2013) indicated that DNA barcoding may be a useful tool for their identification.

In the present study, sequences of mitochondrial COI and nuclear 28S D2 region DNA were acquired from the Hymenoptera parasitoid species emerging from *A. glycines* in North China. We applied DNA barcoding to evaluate the identity of the species on the basis of morphological examination.

Materials and methods

Parasitoid collecting

During 2009–2012, the parasitoids of *A. glycines* were surveyed in four major soybean-planting areas in North China (Beijing, Henan, Liaoning and Heilongjiang provinces). Soybean leaves with aphid mummies and live aphids were collected in the field and brought back to the Key Laboratory of Zoological Systematics and Evolution (IZCAS: Institute of Zoology, Chinese Academy of Sciences). The soybean leaves were placed into plastic cups covered with fine mesh, kept in a growth chamber (25 ± 1°C, 70% relative humidity, and 14 : 10 L : D photoperiod) and checked daily for parasitoid emergence. Emerged parasitoids were promptly killed in 95% ethanol and stored at −20°C. Specimens were identified to genus and provisional morphospecies level, by comparing them with material authoritatively identified and with the aid of taxonomic keys (Starý & Schlesinger, 1967; Andrews, 1978; Shaw & Huddleston, 1991; Gibson & Huber, 1997; Fülöp *et al.*, 2013). After obtaining the molecular results, the specimens were re-examined and their morphological identification reappraised. All specimens (including those vouchers for DNA analysis) in this study were deposited at the Institute of Zoology, Chinese Academy of Sciences (IZCAS).

DNA extraction, amplification and sequencing

Overall, 131 representative specimens of the 11 morphospecies were used for DNA barcoding analysis (see Table S1). The specimens were chosen from different locations to assess the intraspecific variation. DNA was extracted from adult specimens using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. Polymerase chain reactions (PCR) were performed following Chesters *et al.* (2012). The COI gene was amplified using universal primers LCO1490 and HCO2198 (Folmer *et al.*, 1994), and the PCR cycle program for COI followed Hebert *et al*. (2003). The primers for 28S amplification (Table 1) and PCR cycle program followed Zhang *et al*. (2008). PCR products were visualized on a 1% agarose gel, and sequencing was performed in both directions using BigDye v3.1 on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). All sequences have been submitted

to GenBank (Accession numbers: KF597551–KF597680 for 28S, KF597681–KF597796 for COI).

Sequence alignment and molecular analysis

All sequences were verified by NCBI Nucleotide Blast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the BOLD (Barcode of Life Database) species identification tool (http://www.barcodinglife.org). Sequences were aligned using Clustal X (Larkin *et al.*, 2007) and modified 28S sequences manually *via* BioEdit (Hall, 1999).

The Automatic Barcode Gap Discovery (ABGD) species delineation tool (Puillandre *et al.*, 2012) was employed to define provisional species on the basis of COI haplotype groups with default settings except the relative gap width (X = 1). The genetic distances were calculated using the Kimura 2-parameter (K2P) model in MEGA 5.2 (Tamura *et al.*, 2011). Neighbor-joining (NJ) trees (Saitou & Nei, 1987) were reconstructed with 1000 bootstrap replications (Felsenstein, 1985). We use *Aulacus impolitus*e Smith (Aulacidae) as an outgroup based on recent studies of the phylogenetic relationship of Hymenoptera (Heraty *et al.*, 2011; Sharkey *et al*., 2012).

Results and discussion

About 3000 specimens of Hymenoptera parasitoids emerged from the mummies of the soybean aphid. A preliminary morphological classification resulted in 11 morphospecies. Details of these morphospecies are listed in Table S1.

Molecular characterization

Of the 131 specimens examined, the amplification of 28S was successful for 130. COI sequences were obtained from 116 specimens. After the deletion of the terminal ambiguous parts of the aligned data, we obtained matrices of 542 bp and 565 bp for 28S and COI, respectively.

ABGD

Preliminary species delimitation was done by ABGD. The number of species groups varied according to different *a priori* threshold values. However, a major barcode gap was evident at *a priori* genetic distance thresholds of 0.04–0.05, which supports the presence of 14 genetically distinct groups in the data set. The morphospecies of *Aphelinus* sp., *Syrphophagus* sp. and *Alloxysta* sp. were each composed of two barcode

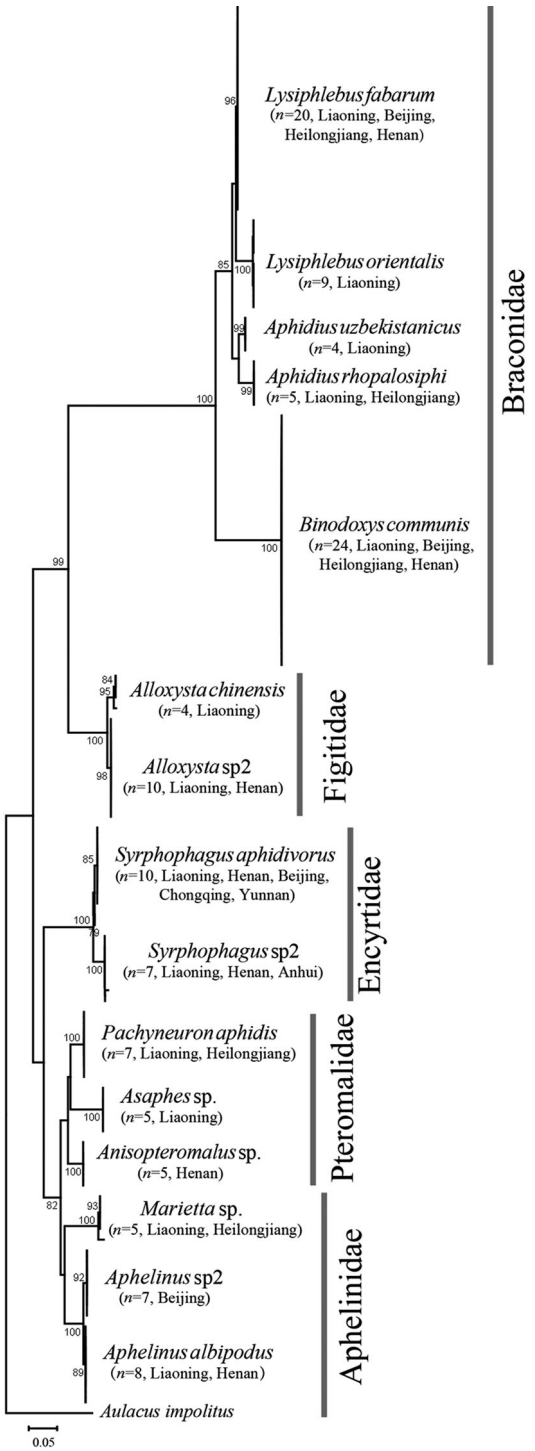

Fig. 1 Neighboring-joining tree of the soybean aphid parasitoids constructed using the COI data set (K2P model, bootstrap = 1000, bootstrap values less than 75% are omitted; species names were chosen according to the synthesis of DNA barcoding results and morphological character examination).

species that might correspond to cryptic species in these genera (Table 2). However, the group of *Aphidius* morphospecies had a deep COI divergence (5.77%), indicating two species of *Aphidius*. Thus, 15 species were involved in our further analysis.

Sequences divergence

The K2P distance indicated a larger interspecific than intraspecific distance for both 28S and COI. The mean interspecific pairwise distance (K2P model) was 30.46% ± 3.42% for 28S (range: 1.30%–55.60%), and 20.41% ± 1.20% for COI (range: 5.10%–34.80%) (Table 3). The mean intraspecific pairwise distance for 28S was 0.09% ± 0.06% (range: 0–0.44%), and 0.36% ± 0.18% (range: 0–1.50%) for COI (Table 4).

NJ tree

Five lineages (corresponding to five families: Braconidae, Figitidae, Encyrtidae, Aphelinidae and Pteromalidae) can be easily observed on the COI NJ tree (Fig. 1) and 28S NJ tree (Fig. 2). Within each family, the species of the same genus clustered together. There are five groups in the lineage of Braconidae, two in Figitidae, two in Encyrtidae, three in Pteromalidae and three in Aphelinidae. In total, 15 distinct species were recognized with high node support (> 90%).

Blast in NCBI and BOLD

The results of sequence comparison against the known sequences in the BOLD system (only COI) and GenBank database (both 28S and COI) are list in Table 2. The COI sequences of six species received 'Top Hits' with different extents of similarity (over 98%) through the BOLD identification system. When blasted in GenBank database, seven species returned close matches for 28S and COI, respectively. Another four species had no matching sequences in both BOLD and NCBI.

A deep inference of species delimitation

Due to the lack of reference sequences and the misidentification of species, identification through DNA markers could be difficult and sometimes misleading (Nilsson *et al*., 2006; Marucci *et al*., 2010). Thus a thorough morphological inference was conducted. Documented data in references have also been used as supplement information

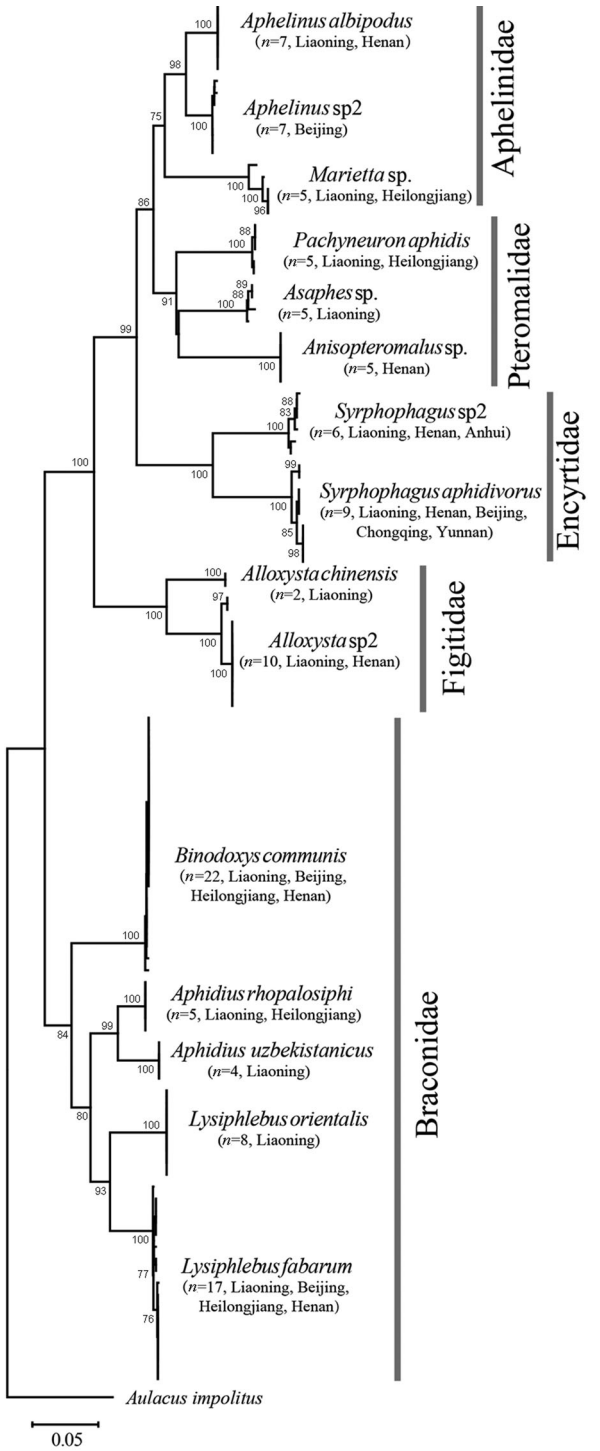

Fig. 2 Neighboring-joining tree of the soybean aphid parasitoids constructed using the 28S data set (K2P model, bootstrap = 1000, bootstrap values less than 75% are omitted; species names were chosen according to the synthesis of DNA barcoding results and morphological character examination).

for species identification. Here the preliminary species names were according to the results of ABGD (Table 2).

Braconidae *Lysiphlebus* sp1 matched both *L. fabarum* and *L. confusus* in BOLD and NCBI. Specimens we collected have shorter lower marginal setae of forewing than *L. confusus* (Chen & Shi, 2001; Rakhshani *et al.*, 2005, 2012). Previous workers have reported that *L. fabarum* is a dominant parasitoid in field surveys (Wu *et al*., 2004a; Xi *et al.*, 2011; Yan *et al*., 2011). Here we recognized it as *L. fabarum*. Although *Lysiphlebus* sp2 had no matching barcodes, we identified it as *L. orientalis* Starý and Rakhshani by a close comparison with characters described by Starý *et al.* (2010).

Binodoxys communis (Gahan) and *Aphidius rhopalosiphi* De Stefani-Perez are well verified by both DNA barcodes and morphological characters.

Aphidius uzbekistanicus Luzhetski shares an identical COI sequence with *A. avenaphis* (Fitch) (Kos *et al*., 2011). *A. uzbekistanicus* was also reported distributed in China (Chen & Shi, 2001). Here, we tentatively regard *Aphidius* sp. H2 as *A. uzbekistanicus*. More detailed taxonomy and biology work should be carried out.

Aphelinidae *Aphelinus* sp1 was recognized as *A. varipes* (Förster) for the COI gene on both the BOLD and NCBI databases. It also had a high similarity with another species, *A. albipodus* Hayat and Fatima, for the 28S gene in NCBI. These two species are hardly distinguishable by morphological characters. A recent study indicated that *A. varipes* and *A. albipodus* are members of a species complex (Heraty *et al.*, 2007). Thus more information (geometric morphometrics, mating tests, etc.) is required to understand these two species. We tentatively identified *Aphelinus* sp1 as *A. albipodus* based on species distribution information and host records (Heimpel *et al.*, 2004; Wu *et al.*, 2004b). *Aphelinus* sp2 has over 5% COI divergence between both *A. varipes* and *A. albipodus*. More specific studies are required to understand the relationship between these three species.

The genus *Marietta* presently includes 49 species (Noyes, 2002). We cannot identify our *Marietta* species using the keys (Hayat, 1998), thus we just named it as *Marietta* sp. pending further taxonomic study.

Encyrtidae *Syrphophagus* sp1 was matched with *S. aphidivorus* (Mayr) for 28S sequences in the NCBI database. Morphological character analyses (Liao *et al*., 1987) and host records (Gao, 1992) confirmed this species as *S. aphidivorus*. *Syrphophagus* sp2 can be separated from *S. aphidivorus* by the color of tibia of the middle leg (dark brown in basal half in *S. aphidivorus*, entirely yellowish in *Syrphophagus* sp2). We tentatively named it as *Syrphophagus* sp2; further taxonomy studies should be carried out.

Pteromalidae *Pachyneuron* sp. has been identified as *P. aphidis* (Bouché) by blast COI sequences in NCBI, confirmed by morphological characters and reference records (it also has been reported as a hyper-parasitoid of soybean aphid). Even after a further morphological examination, the species status of *Anisopteromalus* sp. and *Asaphes* sp. cannot be resolved in the present study.

Figitidae The two morphospecies of *Alloxysta* are both similar to *A. chinensis* Fülöp and Mikó, a new species from China described by Fülöp *et al.* (2013). We identified *Alloxysta* sp1 as *A. chinensis* due to similarity to the original description.

Conclusion

The DNA barcoding approach greatly assisted the identification of the parasitoid complex of the soybean aphid in China. Our results revealed 15 species belonging to 10 genera of five Hymenoptera families. The DNA barcodes show high interspecific distance coupled with low intraspecific distance. However, biological identification through DNA barcoding *per se* cannot be exhaustive. More detailed researches on morphology, behavior and biology are necessary to improve the identification of cryptic species (Desneux *et al*., 2009).

A reliable and comprehensive DNA database of insect pests and parasitoids is required for rapid species identification and understanding the parasitoid community. Accurate identification using DNA barcoding could be a pivotal step in the assessment of their performance and suitability as biocontrol agents in biological control programs (Greenstone, 2006; Gariepy *et al.*, 2007).

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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 Specimens of parasitoids species used in the study (collectors' names are abbreviated as follows: XYQ = Yu-Qiang Xi; LXJ = Xue-Jun Li; LCL = Chun-Lai Li; ZX = Xu Zhang; ZQS = Qing-Song Zhou; ZYZ = Yan-Zhou Zhang; DNA vouchers are in IZCAS and ZYZ identified the species). The DNA barcoding identification was obtained after the interpretation of the comparisons between the sequences generated in this study and the sequences published in the BOLD and NCBI databases.