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Analysis of the Genetic Population Structure of *Cacopsylla chinensis* (Hemiptera: Psyllidae) with Mitochondrial DNA Markers in Saga and Yamaguchi prefectures, Japan

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Abstract

DNA sequence analysis of the mitochondrial cytochrome oxidase I (COI)-leucine tRNA ($tRNA^{Leu}$)-cytochrome oxidase II (COII) regions was performed to elucidate the phylogenetic relationships among 465 summer and winter-form psyllids from Saga and Yamaguchi prefectures in Japan. Multiple alignments of these sequences revealed 2 single-nucleotide polymorphisms (SNPs) at nucleotide positions 78 and 258 in the COI and $tRNA^{Leu}$ regions, respectively. Based on the nucleotide changes at these loci, these 465 insects were grouped into 3 genotypes: 78A/258A, 78G/258G, and 78G/258A. All but 2 of the 225 insects in Saga belonged to the genotype 78A/258A, and the 2 exceptions belonged to the genotype 78G/258G. Conversely, all the insects (105) collected from Shimonoseki city in western Yamaguchi prefecture belonged to the genotype 78G/258G, while eighty percent of the 135 samples from eastern Yamaguchi prefecture belonged to the genotype 78G/258G. Among the other 27 insect samples, 24 were 78G/258A and 3 were 78A/258A. Using POPTREE2, the psyllids were divided into 5 populations associated with the geographical distribution. The results suggest that the genetic population structure of these psyllids varied widely between Saga and Yamaguchi prefectures.

Discipline: Insect pest

Additional key words: cytochrome oxidase I, molecular phylogenetic analysis, Pear psyllids, population genetics

Introduction

Pear psyllids excrete abundant honeydew that causes severe sooty mold damage to pears (*Pyrus* spp.) grown in temperate and subtropical regions worldwide. These psyllids belong to the large genus *Cacopsylla* (Psyllidae, Psyllinae). Li (2011) and Luo et al. (2012) reported that the genus includes 26 pear psyllid species. Severe damage to pears caused by *Cacopsylla chinensis* has been reported in China (Yang & Li 1981). An outbreak of *C. chinensis* also occurred in pear orchards of Western and Central Taiwan (Yang et al. 2004, Lee et al. 2008). Some pear psyllids are not only serious pests in themselves but also vectors of phytoplasma diseases of pears (Jarausch & Jarausch 2010). In 1994, pears with decline symptoms (Pear decline-Taiwan, PDTW) were observed in central Taiwan. In fall, the infected trees showed premature reddening and loss of leaves. Moreover, their leaves remained small and pale the following spring, and few or no shoots developed (Chen et al. 2001). The symptoms of diseased pear trees in Taiwan were apparently associated with phytoplasma that was transmitted by *Cacopsylla chinensis* (Liu et al. 2007, 2011).

In July and August 2011, partial leaf blackening was observed in the orchards of the Japanese pear (*Pyrus pyrifolia* var. culta), located in Imari city in Saga prefecture (Katoh et al. 2013). Since then, etiolation and defoliation have proliferated and we have found many *C. chinensis* during this time (Inoue et al. 2012). The nucleotide sequences of

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the mitochondrial cytochrome oxidase I-leucine tRNA-cytochrome oxidase II (COI-tRNA^{Leu}-COII) region were identical among the Japanese *C. chinensis* from this region (Katoh et al. 2013). Conversely, comparison of nucleotide sequences and phylogenetic analysis differentiated the Japanese *C. chinensis* from the Taiwanese and Chinese *C. chinensis* isolates, with 7-8% nucleotide difference, suggesting that *C. chinensis* possesses a high level of genetic variability and that *C. chinensis* in Saga, Japan belongs to a distinct lineage of Taiwanese and Chinese *C. chinensis*.

In March 2012, the presence of *C. chinensis* was confirmed in other orchards in Imari and Karatsu cities of Saga prefecture. In May 2012, another outbreak of *C. chinensis* occurred at an orchard in Shimonoseki city in the western part of Yamaguchi prefecture, located approximately 150 km northeast of Saga prefecture. Moreover, in July 2012, adult psyllids of *C. chinensis* were also observed in the town Abu of Yamaguchi prefecture. However, little is known about the genetic structure of *C. chinensis* populations in both Saga and Yamaguchi prefectures. Understanding its genetic structure is crucial to estimate the relationships among these populations.

A comparative sequence analysis of the COI-tRNA^{Leu}-COII region was performed to elucidate the genetic structures of *C. chinensis* population in Saga and Yamaguchi prefectures.

Materials and Methods

1. Psyllids and DNA preparation

Adult *C. chinensis* were collected from pear orchards in Saga and Yamaguchi prefectures from January to August of 2012. The specimens were preserved in 99.5% ethanol and stored at 4°C. Total genomic DNA was extracted from the samples using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA), with minor modifications to a previously reported protocol (Katoh et al. 2013).

2. Polymerase chain reaction

The *COI* region, including the 3′ region of *COI*, tRNA^{Leu} and 5′ region of the *COII* gene, was amplified using primers UEA9 (5′-GTAAACCTAACATTTTTTCCTCAACA-3′) and C2-N-3389 (5′-TCATAAGTTCARTATCATTG-3′) (Lunt et al. 1996, Simon et al. 1994). PCR was performed using the GeneAmp PCR system (Applied Biosystems, Foster City, CA, USA) in a 20 μL reaction volume containing 0.5 μL of DNA template, 0.1 μM of each primer, 200 μM dNTP mixture, 1× PCR buffer, and 1.0 unit of *Ex Taq* DNA polymerase Hot Start Version (TaKaRa, Shiga prefecture, Japan). Conditions for PCR amplification were as follows: an initial denaturing step of 95°C for 10 min, 35 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 PCR products were separated using electrophoresis by a 1.5% (wt/vol) agarose gel in a tris-boric acid-EDTA buffer and the PCR products were extracted from the gel using the QIAquick gel extraction kit (Qiagen), according to the manufacturer's instructions.

3. Sequence analysis

Both strands of the DNA fragments were subjected to direct sequencing by the ddNTP termination method (Sanger et al. 1997) using BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems) and ABI 3130xl sequencer (Applied Biosystems). The sequencing reactions contained 1 μL of PCR product, 0.5 μL of BigDye Terminator v3.1 Ready Reaction Mix (Applied Biosystems), 1.75 μL of 5X Sequencing buffer (in kit) and 500 nM primer (either UEA9 or C2-N-3389) and made up the volume to 10 μl. DNA sequences were aligned using the ClustalW program (Thompson et al. 1994), and sequence analysis was performed using GENETYX-windows ver. 11 (Software Development, Tokyo, Japan). The specificity of these sequences was examined by comparison with the nucleotide database at GenBank using BLAST (DNA Data Bank of Japan). The nucleotide sequences of *C. chinensis* collected from pear orchards in Saga prefecture from September to October of 2011 (Katoh et al. 2013) were also used for comparison.

Allele frequencies and the genetic differentiation between populations were analyzed with analysis of molecular variance (AMOVA, Excoffier et al. 1992, Huff et al. 1993) in GenAlEx Ver. 6.5 (Peakall & Smouse 2012). A neighbor-joining tree was constructed in POPTREE2 (Takezaki et al. 2010) using allele frequencies of COI-tRNA^{Leu}-COII.

Results

1. Sequential variation in the mitochondrial COI-tRNA^{Leu}-COII region

The mitochondrial COI-tRNA^{Leu}-COII region is 580 bp in length. Analysis of 465 summer and winter form insects, including the 87 psyllids used in the previous study (Katoh et al. 2013) revealed 2 single nucleotide polymorphisms (SNPs), which were located in nucleotide positions 78 and 258, respectively, of the COI and tRNA^{Leu} regions. Based on the nucleotide changes at these loci, all specimens were classified into 3 genotypes: 78G/258G, 78A/258A, and 78G/258A (Fig. 1). The SNPs at the nucleotide positions 78 and 258 were synonymous and untranslated variation, respectively. All but 2 of the 225 samples in Saga belonged to the genotype 78A/258A, while the 2 exceptional samples from Imari city belonged to the genotype 78G/258G (Fig. 2). Conversely, all 105 samples from Shimonoseki city had the genotype 78G/258G (Fig. 2). In the town Abu, located in the eastern part of Yamaguchi prefecture, 31 insect samples (about two-thirds) showed the profile of 78G/258G, while

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                                                                78 (COI region)
78G/258G    GGACTTACTAATCTCATGAAATATTGTTTCTTCGATCGGGTCTATGATTTCTCTATTTTC
78A/258A    GGACTTACTAATCTCATGAAATATTGTTTCTTCGATCGGGTCTATAATTTCTCTATTTTC
78G/258A    GGACTTACTAATCTCATGAAATATTGTTTCTTCGATCGGGTCTATGATTTCTCTATTTTC
                                          .
                                          .
                                          .                  258(tRNALeu region)
78G/258G    TTCTGAAATTCCTTCAATTTTAAGCAAGTAACTAATGTGTCAGAAGTAAATGTATTAAAC
78A/258A    TTCTGAAATTCCTTCAATTTTAAGCAAGTAACTAATGTGTCAGAAATAAATGTATTAAAC
78G/258A    TTCTGAAATTCCTTCAATTTTAAGCAAGTAACTAATGTGTCAGAAATAAATGTATTAAAC
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Fig. 1. Alignment of partial nucleotide sequences of COI-tRNA^{Leu}-COII region from each of the 3 genotypes found in this study

These sequences were aligned by Clustal W (Thompson et al. 1994). Identical nucleotide sequences are indicated in gray letters, while the SNPs genotyped in this study are indicated in black letters. Nucleotide positions are numbered following the genome sequence of *C. chinensis* isolate "CL-B2". The Genbank accession number is AB364012 (Lee et al. 2008). The sequences of 78G/258G, 78A/258A and 78G/258A are included in COI-tRNA^{Leu}-COII region of *C. chinensis* isolate "T-1fs" (AB920373), "I-1mc" (AB720877, Katoh et al. 2013) and "1-11ns" (AB920374), respectively.

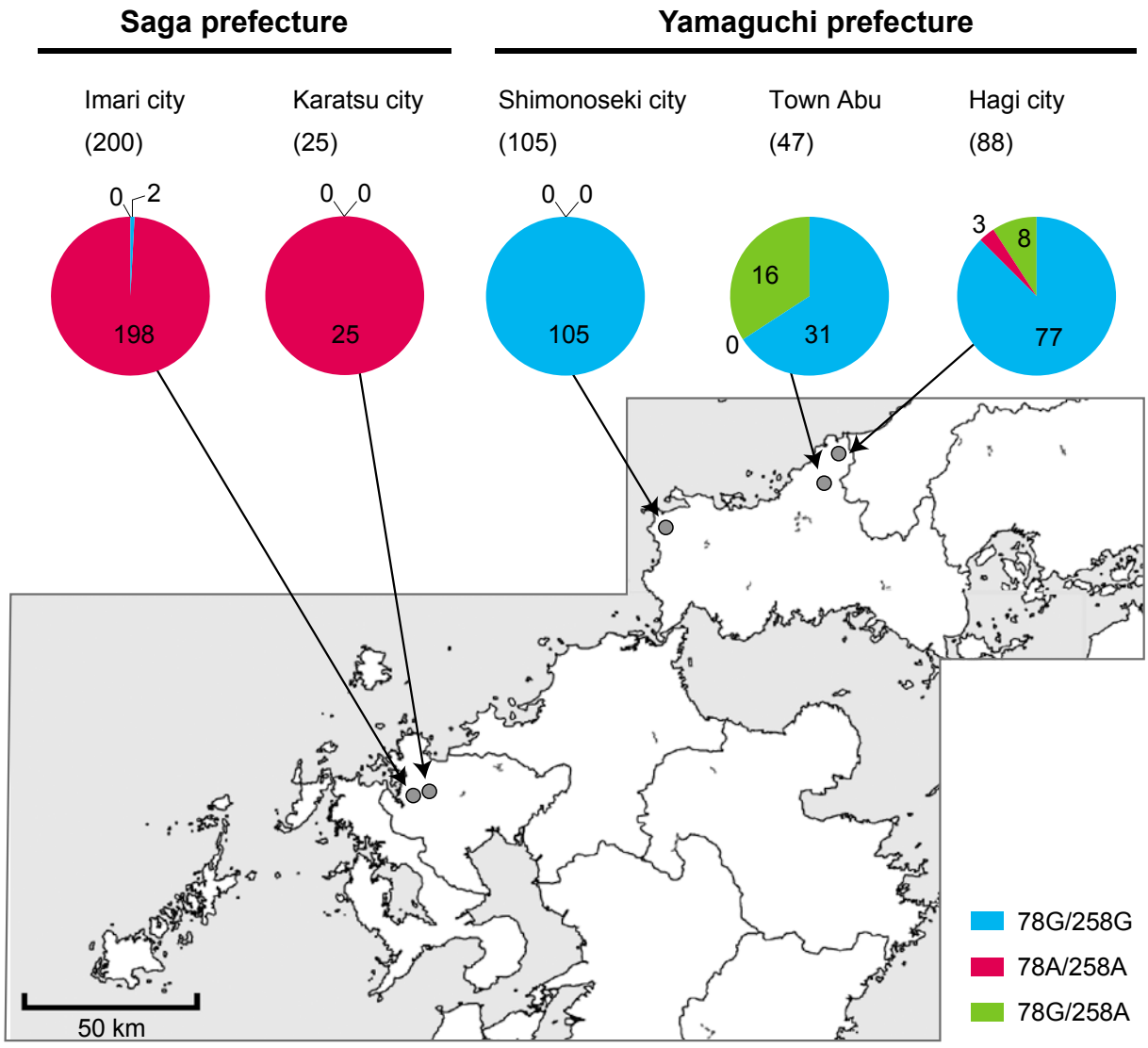

Fig. 2. Distribution and location of pear psyllids analyzed in this study

The number of insects in each population is shown in parentheses.

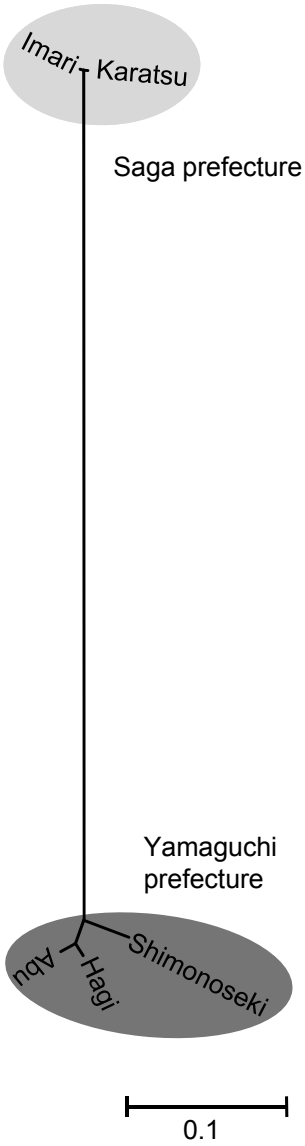

Fig. 3. Neighbor Joining (NJ) tree based on Nei's genetic distances generated from allele frequencies at the COI-tRNA^{Leu}-COII loci

the remaining 16 belonged to the genotype 78G/258A. The population of Hagi city was the most polymorphic (Fig. 2). Nearly 90% of the samples showed the profile of 78G/258G, 3 samples had 78A/258A and 8 samples had 78G/258A (Fig. 2).

2. Specimen-based genetic structure in the landscape

Based on the neighbor-joining method, a phylogenetic tree was constructed using allele frequencies calculated using GenAlEx v 6.5 (Fig. 3). Using the allele frequency data from 5 populations, a neighbor-joining tree based on Nei's D_A distances was constructed (Fig. 3). Five populations were segregated into 2 clades: a clade containing the 2 populations of Saga prefecture and the other clade containing 3 populations of Yamaguchi prefecture. The phylogenetic tree was associated with the geographical distributions of each population (Fig. 3).

Discussion

This is the first report on nucleotide substitution in the COI-tRNA^{Leu}-COII region of Japanese *C. chinensis* population. However, the genetic diversity of the Japanese *C. chinensis* population was much smaller than that of Taiwanese *C. chinensis* population reported by Lee et al. (2008). In particular, the populations of both Karatsu and Shimonoseki cities have no genetic diversity. It is suggested that the *C. chinensis* population of Japan has a shorter invasion period than that of Taiwan, or that invasion to Japan occurred at low frequency. Conversely, the genetic diversity among each population revealed clear geographic patterns. This result indicates that both Karatsu and Shimonoseki populations were influenced by virtually equal genetic drift after divergence. The ancestral population may exist in China and/or elsewhere, prompting the need for additional comprehensive field surveys in these regions. From a big-picture perspective, the genetic population structure of the psyllids between Saga and Yamaguchi prefectures differ significantly. If simultaneous invasion is assumed, the difference would suggest that the populations have been introduced to each of the prefectures separately. It is unlikely that the population of one prefecture subsequently moved to the other prefecture. The three populations within Yamaguchi prefecture have slightly different genetic structures. Our data indicated that the most genetically diverse population infested orchards of Hagi city. A bottleneck effect is also often observed in populations of invasive pests, although this was not revealed in analysis of only two alleles in this study. In future, it will be necessary to find new allele, such as SNPs and SSR, to analyze whether a bottleneck effect exists. The occurrence of *C. chinensis* has not been reported in Fukuoka prefecture, located between Saga and Yamaguchi prefectures, which may be due to the use of different pesticides, or geographical barriers. If this psyllid newly occurs elsewhere in Japan in future, the insights on genotypes reported in this paper could be used to speculate whether they originate from Saga, Yamaguchi or the other foreign lands.

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