TWO PUTATIVE BRIDGEHEAD POPULATIONS OF *APHELINUS MALI* (HYMENOPTERA: APHELINIDAE) INTRODUCED IN CHINA AS REVEALED BY MITOCHONDRIAL DNA MARKER

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ABSTRACT

In China, *Aphelinus mali* (Haldeman) (Hymenoptera: Aphelinidae) was independently introduced as an endoparasitoid of the woolly apple aphid, *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae) from Japan in 1942 and from the former Soviet Union during 1953-1955. However, we do not know which introduction of this endoparasitoid plays important role in the control of *E. lanigerum* in China. To determine the status of this biological control agent in China, we collected 16 populations from 6 provinces (Shandong, Liaoning, Hebei, Shanxi, Xinjiang and Yunnan) and analyzed the 948 mtCOI gene from specimens in these samples. The results revealed that the *A. mali* in China consisted of 2 cryptic mitochondrial clades including 3 haplotypes, which indicated at least 2 independent introductions of the parasitoid into China. Our results showed that each of the populations that had been introduced into Shandong and Liaoning, respectively, had also established in many regions of China , where they play an important role in the control of *E. lanigerum*. Therefore it is very likely that both original introductions have served as bridgeheads to establish other populations in China. Genetic analyses together with field surveys should be helpful in the management of the woolly apple aphid.

Key Words: *Aphelinus mali*, mitochondrial COI gene, bridgehead population

RESUMEN

En China, *Aphelinus mali* (Haldeman) (Hymenoptera: Aphelinidae) fue introducido independientemente como un ectoparasitoide del pulgón lanígero del manzano *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae) de la antigua Unión Soviética durante 1953-1955 y de Japón en 1942. Sin embargo, no se sabe cuál de estas introducciones del ectoparasitoide juega un papel importante en el control de *E. lanigerum* en China. Para determinar el estado de este controlador biologico en China, se colectaron 16 poblaciones en seis provincias (Shandong, Liaoning, Hebei, Shanxi, Xinjiang, y Yunnan) y se analizó el gen 948 COI mitocondrial de los especímenes colectados. Los resultados mostraron que en China hay 2 grupos mitocondriales crípticos que incluyen 3 haplotipos de *A. mali*, lo que suguiere que existieron dos introducciones independientes en China. Nuestros resultados sugieren que las dos poblaciones originalmente introducidas en Shandong y Liaoning se pueden establecer en varias regiones en China y jugar un papel importante en el control de *E. lanigerum*. Por consiguiente, las dos introducciones iniciales pueden haber servido como cabeza de puente para el establecimiento de otras poblaciones en China. Análisis genéticos y estudios de campo serán útiles en el manejo del pulgón lanígero del manzano.

Palabras Clave: *Aphelinus mali*, gene COI mitocondrial, población cabeza de puente

The endoparasitoid *Aphelinus mali* (Haldeman) (Hymenoptera: Aphelinidae) was first discovered in eastern North America, the same region of origin as its host, the woolly apple aphid, *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae) (Mueller et al. 1992; Beers 2012; Lessando 2012; Lavandero & Tylianakis 2013). In the early 1920s, the potential role of *A. mali* in biological control was noted and a concerted plan for its rearing and introduction was implemented (Howard 1929). During the 20th century, *A. mali* was introduced into 51 countries; the parasitoid successfully established populations in 42 of these countries, which were located in virtually all biogeographical zones of the world (Zhou et al. 2010).

During the 1940s-1950s, *A. mali* was introduced as an endoparasitoid of *E. lanigerum* in China, i.e., *A. mali* was introduced into Dalian, Liaoning from Japan in 1942, and into Qingdao, Shandong from the former Soviet Union during 1953-1955 (Long et al. 1960). Since then, *A. mali* has spread into most regions of China including Shandong, Liaoning, Shanxi, Yunnan, Hebei, Henan, and Xinjiang provinces (Zhou et al. 2010; the present study). However, we did not know which introduction of this endoparasitoid played an important role in the control of *E. lanigerum* in China. The information might be helpful in the management of the *E. lanigerum*, which is a severely damaging invasive species in China. For instance, the *A. mali* populations with strong adaptability may be used or re-introduced for the control of the *E. lanigerum*.

To determine the genetic structures of various *A. mali* populations and their status as a biological control agent in China, we collected 16 populations from 6 provinces (Shandong, Liaoning, Hebei, Shanxi, Xinjiang, and Yunnan) and analyzed the mtCOI gene of specimens from each of these samples.

Materials and Methods

Sample Collection and Species Identification

Adults of the woolly apple aphid, *E. lanigerum*, were collected from apple trees in 6 provinces in China during 2007, 2008 and 2012. The sampled localities cover a great part of the invasion areas in China, ranging from Huludao, Liaoning (HLD) in the northeast to Zhaotong, Yunnan (ZT) in the southwest. Samples from the initial areas of introduction in Dalian, Liaoning and Qingdao, Shandong (DL and QD) were also collected. The collected specimens of *E. lanigerum* were placed in Petri dishes and kept at room temperature until *A. mali* eclosion. The emerging adult *A. mali* were collected and stored in 95% ethanol at -20 °C until DNA extraction. Before storage in alcohol, each individual was examined and identified unambiguously by a microscope.

DNA Extraction, PCR Amplification, and Sequencing

Genomic DNA was extracted from individual female adults of *A. mali* by the procedure described by Frohlich et al. (1999). The lysate was stored at -20 °C and was used as DNA template in PCR amplification. All 948 individual DNA samples were amplified using the newly designed primers F (5'-TCTCATATAATTTGTAATGAAAG-3') and R (5'-TGATAACTAGGAGGAAAATTTAT-3') to yield a 648-bp fragment of the mtCOI gene. PCRs were performed on a TP600 machine (TaKaRa) in a 25-μL reaction volume containing 0.25 μL of EasyTaq polymerase, 2.5 μL of 10 × EasyTaq Buffer ($+Mg^{2+}$), 0.5 μL of dNTPs (Sangon), 3 μL of DNA (concentration not estimated), and 0.5 μL of each oligonucleotide primer. The thermal profile included an initial denaturation step at 94 °C for 4 min; followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 54 °C for 45 s, and extension at 72 °C for 1 min; and a final extension at 72 °C for 7 min. PCR products were run on a 1.5% agarose gel stained with ethidium bromide and then were sequenced in both directions using the same primer pairs. Sequences were aligned with Clustal W (Thompson 1994) and were then checked for indels and nuclear copies.

Table 1. Codes, names, geographical coordinates, number of individuals, and sampling dates for 16 Chinese populations of *Aphelinus mali* collected for this study.

Code	Location	Longitude	Latitude	Number of individuals used	Sampling dates
DL	Dalian, Liaoning	39°01'	121°44'	107	Sep 2007
HLD	Huludao, Liaoning	40°36'	120°22'	51	Sep 2007
CZ	Changzhi, Shanxi	36°11'	113°6'	228	Jul 2008
JZ	Jinzhong, Shanxi	37°41'	112°44'	10	Jul 2008
YC	Yuncheng, Shanxi	35°1'	111°0'	33	Jul 2008
QHD	Qinhuangdao, Hebei	39°56'	119°35'	12	Oct 2007
SJZ	Shijiazhuang, Hebei	38°2'	114°30'	14	Nov 2007
BD	Baoding, Hebei	38°52'	115°27'	10	Nov 2007
YL	Yili, Xinjiang	44°24'	84°48'	8	Aug 2008
ZT	Zhaotong, Yunnan	27°12'	103°25'	74	Sep 2012
HZ	Heze, Shandong	35°8'	115°15'	22	Aug 2012
LC	Liaocheng, Shandong	36°15'	115°34'	38	Oct 2012
TA	Taian, Shandong	36°11'	117°07'	121	Aug 2012
WF	Weifang, Shandong	36°25'	119°3'	90	Sep 2012
YT	Yantai, Shandong	37°19'	121°14'	100	Dec 2012
QD	Qingdao, Shandong	36°19'	120°23'	30	Jul 2012

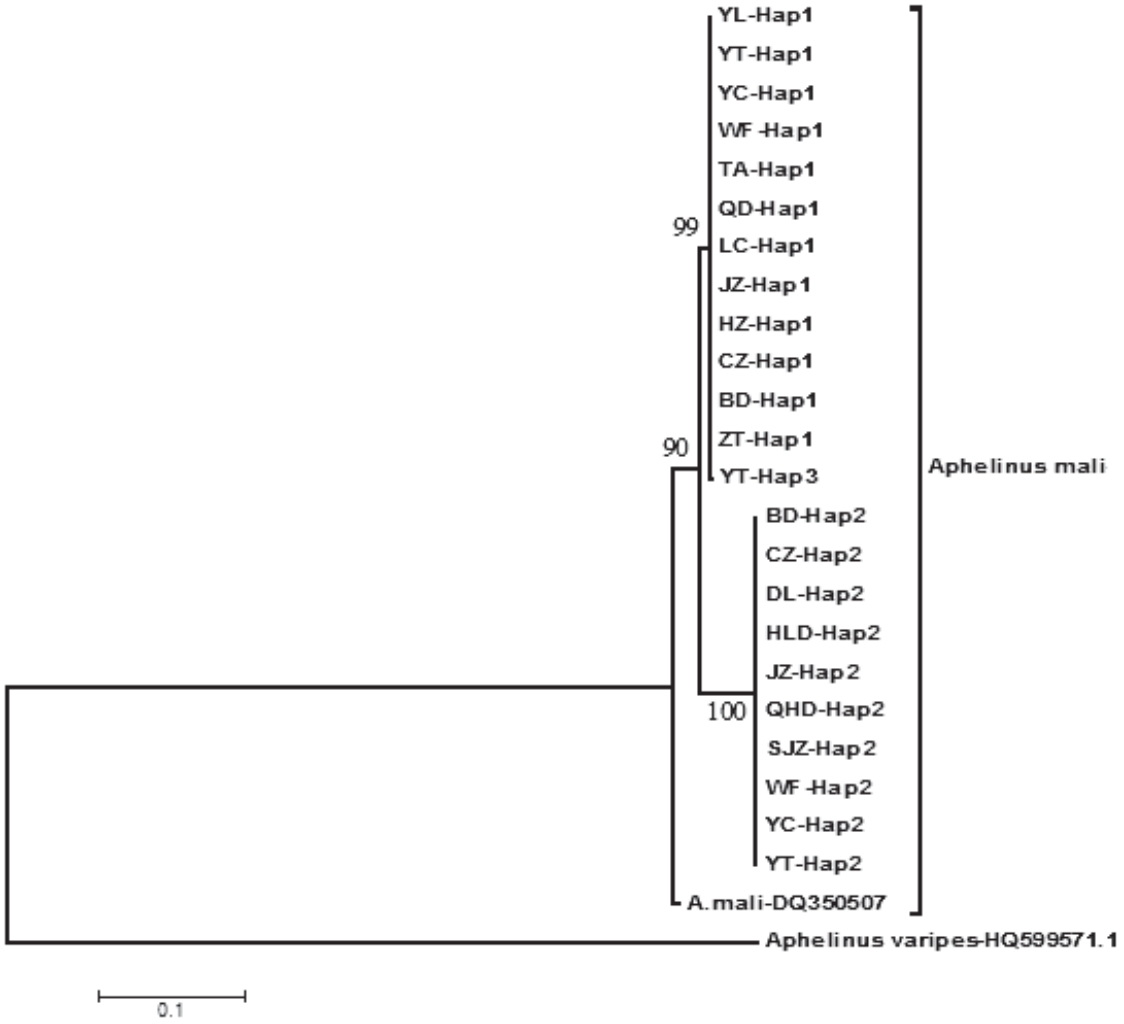

Fig. 1. A Maximum Likelihood tree of *Aphelinus mali* with the *Aphelinus varipes* mtCOI sequence as an outgroup. Bootstrap values were computed over 1000 replications. The sequence code consists of the location code (shown in Table 1) plus the haplotype. The data shown that the *A. mali* populations in Shandong Province and in neighboring regions mainly belong to the same clade (SD clade) and the *A. mali* population in Liaoning Province and in neighboring regions mainly belong to another clade (LN clade).

Haplotype Determination and Phylogenetic Analyses

The mtCOI sequences of all individuals were manually edited using DNASTAR and were aligned using MEGA 5. Only 1 sequence was selected from the same sequences from each location to conduct a phylogenetic analysis. We constructed a phylogenetic tree using the Maximum Likelihood (ML) method in MEGA5 with the *Aphelinus varipes* mtCOI sequence (GenBank No. HQ599571) as an outgroup. Also we downloaded the entire sequence of *A. mali* from GenBank and only 1 sequence was obtained (GenBank No. DQ350507). We determined the type of the clades based on the phylogenetic tree.

Results

Geographical Distribution of *Aphelinus mali*

We genotyped 948 *A. mali* adult females from 16 populations (6 populations from Shandong; 3 each from Shanxi and Hebei, 2 from Liaoning; and 1 each from Xinjiang and Yunnan), yielding an average sample size of 59 individuals per population (Table 1).

Haplotype Composition of *Aphelinus mali* Based on the Mitochondrial Gene

Within the mtCOI sequences, 31 positions were polymorphic representing 1 singleton variable

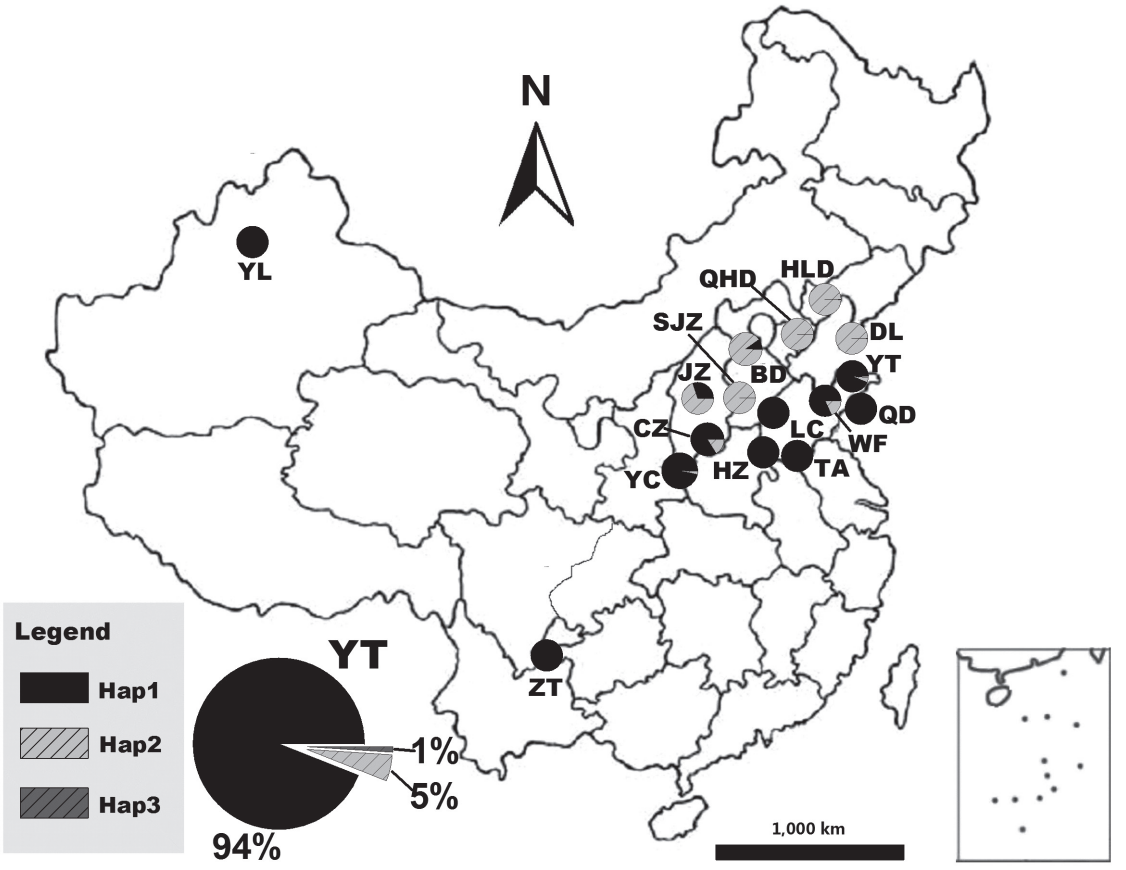

Fig. 2. Sampling sites and haplotype frequencies in the 16 populations of *Aphelinus mali* in China. Three haplotypes were identified in China: Hap1 (dark red), Hap2 (orange), and Hap3 (dark yellow). The circular symbol for each population indicates the proportion of each haplotype in that population.

site and 30 parsimony informative sites. These polymorphic sites defined 3 haplotypes (coded as Hap1–Hap3; GenBank numbers: KF039708–KF039710) within the 948 individuals from 16 localities across China. Hap1 was found in 679 individuals from 12 locations, and Hap2 was found in 268 individuals from 10 locations across China. However, Hap3 was only found in 1 individual from Yantai, Shandong (YT). All of the COI haplotypes defined 2 main clades: SD and LN (Fig. 1). The SD (Shandong) clade was mainly distributed in Shandong and neighboring regions and characterized by Hap1 while the LN(Liaoning) clade was mainly distributed in Liaoning and neighboring regions by Hap2 (Fig. 2).

Discussion

Our analyses show that the *A. mali* populations in Shandong Province and neighboring regions mainly belong to the same clade (SD clade) and that the *A. mali* population in Liaoning Province and neighboring regions mainly belong to another clade (LN clade). The distribution of mtCOI haplotypes and clades of *A. mali* (Fig. 1) is consistent with previous reports concerning the intentional introduction of *A. mali* into China (see Long et al. 1960).

Combined with the historical records of the initial introductions of *A. mali* and the haplotype data in this study, our results strongly suggest that each of the 2 populations that had been introduced into Shandong (QD) and Liaoning (DL) can establish in many regions of China and play an important role in the control of *E. lanigerum* in China. Indeed both original introductions appear to have served either as bridgeheads to establish *A. mali* in adjacent areas, or as source populations to establish *A. mali* in distant areas in China. This should be further explored using nuclear markers. The role of initially introduced population as a bridgehead has also been shown for several invasive insect pest species (Miller et al. 2005; Ciosi et al. 2008; Ascunce et al. 2011; Lombaert et al. 2010; Lombaert et al. 2011; Kajita et al. 2012; Yang et al. 2012). Moreover, based on

genetic variation of the adventive western flower thrips, *Frankliniella occidentalis* (Pergande), in China, Yang et al. (2012) revealed that the introduced population in Kunming probably served as a bridgehead to other populations in China.

Within the 16 populations, 6 were mixed populations that contained more than 1 haplotype. Hybridization or gene flow may have occurred between the 2 mitochondrial clades, which should be further explored using nuclear DNA markers. We suggest that the genetic introgression of *A. mali* clades may have facilitated the adaptation of the species to conditions in China. Prior studies demonstrated that such genetic introgressions may have facilitated adaptation by allowing the appearance of new gene combinations in *Harmonia axyridis* Pallis (Coccinellidae) (Lombaert et al. 2011; Facon et al. 2011). The biological and ecological effects of genetic admixture should also be further explored for *A. mali* in the future research.

Endnotes

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