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Discrimination of Reproductive Forms of *Thrips tabaci* (Thysanoptera: Thripidae) by PCR With Sequence Specific Primers

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ABSTRACT In agriculture, although it is important to identify species of pest insects, the morphological identification is often difficult. DNA genotyping is useful for the identification of species in morphologically indiscriminable species. *Thrips tabaci* (Lindeman) can be divided into two reproductive forms (arrhenotoky and thelytoky, each of which different in pesticide resistance) but morphological discrimination is not possible. Here, we establish a simple method to discriminate the strains based on their mitochondrial DNA sequences. Phylogenetic analysis including the *T. tabaci* and congeneric species provided ancestor sequences of each strain of *T. tabaci*. Based on the ancestor sequences, we developed a primer set that include strain specific primers on sense strand and common primer on anti sense strand. Using this primer set, the strains of 196 individuals of *T. tabaci* were successfully assigned to each of genotypic forms. As the phylogeny and ancestor sequences were based on worldwide samples, this method will work well on most populations around the world.

KEY WORDS *Thrips tabaci*, phylogeny, mitochondrial DNA, reproductive form, PCR-SSP

Morphological discrimination of multiple strains within a species is often difficult when there are few morphological differences among them. In a case of species identification, DNA genotyping was used to identify the species of morphologically indiscriminable ones. For example, although it is hard to identify species of juveniles of thrips (Thysanoptera: Thripidae), we can identify them based on sequences of mitochondrial DNA (Moritz et al. 2000, Brunner et al. 2002, Toda and Komazaki 2002, Walsh et al. 2005, Hoddle et al. 2008, Mound and Azidah 2009, Glover et al. 2010, Karimi et al. 2010). Such molecular techniques will be also useful for the discrimination of the strains.

Onion thirps, *Thrips tabaci* Lindeman, is distributed worldwide and an economically important pest because they damage on many crops such as onion, leek, cabbage, tobacco, and so forth. Based on mitochondrial DNA sequences, *T. tabaci* was classified into three strains; one is only found on tobacco (Brunner et al. 2004), the others are polyphagous and are divided into two reproduction forms; arrhenotoky and thelytoky (Toda and Murai 2007). As the tobacco strain is arrhenotoky, thelytoky will be a derived character in the polyphagous strains (Toda and Murai 2007). Until 1990, all the individuals of *T. tabaci* in Japan had been thought as the thelytokous strain, but the arrhenotokous strain was found from several areas (Murai 1990, Toda and Murai 2007). The two strains have different ecological characters; the ability to transmit tomato spotted wilt virus (Wijkamp et al. 1995; Chatzivassiliou et al. 1999, 2002), host preference (Chatzivassiliou et al. 2002), and Pyrethroid resistance (Toda and Morishita 2009). Thus, discrimination of the strain is agriculturally important but there is no simple method for discrimination among the strains.

In this study, we aim to establish a primer set for a polymerase chain reaction (PCR) to discriminate the arrhenotokous and thelytokous strains of *T. tabaci*. The mitochondrial DNA sequences are informative to discriminate the strains because a strict relationship between reproductive modes and the mitochondrial DNA sequences was demonstrated (Toda and Murai 2007). However, as DNA sequencing is costly, we develop a simple method using a PCR with strain specific primers (PCR-SSP). Strain specific primers are set on a strand and a common primer is on the other strand. These primers enable us to discriminate the strains based on the fragment length of PCR products. This PCR-SSP method can discriminate the strains with a lower cost than DNA sequencing. Our method is also less time consuming than sequencing and enables handling of a large number of samples. Using individuals from Hokkaido prefecture in Japan, we examine effectiveness of the proposed method.

Materials and Methods

In July 2009, 196 adults of *T. tabaci* were sampled from five onion fields in Naganuma town Hokkaido

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Prefecture Japan. Collected individuals were preserved in 70% ethanol until DNA extraction. Number of males and females in each sampling field were counted under a binocular microscope (Olympus, SZH-ILLD). DNA was extracted by a modified Chelex method (Walsh et al. 1991). In a 1.5 ml micro centrifuge tube, a dried individual was mixed with 2 μl of proteinase K (20 mg/ml) and 100 μl of 5% Chelex solution (10 mM Tris-HCl pH 8.0 buffer), and incubated at 55°C for over 12 h. Then, the solution was boiled at 98°C for 10 min to inactivate the proteinase K. Water layer on top of Chelex layer was used as a template DNA.

We obtained sequences of cytochrome oxidase subunit I (COI) of *T. tabaci* and other species in the genus *Thrips* from DNA databases (NCBI, GenBank, and DDBJ) by Basic Local Alignment Search Tool (BLAST). The obtained sequences were aligned using ClustalW (ver. 1.8.3; Thompson et al. 1994). We used only overlapped region among the sequences in further analyses. Some sequences were excluded from phylogenetic analysis because these sequences are completely same with other sequences in the region we used. We inferred phylogenies using MEGA (ver. 5.05; Tamura et al. 2011). Three different methods (neighbor joining, NJ; maximum parsimony, MP; and maximum likelihood, ML) were used to infer phylogeny. In NJ, the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004). In MP, the Close-Neighbor-Interchange algorithm (Nei and Kumar 2000) was used to search the MP tree. In ML, General Time Reversible model plus Gamma distributed plus Invariable rate (GTR + G + I) was used and the model is estimated based on AICc (Akaike Information criterion, corrected). In ML analysis, the NJ tree was used as an initial tree in the heuristic search. After the phylogenetic analysis, to develop strain specific primers, we estimated the ML sequence of common ancestor of each strain from the ML tree with the same evolutionally model to infer the ML tree. For PCR-SSP, three primers were designed on the COI sequences of ancestors; one is consensus sequence on an antisense strand (TCOR: 5′-attgcgtaaattattcctaaaagtcca-3′) and the others are strain specific sequence on a sense strand (arrhenotokous specific primer TCOS: 5′-aacagcTattctCcttctttatctC-3′ and thelytokous specific primer TCOC: 5′-gaacagtatatccacctttatcaacG-3′; the capital letters indicated each strain specific nucleotide). The strain specific primers were designed within preserved regions that were not substituted within the phylogenetic group of each strain. Using these primers, we determined the strain of all 196 adults that we sampled. The composition of the reaction mixtures was as follows, each 10 μl of reaction mixture contained 5 pmol of the consensus primer and 2.5 pmol of the strain specific primers, 5 μl of 2 × MightyAmp Buffer, 0.1 μl of MightyAmp DNA Polymerase (TaKaRa; 1.25 U/μl) and 0.5 μl template DNA. A 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) was used for PCR that was carried out by the following temperature cycle, 2 min at 98°C followed by 35 cycles of 10 s at 98°C and 1 min at 60°C. To complete extension, one step of 1 min at 68°C was added. The fragment length of PCR products was detected by 1% agarose gel electrophoresis with 5 μl of 100 bp DNA Ladder (TaKaRa) and staining with ethidium bromide.

To examine the accuracy of the PCR-SSP, we sequenced a COI region using sampled individuals and compared the results of the PCR-SSP with it of the sequencing. DNA of three males and 40 females (20 females from each strain that was judged by the PCR-SSP) were amplified by a primer pair designed in the preserved regions (TCOFn2: 5′- ctttttacccccttctctggg-3′ and TCOR: 5′-attgcgtaaattattcctaaaagtcca-3′). PCR cycle consisted of 1 min at 94°C, followed by 40 cycles of denaturation for 5 s at 98°C and an annealing and extension of 1 min at 60°C, and one step of 1 min at 72°C to complete extension at the end. Each of the 10 μl reaction mixtures contained 5 pmol of each primer, 1 μl of dNTP mixture (2.5 mM each), 1 μl x10 Ex *Taq* buffer, 0.1 U of Ex *Taq*DNA polymerase (TaKaRa), 5.5 μl of sterilized water, and 2 μl of template DNA. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and were eluted into 10 μl of sterilized water. The products were sequenced using DTCS-Quick Start Kit (Beckman Coulter, Fullerton, CA), a CEQ2000 XL automatic DNA sequencer (Beckman Coulter) and the primer TCOR. After the sequencing, to confirm an effect of the obtained sequences to the topology and the sequences of common ancestors, we inferred the phylogeny again using the obtained sequences to estimate the sequences of common ancestors of strains by the same method above.

Results

All three methods (NJ, MP, and ML) showed that *T. tabaci* are divided into three monophyletic groups that corresponded to the tobacco strain and the polyphagous thelytokous and arrhenotokous strains (Fig. 1). BLAST found over 100 sequences sampled from Japan (Inoue and Sakurai 2007, Toda and Murai 2007); India (Asokan et al. 2007); the United Kingdom, Bosnia-Hertzegovina (Glover et al. 2010); Switzerland, Greece, Bulgaria (Brunner et al. 2004); and Iran (Fekrat et al. 2009). Three haplotypes (AB665089-AB665091) were newly found from our samples in the sequencing test. Excluding sequences that are the same with another sequence in the region we analyzed, the final phylogenetic tree were inferred from 34 sequences and 342 nucleotide positions with 65 polymorphic sites (19%).

There were 11 polymorphic sites between the sequence of common ancestor of thelytokous strain and that of arrhenotokous strain. In both of the strains, each nucleotide site in the common ancestor's sequences was estimated to be a specific nucleotide with high probability (over 95%). The products of PCR-SSP were 261 bp for the arrhenotokous strain and 451 bp for the thelytokous strain (Fig. 2). The PCR-SSP indicated that both strains cohabit in all the five fields,

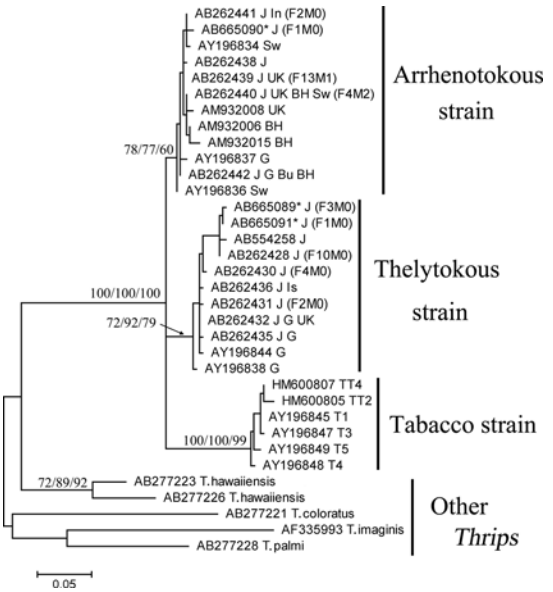

Fig. 1. Molecular phylogenetic analysis of *T. tabaci* and the congeneric species by ML method using GTR + G + I model. The tree of the highest log likelihood (−1643.4543) is shown with single representative accession number and country codes of samples (J, Japan; In, India; UK, the United Kingdom; BH, Bosnia-Herzegovina; G, Greece; Sw, Switzerland; Bu, Bulgaria; Ir, Iran). Asterisks after the accession number indicate novel sequences we found. In parentheses, number of females and males that we found in the sequencing analysis was shown. The bootstrap support values of each main branch estimated by NJ, MP, and ML method are shown on the branches. A discrete γ distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 0.8479)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 43.3421% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

and that three males and 47 females are the arrhenotokous strain (Table 1). This result is a first record of males and arrhenotokous females in Hokkaido. Our sequencing analysis showed that the accuracy rate of the PCR-SSP was 100% (n = 43; 3 males and 40 females). Reinferred phylogenetic trees were basically same topology with the firstly inferred trees, meaning that each the strain constructed each monophyletic clade (Fig. 1). In each the strain, the sequence of the reinferred common ancestor is identical with the firstly inferred ancestral sequence.

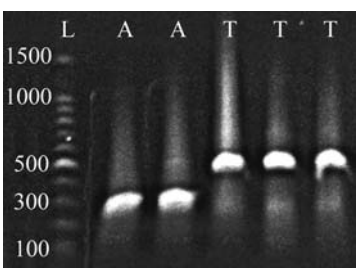

Fig. 2. PCR products of PCR-SSP and 100 bp ladder (Takara) in 1% agarose gel. The numbers in left side indicates fragment length of each band in the 100 bp ladder. L: 100 bp ladder (10 fragments between 100 and 1 kb in multiples of 100 bp and an additional fragment at 1.5 kb), A: products of arrhenotokous strain (261 bp); T: products of thelytokous strain (451 bp).

Table 1. Number of individuals discriminated by the PCR-SSP to each strain

Field ID	Arrhenotokous strain		Thelytokous strain	
	Males	Females	Males	Females
Ho1	1	2	0	29
Ho2	1	8	0	19
Ho3	0	11	0	32
Ho4	0	24	0	51
Ho5	1	2	0	15

Discussion

The accurate discrimination of strain is important to manage pest insects because such strains are often different in economically important characters like pesticide resistance. In *T. tabaci*, several pyrethroid resistance genotypes were found and distributed differently in each strain (Toda and Morishita 2009). As there is no information to discriminate the strains from morphological characters, discrimination based on molecular technique is required. There are strain-specific differences in the mitochondrial DNA sequences between the arrhenotokous and the thelytokous strains (Toda and Murai 2007). However, the DNA sequencing requires expensive regents and a DNA sequencer. This study provided a simple discrimination method that is far less costly in both money and time than the DNA sequencing. Our method will be useful for the pest management of *T. tabaci.*

The PCR-SSP shows coexistence of the strains within each field (Table 1). Because there is a possibility of coexistence of both strains, existence of males in a field does not mean that all females in the field are arrhenotokous strain. Only because males are sampled from a field, choice of a management only for the arrhenotokous strain has a possibility of failure. There is a possibility of existence of thelytokous strain even in the field with males. To choice an adequate management for each field, discrimination of the strains of females sampled from each field is important. This coexistence of the strains may be caused by wide migrations of each strain. Sampling from wide area and continued observation of frequency of the strains will clarify population dynamics and migration route of the strains. The PCR-SSP provides fundamental information for these further investigations to explore population dynamics of each strain.

There is a wide variety of haplotypes within the strains in a country and occurrence of similar haplotypes over several countries. This was confirmed by our phylogenetic analysis (Fig. 1). This result implies that individuals of *T. tabaci* migrate over countries.

The worldwide migration and sexual reproduction in the arrhenotokous strain will disrupt a close relationship between biological characters and mitochondrial haplotypes because such a migration manner destroys a combination of mtDNA and genomic DNA. On the one hand, in thelytokous strain a mitochondrial haplotype is tightly linked with a genomic genotype, and thus the adequate way of management can be selected based on the strain. However, in arrhenotokous strain, the mitochondrial haplotypes of individuals found in specific fields may not be linked with an adequate management that depends on genomic genotypes. However, we may choose a better management method than to the thelytokous strain if we know that the observed thrips are arrhenotoky strain because they are different at pyrethroid resistance ability (Toda and Morishita 2009). Thus, discrimination between the strains is useful in pest management.

T. tabaci may provide important insights in evolutionally biology. The sex ratio in the arrhenotokous strain was highly female-biased (3 males to 47 females; Table 1). If there is a competition within the males produced by a single mother, their female-biased sex ratios may be a result of evolution according to a prediction of local mate competition (Hamilton 1967). The sex ratios in the arrhenotokous strain give a chance to verify the prediction by an investigation of mating system in the arrhenotokous strain. Evolution of reproductive mode is one of the important issues in evolutionary biology (Maynard Smith 1978, Hamilton 1980, Meirmans 2009, Otto 2009). In *T. tabaci*, the several different characters between the strains imply the genetic differentiation between them. The phylogenetic analysis in this study showed two clusters corresponding to the two strains, which also suggests genetic differentiation of the two strains. Because the tobacco strain is known as arrhenotoky, the thelytoky is likely to be a derived character (Toda and Murai 2007). This situation will be suit to study the impact of thelytokous cohabitants on arrhenotokous ancestor. Does the evolution of thelytokous strain without a cost of sex lead to extinction of the arrhenotokous strain? (Otto 2009) Discrimination of the strain is fundamental to answer such questions. Therefore, our method will become important technique in the further investigations.

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