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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 biológico 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 cripticos que incluyen 3 haplotipos de A. mali, lo que sugiere 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; Lessandro 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 (DL) and Qingdao, Shandong (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 (+Mg²⁺), 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       |
| YD   | Qingdao, Shandong | 36°19'   | 120°23'  | 30                         | Jul 2012
The mitochondrial COI 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...
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 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; Asuncion 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

Rui-Ming Zhang and Hong-Xu Zhou contributed equally to this work. We are grateful to Dr. Gabor Lovel (Department of Agroecology, Aarhus University) and Blas Lavandero (Instituto de Biología Vegetal y Biotecnología, Laboratorio de Interacciones Insecto-Planta, Universidad de Talca, Chile) for his detailed comments on this manuscript. This work was supported by the National Key Basic Research Development Plan Project (2013CB127600), the National Natural Science Foundation of Qingdao (13-1-3-108-nsh), and the Taishan Science and Technology Development Planning Project (31371994), the Special Fund for Agro-scientific Research in the Public Interest (201103026-5-2), the Science and Technology Development Planning Program of Qingdao (13-1-3-108-nsh), and the Taishan Mountain Scholar Constructive Engineering Foundation of Shandong, China.

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