Identification of two invasive *Cacopsylla chinensis* (Hemiptera: Psyllidae) lineages based on two mitochondrial sequences and restriction fragment length polymorphism of COI amplicon

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Running Title: Mitochondrial DNA identification of two invasive *C. chinensis* lineages
Abstract

The occurrence of pear decline, a disease found in some pear orchards of Taiwan in recent years, is accompanied by an outbreak of *Cacopsylla chinensis* (Yang and Li). Two major morphological forms (summer and winter forms) with a variety of intermediate body color and two phylogenetic lineages of this psyllid have been described. The work herein used sequences of mitochondrial COI and 16S rDNA regions to delineate the genetic differentiation of this color-variable insect and to elucidate their relationship. Sequence divergence and phylogenetic analysis have shown that *C. chinensis* individuals could be divided into two lineages with 3.3% and 2.3% divergence of COI and 16S rDNA, respectively. All specimens from China were found to belong to lineage I. Restriction fragment length polymorphism (RFLP) analysis of COI with restriction enzymes *Acu*I, *Asel*, *Bcc*I, and *Fok*I on 263 specimens of six populations from Taiwan produced two digestion patterns, which are in agreement with the two lineages described above. Both patterns could be found in each population, with most individuals belonging to lineage I and 5-21% of the individuals belonging to lineage II. Since these two lineages included summer as well as winter morphological forms, the lineage differentiation is apparently not related to morphological characters of this psyllid. Since the invasive records are not in favor of a sympatric differentiation, this psyllid is more likely introduced as different populations from countries in temperate regions.

Keywords: *Cacopsylla chinensis*, invasive species, cytochrome oxidase I, 16S rDNA, restriction fragment length polymorphism
Introduction

Pear psyllid is one of the menacing pests in pear orchards in temperate and subtropical regions. High population density of this insect can cause premature leaf and fruit drop, diminish plant growth, and reduce fruit sizes (Burckhardt 1994). Furthermore, some of these psyllids are vectors of phytoplasma, the pathogen causing pear decline. Pear psyllid of Cacopsylla consists of two subgenera, Hepatopsylla and Thamnopsylla, and species of the former are multivoltine with seasonal dimorphism depending on photoperiod and temperature (Wong and Madsen 1967). For Hepatopsylla psyllids, such as C. pyricola, C. pyri, and C. chinensis, the summer form is light yellow or green in color, small in size with clear wings, and the winter form is dark, significantly larger in size with a cloud-bearing cell in the forewing (Wong and Madsen 1967, Burckhardt and Hodkinson 1986, Yang and Li 1981, Yang et al. 2004). Seasonal dimorphic pear psyllids of C. pyri, C. pyricola, and C. bidens have been considered as separate species (Burckhardt and Hodkinson 1986).

Several psyllid species of Cacopsylla have been reported causing serious damage to pear orchard throughout Europe, North America and Asia, such as C. pyri and C. pyricola (Jensen et al. 1964, Burckhardt 1994, Agusti et al. 2003, Civolani and Pasqualini 2003), and in China, such as C. chinensis (Yang and Li 1981). However, none of these were recorded in Taiwan in last century. Only until recent years, pear decline disease was found in orchards of western and central Taiwan with a simultaneous outbreak of psyllids. Yang et al. (2004) identified these psyllids as C. chinensis (Yang and Li), which was first reported as a pear pest in northern China by Yang and Li (1981). Differences of several morphological characters have been noted for C. chinensis collected from Taiwan and
China, including the shape of genitalia, body size, and forewing length (Yang et al. 2004, Yang and Li 1981).

Using mitochondrial 16S rDNA sequences, in a preliminary study, Lee et al. (2007) reported that psyllids fell into two phylogenetic lineages: most individuals, both summer and winter forms, belonged to the lineage I, and only a few individuals of summer form belonged to lineage II. Both lineages were identified in populations from some areas.

Molecular markers have been valuable for species and population identification, especially when a large number of closely related samples need to be processed. In this study, sequences of cytochrome oxidase I region (COI) and 16S rDNA were used to delineate the differentiation patterns of the two \textit{C. chinensis} lineages. It would be interesting to further examine the possibility of sympatric differentiation or multiple invasions. Moreover, restriction fragment length polymorphism (RFLP) of COI was used to determine the composition of these two lineages in \textit{C. chinensis} populations, and to elucidate the relationships between phylogenetic lineages and their seasonal forms.

**Materials and Methods**

**Sample collection**

\textit{C. chinensis} adults and nymphs were obtained from the pear orchard, pertinent collection information is given in Table 1 & Figure 1. Some specimens were acquired from Beijing (BA) and Xizang (CuA) province of China. \textit{C. qianli} from Meifong, and \textit{Psylla alniformosanaesuga} and \textit{P. lanceolata} from Dayuling of Hualien County (DA-1 and DA-2) were used as outgroups for comparison. Specimens were collected between 2002 and 2006, preserved in 95% alcohol, and stored at -20 °C in the Department of
Entomology, National Chung Hsing University. Pear psyllids were identified according to Yang et al. (2004), Yang (1984), and Yang and Li (1981).

**DNA extraction and amplification**

Total genomic DNA was extracted from three legs of single psyllid using Wizard Genomic DNA purification kit (modified from Yeh et al. 2004). Voucher specimens are stored at -20°C in the Department of Entomology, National Chung Hsing University. The isolated DNA was resuspended in 100μl ddH2O and stored at 4°C. Partial sequence of mitochondrial 16S rDNA gene was amplified and sequenced by primers 16SR21 and 16S22 (Yeh et al. 1997). The COI region, including the 3' region of COI, the leucine tRNA, and 5' region of the COII gene, was amplified using primers UEA9 and C2N-3389 (Lunt et al. 1996, Simon et al. 1994). Conditions for PCR amplification were as follows: an initial denaturing step of 95°C for 10 min; 41 cycles of 95°C for 50 s, 45°C for 1 min, and 72°C for 1 min; and a final extension step of 72°C for 10 min. The amplified products were stored at 4°C.

DNA was purified directly from the amplified product using a PCR purification kit (Quiagen, German), or after resolving on agarose gel, excised and extracted with the Qiaquick gel extraction kit. DNA products were sequenced using Taq dye terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI 377A sequencer.

**Sequence alignment and phylogenetic analysis**

Sequences were aligned using the pileup program of the Genetic Computer Group software package (GCG, version 10.3), then checked manually. Phylogenetic analysis was performed by the Neighbor-Joining method (Saitou and Nei 1987) where the pairwise distance estimates were based on 2-parameter models supplied in software MEGA3.
Restriction Fragment Length Polymorphism (RFLP) of COI Region

Restriction sites in COI region were predicted using mapplot program of GCG, and AcuI, AseI, BccI and FokI were selected on account of the length of digested fragments. Two hundred and sixty three representative individuals from six seriously damaged orchards were examined for their polymorphism; and the COI region of each specimen was digested at least by two of these restriction enzymes. Restriction enzyme was diluted in 10X reaction buffer to a final concentration of 2U/µl, and 5µl of PCR product was added to a final volume of 10µl; and the digestion was kept at 37°C for 2 hr. The resulting DNA fragments were visualized with ethidium bromide staining after 1.5% agarose gel running.

Results

Sequence variations of mitochondrial 16S rDNA and COI regions

Sequence analyses of 38 psyllids, including 32 individuals of C. chinensis and six outgroup individuals, showed that within 512 bp of the partial 16S rDNA gene, 38 singletons were found in 84 variable positions. Base compositions of this gene among these psyllids were similar, with average A, T, C, and G of 35.1%, 38.4%, 9.8%, and 16.7%, respectively. According to their 16S rDNA sequences, the 32 C. chinensis could be divided into two groups: 27 individuals including three specimens from China as one group, showing 2.3% divergence from a second group consisting of the remaining five individuals. Sequence divergence within the first and second groups was 0-0.3% and 0-0.2%, respectively; and between C. chinensis and outgroup C. qianli and Psylla species
Base compositions of COI region among the pear psyllids were not significantly different, and the average A, T, C, and G contents were 35.5%, 40.6%, 14.9%, and 9.0%, respectively. The 32 individuals of *C. chinensis* fell into two groups which are identical to those for 16S rDNA gene. While there was a 3.3% divergence between the two groups, the intragroup divergences were 0-0.6% and 0-0.2%. The sequence divergence between *C. chinensis* and outgroup *Psylla* species and *C. qianli* was 11.2% to 13%.

**Phylogenetic analysis of mitochondrial 16S rDNA and COI regions**

The phylogenetic trees representing 1000 bootstrap replications for 16S rDNA (Fig 2A) and COI (Fig 2B) of these 38 psyllid specimens have displayed similar evolutionary patterns. With members of each species grouped together and receiving significant bootstrap possibilities, four distinct clusters, i.e. *C. chinensis*, *C. qianli*, *P. lanceolata*, and *P. alniformosanaesuga* have been observed. *C. chinensis* is comprised mainly of lineage I, which is differentiated to lineage II consisting of individuals TD-A, HD, JA-1, JA-2, and JA-3. No significant differentiation was detected within each *C. chinensis* lineage.

**PCR-RFLP of COI region**

Fig. 3 contains the representative PCR-RFLP patterns of COI fragment (672 bp) of *C. chinensis*. While the results of only seven individuals are shown in this figure, the restriction patterns produced by four different enzymes, *Acu* I, *Ase* I, *Bcc* I and *Fok* I are identical for all 263 specimens of six populations (Table 2). They can be separated into two clear-cut patterns, which agreed fully with lineage I and lineage II of *C. chinensis* in the phylogenetic study. While both lineages (patterns) were detected in the six populations examined, only 5 to 21% of the specimens belonged to lineage II (Table 2).
Since both lineages I and II contain both summer and winter forms, the lineage
differentiation is apparently not related to morphological characters of the psyllid.

Discussion

Taxon identification based on DNA sequences, especially that of COI amplicon in
mitochondria, can facilitate the recognition of known species and the discovery of new
divergence greater than 2% has been found in 98% pair-wise comparisons of congeneric
species for 11 animal phyla (Hebert et al. 2003b). In insects, however, recent studies have
shown that no definite percent sequence difference of COI could be obtained within
and/or among species (Cognato 2006, Meir et al. 2006, Roe and Sperling 2007).

In a study also using COI region to examine the diversity of 23 legume-feeding
arytaininae psyllids, Percy (2003) reported an intraspecies divergence of <2.8%, except
four of the species, i.e., Livilla monospermae, Arytaina devia, Arytinnis dividens, and A.
occidendalis, which showed a maximum divergence of 4 to 10% in populations from
isolated islands (Percy 2003). Recent studies also suggest that multiple genes, including
at least one nuclear amplicon, will be more accurate and effective in species
recognition (Rubinoff et al. 2006, Vences et al. 2005, Rubinoff 2006). Although the
COI divergence between the two psyllid lineages, i.e., 3.3%, is high, it is still premature
to discuss the taxonomic status of C. chinensis when only mitochondrial sequence is
examined in this psyllid.

Yang and his colleagues (Yang 1984, Fang and Yang 1986, Lauterer et al. 1988, Fang
1990, Chou and Fang 1994) in their wide and thorough survey of psyllids in Taiwan did
not record *C. chinensis*. Then, Yang et al. (2004) reported a new record of *Cacopsylla* species from pear orchards, implying that *C. chinensis* had been introduced into Taiwan recently. As stated above, among the 32 *C. chinensis* specimens examined, only five individuals belong to lineage II; and the remaining 27 specimens, which include three individuals from China, fall into lineage I. Since a sympatric differentiation is not expected to occur in such a short time interval, the two lineages of *C. chinensis* are more likely the results of multiple invasions from countries in temperate regions.

In terms of RFLP patterns of COI gene, *C. chinensis* samples in this study comprise mainly lineage I, with the coexisting lineage II constituting ca. >13% and <8% of samples from infected areas in western and eastern parts of Taiwan, respectively (Fig. 1). With the central mountain range of 3,000m altitude effectively preventing natural dispersal, finding only a low proportion of samples from central and eastern Taiwan belonging to lineage II, implies that the dispersal of *C. chinensis* from the west to the central/east parts might not have been random. Field survey reported the first sighting of pear psyllids in eastern areas in 2005, and none has been observed in southern Taiwan. Therefore, in the past 5 years or so, the founder effect of an artificial transplant of this psyllid to Lishan (in central Taiwan) and Taitung (in eastern Taiwan) might have led to the low frequency of lineage II.

This study, using partial regions of mitochondrial COI and 16S rDNA, has obtained some basic information on the genetic differentiation and composition of *C. chinensis* which has been introduced into Taiwan in the past decade or so. Future work will use nuclear sequences and *C. chinensis* specimens from countries of temperate regions to gain more insight into the taxonomic status and the colonization/dispersal history of this
important insect pest.

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Figure legend

Fig. 1 Distribution and location of pear psyllids analyzed in this study. Individuals come from more than one pear orchard in the neighborhood areas were present as the same population. Abbreviations of collecting localities and frequencies of RFLP pattern I (light) and II (dark) were shown of each locality. The Central Mountain Range was shown with the light gray representing the altitude between 500 and 1,000 m and dark gray representing an altitude of over 1,500 m.

Fig. 2 Phylogenetic tree constructed from COI (A) and 16S rDNA (B) sequences with neighbour-joining method. Bootstrap values are shown beneath the branch. Individuals of *Cacopsylla chinensis* were divided consistently into two lineages.

Fig. 3 Polymorphism of restriction fragment length of molecular COI amplicon. The PCR products of seven individuals were selected and digested by enzymes of *Acu*I (A), *Ase*I (B), *Bcc*I (C), and *Fok*I (D) enzymes, respectively. Digested patterns revealed that five individuals were belonging to lineage I and the other two were lineage II.