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Discrimination among Japanese Species of the *Orius* Flower Bugs (Heteroptera: Anthocoridae) Based on PCR-RFLP of the Nuclear and Mitochondrial DNAs

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Abstract

A PCR-RFLP-based method of species identification was considered for 5 Japanese species of *Orius* Wolff flower bugs, *Orius strigicollis* (Poppius), *O. minutus* (Linnaeus), *O. sauteri* (Poppius), *O. nagaii* Yasunaga, and *O. tantillus* (Motschulsky). Nucleotide sequences of the internal transcribed spacer 1 of the nuclear ribosomal gene and a portion of mitochondrial cytochrome oxidase subunit I gene were compared among species and recognition sites of diagnostically useful restriction enzymes were examined. The PCR-RFLP analysis using 108 individuals of 26 laboratory strains confirmed that the 5 species could be correctly identified by banding patterns generated using these DNA regions. Because our PCR primers can amplify DNA fragments from DNA template extracted from both freshly killed insects and dried specimens stored at room conditions, the PCR-RFLP-based method was considered useful for analyses of field populations in which researchers store and accumulate field-collected samples before they perform laboratory examinations.

Discipline: Biotechnology
Additional key words: natural enemy, predatory bugs, species identification, internal transcribed spacer 1, cytochrome oxidase subunit I

Introduction

The anthocorid genus *Orius* is known as a group of natural enemies predating on economically important herbivorous insect pests such as thrips, aphids, psyllids, and mites. Many researchers have analyzed the ecology of Japanese species, and showed that they are highly useful as biological control agents[4]. However, because they are closely allied morphologically and frequently live together on the same plants[6], it has been difficult in field research to evaluate each species as a biological control agent. Especially, because of the scarcity of clear morphological differences among closely related species, morphological criteria have not been established for nymphal stages and female adults that are also predators of agricultural pests. Such situations strongly necessitate the development of new criteria for rapid and accurate identification of *Orius* species that can be applied in field research.

Recently, several researchers have analyzed nucleotide sequences of PCR-amplified mitochondrial and nuclear DNAs to infer phylogenetic relationships among *Orius* species[1,2]. However, there has not yet been any research performed to examine the usefulness of these sequences for species identification. In this study, we considered the PCR-RFLP-based method of *Orius* species identification. We firstly analyzed nucleotide sequences of the internal transcribed spacer 1 (ITS 1) of the nuclear ribosomal RNA gene (rDNA) using 5 Japanese species of predatory flower bugs, *Orius strigicollis*, *O. minutus*, *O. sauteri*, *O. nagaii*, and *O. tantillus*. Then, we compared

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the sequences to determine recognition sites of diagnostically informative restriction enzymes. In addition, we also analyzed a portion of the mitochondrial cytochrome oxidase subunit I gene (COI) using previously reported sequences of the 5 species[2]. PCR-RFLP analyses of 26 laboratory strains revealed that all the species could be clearly identified by the banding patterns generated using one of the two DNA regions and a small number of restriction enzymes.

Materials and methods

Materials used in this study are listed in Table 1. Total DNA was extracted from individual adults using a GenomicPrep™ Cells and Tissue DNA Isolation Kit according to the manufacturer's instructions (Amersham Pharmacia Biotech), with the exception that the RNase treatment was omitted. In addition to 90 individuals of freshly killed insects, 18 dried specimens stored at room conditions for one month (2–5 individuals /species) were used for DNA extraction. Nucleic acids obtained were dissolved in 50 μL (freshly killed insects) or 20 μL (dried specimens) of sterile distilled water and used as a template for the PCR.

ITS 1 and the flanking regions were amplified using PCR primers, 5'-ACCGCCCGCGCTACTACCGAT-3' and 5'-TGTTCATGTGTCCTGCAGTTCACA-3'. They were designed based on the similarity among the aligned homologous sequences of the 28S and 5.8S rDNAs reported for *Drosophila melanogaster* (M21017), *Aedes albopictus* (L22060) and several other insect species. The amplification was carried out according to the method described in Muraji and Nakahara[3]. The PCR-amplified fragment was ligated into *Eco* RV site of the plasmid DNA, pBluescript II KS+ (Stratagene), using a DNA blunting kit (TAKARA BIO). The plasmid DNA was amplified and purified by usual methods[5], and used as a template for nucleotide sequencing using an ABI PRISM 377 DNA sequencer. The sequences obtained were submitted to DDBJ / EMBL / GenBank nucleotide sequence databases (Accession numbers; AB120700–AB120704).

Nucleotide sequences were aligned using a computer program Genetyx Mac version 10.1 (GENETYX Corp.) and then modified by visual manipulation. This program was also used to examine recognition sites of various restriction enzymes. For analyses of COI, nucleotide sequences were obtained from the nucleotide sequence databases. Accession numbers of the sequences were; AB021144, AB021145, AB021146, AB021147, and AB021148 [2].

For analyses of restriction fragment patterns, ITS 1 was amplified using PCR primers described above. A portion of COI was amplified using primers, 5'-AGCA GGAATTTCTTCAATTTT-3' and 5'-CTGTAAATAT-GTGATGTGCTC-3' [2]. The sequence of the PCR-amplified COI segment approximately corresponds to those used in the sequence analyses mentioned above. PCR products were treated with a restriction enzyme and electrophoresed on 2 or 1.7% MetaPhor™ agarose (BioWhittaker Molecular Applications) gel in 1 × TBE buffer at 7 V/cm for 2 h, then visualized by staining with ethidium bromide.

Table 1. Materials used in this study

Species and strains
Orius (Heterorius) minutus (Linnaeus)
Obihiro, Hokkaido Pref. (3)
Sapporo, Hokkaido Pref. (3)
Morioka, Iwate Pref. (6)
Iwaki, Fukushima Pref. (1)
Tsuchiura, Ibaraki Pref. (5)
Tsukuba, Ibaraki Pref. (3)
Kagoshima, Kagoshima Pref. (2)
Orius (Heterorius) strigicollis (Poppius)
Tsuchiura, Ibaraki Pref. (6)
Nagakute, Aichi Pref. (3)
Kurashiki, Okayama Pref. (7)
Nangoku, Kouchi Pref. (3)
Kagoshima, Kagoshima Pref. (7)
Naha, Okinawa Pref. (3)
Orius (Heterorius) sauteri (Poppius)
Obihiro, Hokkaido Pref. (3)
Sapporo, Hokkaido Pref. (3)
Hirosaki, Aomori Pref. (3)
Iwaki, Fukushima Pref. (2)
Tsuchiura, Ibaraki Pref. (9)
Tsukuba, Ibaraki Pref. (3)
Nagakute, Aichi Pref. (3)
Nangoku, Kouchi Pref. (3)
Orius (Heterorius) nagaii Yasunaga
Tsuchiura, Ibaraki Pref. (9)
Tsukuba, Ibaraki Pref. (3)
Nangoku, Kouchi Pref. (3)
Orius (Paraorius) tantillus (Motschulsky)
Naha, Okinawa Pref. (5)
Okinawa, Okinawa Pref. (7)

Numerals in parentheses indicate the number of individuals used in the PCR-RFLP analysis.

Results and discussion

1. Variation in the ITS 1 of the nuclear rDNA

Fig. 1 shows PCR products of ITS 1 amplified from representative insects of the 5 species. Our PCR primers successfully amplified DNA fragments from all the samples (n = 108) including those extracted from dried specimens. In Fig. 1, the length of amplified fragments varied among different species. However, no apparent intraspecies variation was observed among samples used in this study (data not shown). The lengths of PCR products determined by DNA sequencing were: *O. minutus*; 923 bp, *O. strigicollis*; 689 bp, *O. sauteri*; 667 bp, *O. nagaii*; 620 bp, and *O. tantillus*; 494 bp. In the analysis of nucleotide sequences, the computer program could not clearly align the sequences among different species, although the 5 species shared the same sequences in several portions. In the course of sequence alignment by visual manipulation, we found that the sequences contain various numbers of repetitive sequences (2 bp–222 bp / repeated unit) and that positions of several homologous sequences differed among different species (data not shown).

In Fig. 1, several species showed DNA bands of similar length, especially between *O. strigicollis* and *O. sauteri*. Such a difference could be easily ambiguous depending on the conditions of agarose gel electrophoresis and was not considered useful as a criterion for species identification. Therefore, we tried to make more discrete banding patterns by applying PCR-RFLP analysis. To do this, we examined the nucleotide sequences to detect positions of recognition sites of 50 different restriction enzymes and compared the estimated number and length of restriction fragments among different species. As a result, we found several restriction enzymes, *Hae* III, *Taq* I, and *Msp* I, that can express differences in nucleotide sequences as differences in the restriction fragment patterns among the 5 species (Table 2). Fig. 2 shows banding patterns of representative insects generated using the restriction enzyme *Hae* III. When com-

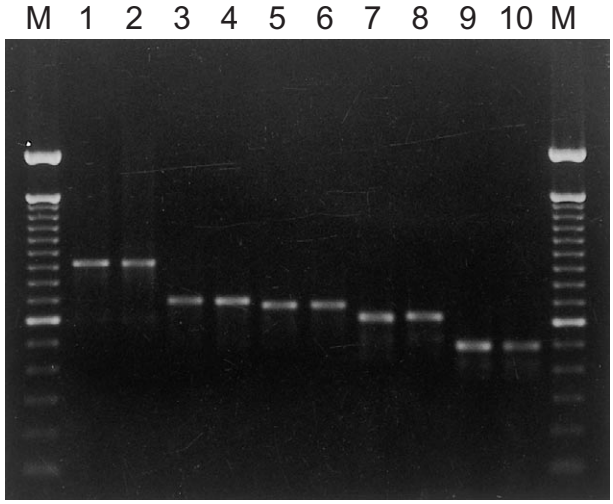

Fig. 1. PCR amplified fragments of ITS 1 obtained from Japanese species of *Orius* flower bugs
Lane 1, 2: *O. minutus*, Lane 3, 4: *O. strigicollis*, Lane 5, 6: *O. sauteri*, Lane 7, 8: *O. nagaii*, and Lane 9, 10: *O. tantillus*. Lane M: DNA size marker (100 bp ladder).

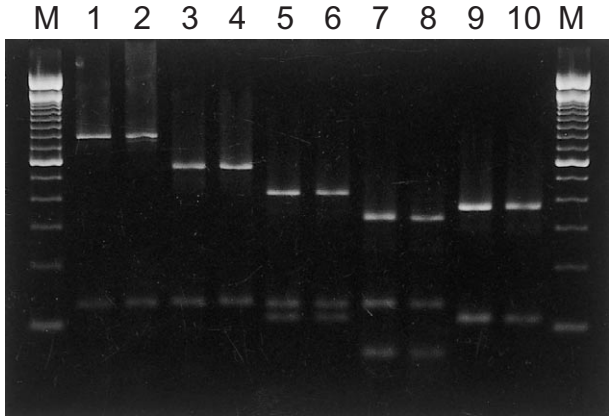

Fig. 2. Banding patterns of the PCR amplified ITS 1 fragments treated with the restriction enzyme *Hae* III
Lane 1, 2: *O. minutus*, Lane 3, 4: *O. strigicollis*, Lane 5, 6: *O. sauteri*, Lane 7, 8: *O. nagaii*, and Lane 9, 10: *O. tantillus*. Lane M: DNA size marker (100 bp ladder).

Table 2. Restriction fragment lengths of PCR amplified section of ITS 1 and COI treated with several restriction enzymes separately

Species	ITS 1			COI	
	Hae III	*Msp* I	*Taq* I	*Hin* fI	*Fok* I
O. minutus	780, 129, 14	393, 257, 225, 48	338, 225, 184, 120, 56	252, 176	181, 179, 68
O. strigicollis	544, 131, 14	379, 263, 47	443, 147, 56, 43	252, 176	249, 179
O. sauteri	417, 128, 108, 14	619, 48	611, 56	176, 168, 84	249, 179
O. nagaii	341, 130, 68, 67, 14	338, 234, 48	564, 56	428	249, 179
O. tantillus	369, 111, 14	270, 177, 47	437, 57	294, 134	249, 179

The lengths were determined based on nucleotide sequences obtained for each species.

pared with Fig. 1, banding patterns differed more discretely among different species. No apparent difference was observed in the number and length of restriction fragments among individuals within the same species (n = 108, data not shown).

2. PCR-RFLP of the mitochondrial COI

In the case of the mitochondrial COI gene, the length of PCR products did not vary among different species. The length of PCR products estimated from the nucleotide sequences was 428 bp. In the analysis of restriction fragments expected from the sequences, we found at least two restriction enzymes are needed to discriminate among all the species simultaneously (Table 2). Fig. 3 shows banding patterns of representative insects generated using the PCR products treated with enzymes *Hin* fI (a) and *Fok* I (b) separately. In this figure, 3 of the 5 species are identified by banding patterns treated with *Hin* fI (*O. sauteri*, *O. nagaii*, and *O. tantillus*), and the remaining 2 species are discriminated by analysis using *Fok* I (*O. strigicollis* and *O. minutus*). Among specimens used in this study (n = 108, including both freshly killed insects and dried specimens), no intraspecies variation was observed in banding patterns generated using these enzymes (data not shown). Results of species identification were consistent in all the specimens between analyses using the mitochondrial COI gene and ITS 1 of the nuclear rDNA, indicating that the two DNA regions are equally useful as criteria for species identification.

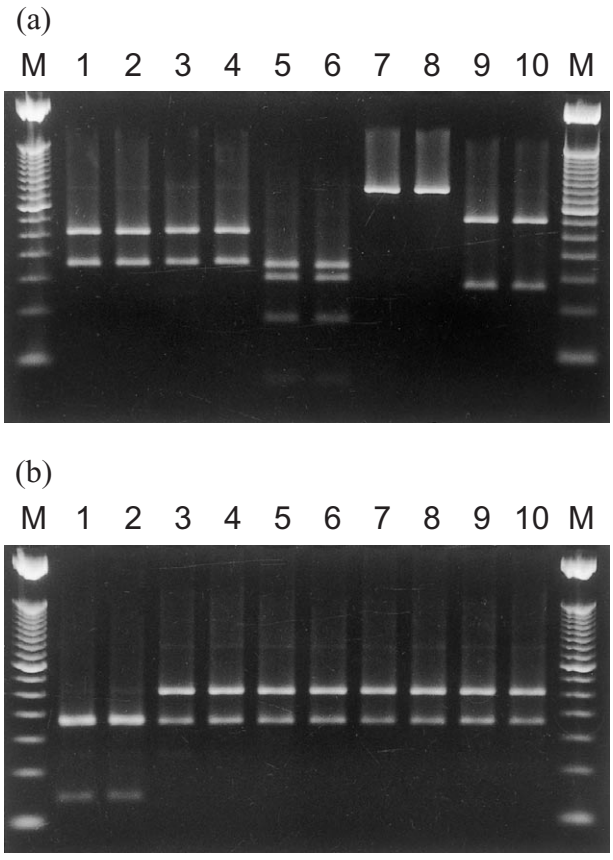

Fig. 3. Banding patterns of the PCR amplified section of the mitochondrial COI gene treated with *Hin* fI (a) and *Fok* I (b)

Lane 1, 2: *O. minutus*, 3, 4: *O. strigicollis*, 5, 6: *O. sauteri*, 7, 8: *O. nagaii*, 9, 10: *O. tantillus*, and M: 50 bp ladder.

3. Conclusion

In this study, we showed that the PCR-RFLP-based method is highly useful for the identification of Japanese *Orius* species. The method provided new criteria for identification of female adults and immature stages that could not be correctly identified by morphological characteristics. Because, in *Orius* species, both adult and nymphal stages are predators of agricultural insect pests, the method is especially useful for precise evaluation of nymphal stages of each species as biological control agents.

In the case of ecological studies of field populations, researchers frequently accumulate and store field-collected samples in a laboratory for several weeks before species identification. DNA samples extracted from such specimens have usually deteriorated and they are sometimes difficult to use as substrates for PCR-RFLP analysis. However, we confirmed that our method could successfully amplify both ITS 1 and COI sections from dried specimens stored at room conditions for one month. Thus, the method can be effectively used in analyses of field populations treating a large number of insects collected in the field and stored in a laboratory for long periods of time.

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