RES. BULL. PL. PROT. JAPAN No. 38: 73~80 (2002)

Short Communication

Identification of the *Bactrocera dorsalis* Complex (Diptera: Tephritidae) by PCR-RFLP Analysis

III. Discrimination between *B. philippinensis* and *B. occipitalis*

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Abstract: Applicability of the polymerase chain reaction fragment length polymorphism (PCR-RFLP) for discrimination between two Philippine species of fruit flies, *Bactrocera philippinensis* and *B. occipitalis*, was examined. To determine restriction enzymes that can be used in PCR-RFLP analysis, a 0.48 kb-long portion of the PCR amplified 16S rRNA gene of the mitochondrial DNA was sequenced. Comparison of the sequences suggested that the two species can be differentiated by electrophoresis patterns of the DNA fragment treated by the restriction enzyme *Hin*fI. In order to confirm the usefulness of the banding patterns for the species discrimination, relationships between banding patterns and previously proposed morphological diagnostic characters, such as lengths of male aedeagus, female aculeus, and CuA1 vein, were examined using a total of 130 individuals collected in the field and at quarantine inspection sitxes. In the case of male adults, PCR-RFLP patterns agreed very well with the result of species discrimination based on the aedeagus length. PCR-RFLP analyses also clearly divided female adults into two different groups of individuals. Such a grouping roughly agreed with that based on the aculeus/CuA1 value. However, because there were several individuals that showed intermediate values between the two groups, we could not confirm the accuracy of the species discrimination of female adults based on PCR-RFLP analyses. To do this, further analyses using mtDNA, nuclear DNA, and other morphological markers are needed.
Key words: *Bactrocera philippinensis*, *Bactrocera occipitalis*, PCR-RFLP, mitochondrial DNA, discrimination

Introduction

The *Bactrocera dorsalis* complex includes 66 species of fruit flies that are distributed mainly in the Asia and Pacific regions (WHITE and HANCOCK, 1997). Some of these species are known as pests of quarantine importance in the world and are also frequently intercepted at import quarantine sites in Japan. Because of their morphological similarity and individual variation in morphological diagnostic characters (DREW and HANCOCK, 1994), it is sometimes impossible to differentiate among species of this group by means of previously proposed diagnostic keys based on morphological characters (WHITE and HANCOCK, 1997). Recently, IWAIZUMI *et al.* (1997) indicated that the length of genital segments are different among closely related species, *B. dorsalis*, *B. papayae*, *B. carambolae*,

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Table 1. Samples used in this study

Locality	Sex	Number of individuals	Date of capture
The Philippines (New Tokyo International Airport)*	female	75	1998–1999
Davao City, The Philippines	male	57	December 2000

* The individuals were intercepted at New Tokyo International Airport, Chiba, Japan.

B. philippinensis and *B. occipitalis*. IWAHASHI (1999a, 1999b) also showed the length of male aedeagus can be used as a criterion for discrimination between *B. occipitalis* and *B. philippinensis* and between *B. carambolae* and *B. papayae*. However, the length of such a character varies considerably among individuals, and specimens frequently showed intermediate values between different species (KOGANEZAWA and IMAMURA, 2000; NAKAHARA *et al.*, 2001). In addition, such a criterion can not be applied to the species identification of immature stages that are frequently detected at quarantine inspection sites. In order to develop a more accurate and simple method of species identification of the *B. dorsalis* complex, we have been investigating DNA polymorphisms of the mitochondrial DNA (mtDNA) by restriction fragment length polymorphism in PCR amplified DNA fragment (PCR-RFLP). In the present study, we compared nucleotide sequences and PCR-RFLP patterns of a portion of the mitochondrial 16S rRNA gene (16S rDNA) between *B. philippinensis* and *B. occipitalis*, that are the most serious pest species in Philippines. Using a total of 122 individuals, the relationship between PCR-RFLP pattern and previously recognized morphological diagnostic characters were examined. Based on the results, applicability of the PCR-RFLP method for the species discrimination was discussed.

Materials and Methods

Specimens

Materials used in this study were listed in Table 1. These samples were discriminated by morphological diagnostic characters according to the key reported by WHITE and HANCOCK (1997). A total of 75 female adults were bred from fruits, Red mombin (*Spondias mombin*), Mango (*Mangifera indica*), and Guava (*Psidium guayaba*), that were imported from the Philippines and intercepted at New Tokyo International Airport. These flies were kept in a freezer temporarily, and then stored in 99.5% ethanol until DNA extraction. Fifty seven male flies were collected in the field at Davao city, the Philippines, using methyl eugenol traps. They were stored in 99.5% ethanol.

DNA extraction

DNA template used in nucleotide sequencing and PCR-RFLP analysis was extracted from individual insects using GenomicPrep Cell & Tissue DNA Isolation Kit (Amersham Pharmacia). Because male aedeagus, female aculeus and wings were used for measurement, the DNA was extracted from the remainder, mainly head and legs. Extracted DNA was dissolved in sterilized distilled water.

PCR

A portion of the mitochondrial 16S rDNA was amplified by PCR. The amplification was performed in 20 (for PCR-RFLP analysis) or 50 μl (for direct sequencing) volume containing 1×PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$), 2.5 mM each dNTPs, 2–40 $\mu g/ml$ DNA template, 10 mM each of primer, and 5 unit/μl of Taq DNA polymerase (Takara Shuzo). Two primers, MT342 (5'TAATCCAACATCGAGGTCGC 3') (NAKAHARA, unpublished) and MT34 (5'CGCCTGTTTAACAAAAACAT 3') (Muraji, unpublished) were used for amplification of the mitochondrial DNA fragment. Temperature cycling was carried out in an Astec program temp control system (Model PC-800). After an initial heating step at 92℃ for 2 min, samples were incubated 35 cycles of 90℃ for 30 sec, 47℃ for 30 sec, and 65℃ for 1.5 min, and then incubated at 65℃ for 5 min. Two micro litters of PCR products were electrophoresed on a 1.5% agarose gel and the size of amplified DNA fragment was examined.

Sequencing of the PCR product

PCR products obtained from several individuals collected at Davao city were purified using by GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia) and used as a template for nucleotide sequencing. The DNA was labeled with Thermo Sequenase Dye Terminator Cycle Sequencing Pre-mix Kit (Amersham Pharmacia) and electrophoresed on an Applied Biosystems 373S DNA sequencer (Applied Biosystems). In addition to MT342 and MT34, two primers, 16Fa (5'CCGGTTTGAACTCAGATCATGT 3') and 16Fb (5'TCCAACCGTTCATACCAGCCTTCA 3') (MURAJI *et al.*, 2000), were used for nucleotide sequencing. Nucleotide sequences obtained were aligned with those of previously reported *Bactrocera* species (Accession number AB035111, AB048741, AB048742 and AB 048743) (MURAJI and NAKAHARA, 2001) using a computer program GENETYX MAC V.10 (Software Development Co.) and recognition sites of restriction enzymes that can be used for species discrimination were examined.

Restriction analysis

For 132 individuals, electrophoresis patterns of PCR amplified fragment treated with the restriction enzyme *Hin*fI were examined. For each individual, 3 μl of PCR product was mixed with 0.5–1.0 μl of the restriction enzyme *Hin*fI, 1.0 μl of 10×buffer (Nippon Gene), and 5.5 μl sterilized distilled water in a reaction tube and then incubated at 37℃ for 2 hour. The digested DNA was resolved by electrophoresis using 3.0% agarose gel (MetaPhor ™ Agarose, FMC). A 100 bp-ladder (Gibco BRL) was used as a molecular size standard. Electrophoresed gel was stained with Ethidium Bromide (0.5 $\mu g/ml$) and banding patterns were photographed under Ultra Violet light (230 nm).

Measurement of morphological characters

The lengths of male aedeagus, female aculeus, and CuA1 vein were measured using insects whose mtDNA type was determined by PCR-RFLP analyses. To do this, male aedeagus and female aculeus were removed from insect body, put on the sticky tape, and the angle of these segments was adjusted for measurement under a digital microscope

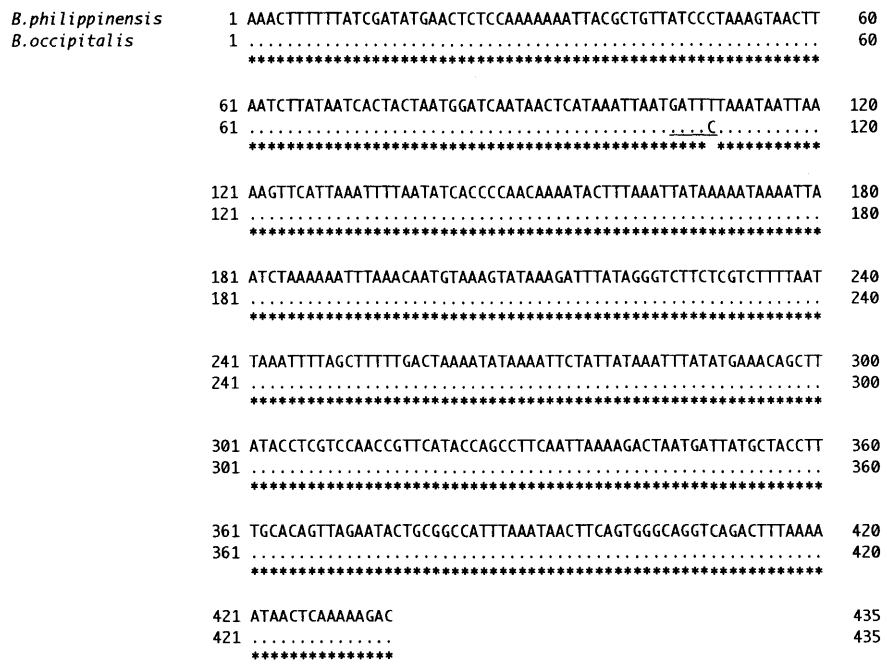

Fig. 1. Partial nucleotide sequences of 16S rDNA of *B. philippinensis* and *B. occipitalis*. Underlined part indicates the diagnostic sequences of *Hin*fI. Asterisk indicates bases identical to 2 inidividuals.

(Keyence).

Results and Discussion

DNA amplification and nucleotide sequencing

PCR primers, MT342 and MT34, successfully amplified a 0.48 kb-long fragment of the 16S rDNA from individual insects. Nucleotide sequences of 475 bp-long obtained in this study agreed well with previously reported sequences of *B. philippinensis* and *B. occipitalis* (Accession numbers of DNA data base Japan: AB035111 and AB048741) (MURAJI and NAKAHARA, 2001). Figure 1 presents a comparison of the sequences between *B. philippinensis* and *B. occipitalis*. This figure shows that the sequences were highly homologous (99.8%) between the species and differences were seen only in 1 nucleotide site. In the case of *B. occipitalis*, analysis of the sequence using a computer program GNETYX revealed that such a nucleotide site was included in the recognition sequence (GANTC) of the restriction enzyme *Hin*fI No such recognition sites were detected in the sequence of *B. philippinensis*. This phenomenon was confirmed in all of the sequences obtained in this and our previous studies (6 sequences in total).

RFLP analysis

Restriction fragment patterns generated using *Hin*fI were shown in Fig. 2. In this picture, two cleavage fragments (about 0.35 and 0.13 bp) were observed in *B. occipitalis*, whereas only a single band was observed in *B. philippinensis*. These results corresponded

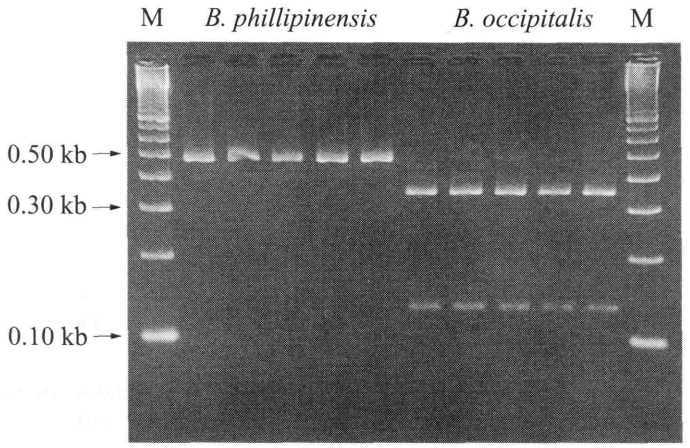

Fig. 2. *Hin*fI digests of PCR products from *B. philippinensis* and *B. occipitalis* on 3.0% Meta Phor agarose gel, M: 100 bp ladder.

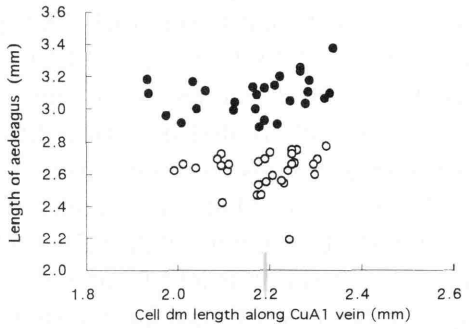

Fig. 3. Correlation of morphological characters with RFLP patterns in males trapped in Davao. Open and solid circles indicate each RFLP pattern, ○: *B. occipitalis*, ●: *B. philippinensis*.

to banding patterns estimated from the nucleotide sequences. As long as samples used in this picture, intraspecific variation was not found in each of two species. These results suggest PCR-RFLP patterns to be a useful marker for discrimination between *B. philippinensis* and *B. occipitalis*.

Correlation of morphological character and RFLP pattern

In order to confirm the usefulness of PCR-RFLP patterns for the species discrimination, we further analyzed relationship between banding patterns and previously proposed morphological diagnostic characters. To do this, both PCR-RFLP pattern and morphological characters, such as male aedeagus, female aculeus, and fore wing CuA1 vein, were measured using 122 flies. Results were summarized in Fig. 3 and 4. In the case of males trapped in Davao (Fig. 3), plots were clearly divided into two groups. Although values of morphological traits were overlapped between these groups (aedeagus; *B. philippinensis*: 2.89–3.38, 3.09±0.12, *B. occipitalis*: 2.19–2.77, 2.62±0.12. CuA1; *B. philippinensis*: 1.93–2.34, 2.17±0.12, *B. occipitalis*: 1.99–2.32, 2.18±0.09, range and mean±S.D., mm), such a grouping exactly agreed with those determined by PCR-RFLP patterns. In the case of females

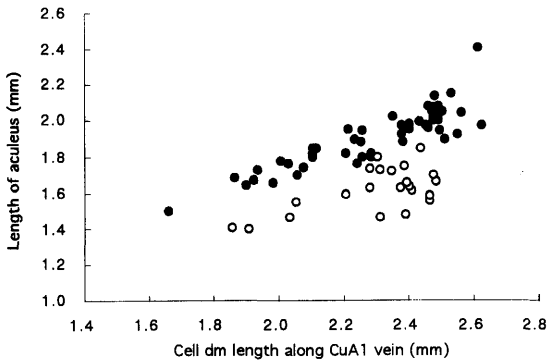

Fig. 4. Correlation of morphological characters with RFLP patterns in females intercepted at New Tokyo International Airport. Open and solid circles indicate each RFLP pattern, ○: *B. occipitalis*, ●: *B. philippinensis*.

intercepted at New Tokyo International Airport (Fig. 4), both aculeus and CuA1 lengths were largely overlapped between insects of different mtDNA types (aculeus; *B. philippinensis*: 1.50–2.41, 1.91±0.16, *B. occipitalis*: 1.40–1.84, 1.62±0.12. CuA1; *B. philippinensis*: 1.66–2.63, 2.3±0.22, *B. occipitalis*: 1.86–2.48, 2.3±0.18, range and mean±S.D., mm). Although plots seem to be distributed being subdivided into two different portions, there were several individuals that positioned at ranges between different mtDNA types. The values of aculeus/CuA1 ratio were also overlapped between females of different mtDNA types (*B. philippinensis*: 0.75–0.92, 0.83±0.04, *B. occipitalis*: 0.62–0.78, 0.71±0.05, range and mean± S.D.). In this study, we revealed that the PCR-RFLP can clearly separate Philippine fruit flies into two discrete groups. In the case of male adults, such groups coincide well with those separated by the length of aedeagus which is known as a morphological criterion that discriminate between *B. philippinensis* and *B. occipitalis* (IWAIZUMI *et al.*, 1997; IWAHASHI, 1999b). Thus, the PCR-RFLP of the mtDNA fragment is confirmed to be a useful marker to discriminate between the Philippine species. In the preliminary experiments, the PCR primers effectively amplified the mtDNA fragment from immature stages such as eggs, larvae, and pupae. Therefore, the species discrimination method proposed in this study can be applied wide variety of samples collected in the field and at quarantine inspection sites.

On the other hand, the morphological characters did not separate female adults into two groups, although PCR-RFLP analyses clearly divided them into two groups of different mtDNA types (Fig. 4). Thus, the usefulness of the PCR-RFLP method could not be confirmed as regard with female adults. However, because results of both PCR-RFLP and morphological analyses roughly agreed with each other except in several insects that showed intermediate values of morphological traits, PCR-RFLP analysis can be applied for practical use. In order to test whether PCR-RFLP method can correctly discriminate between the two species even in the case of females showing intermediate morphological traits, it is needed to perform detailed analyses using morphological characters other than aculeus and fore wing CuA1 vein. For several species of the *B. dorsalis* complex, it was reported that heterospecific matings occur under laboratory condition (IWAIZUMI *et al.*,

1997). It may be possible that females of intermediate morphological traits are due to hybridization between the two species. The method proposed in this study can also be used to clarify such a phenomenon under the field conditions when used with genomic DNA markers such as RAPD-PCR (SCHNELL *et al.*, 1996), microsatellite DNA (KINNEAR *et al.*, 1998), and the introns of the actin gene (HE and HAYMER, 1997).

Acknowledgments

We thank Mr. I. MIYAZAKI of Naha Plant Protection Station and colleagues of Narita Branch of Yokohama Plant Protection Station for providing materials used in this study. We also thank Mr. R. IWAIZUMI and Mr. K. OMURA of Yokohama Plant Protection Station for useful suggestions.

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和 文 摘 要

PCR-RFLP によるミカンコミバエ種群 *Bactrocera dorsalis* Complex (Diptera: Tephritidae) の識別

III. *B. philippinensis* と *B. occipitalis* の識別

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フィリピンに分布するミカンコミバエ種群種 *Bactrocera philippinensis*, *B. occipitalis* について PCR-RFLP による種識別の可能性を調査した。はじめに，PCR-RFLP に利用可能な制限酵素を決定するため，2種ミバエのミトコンドリア DNA 16S rRNA 遺伝子約 0.48 kb を増幅し，塩基配列の比較を試みた。次に，2種を識別できる可能性が示唆された制限酵素 *Hin*fI のバンドパターンの有効性を確認するため，フィリピン共和国ダバオで捕獲された個体及び検疫現場で輸入禁止生果実から発見された合計 130 個体の形態的特徴（オスのペニス長，メスの産卵管長及び前翅 CuA1 脈長）とバンドパターンの関係を調査した。その結果，ダバオで捕獲されたオスのバンドパターンによる識別結果は，ペニス長による結果と一致した。また，メスでは2種の中間的な数値を示す一部の個体で PCR-RFLP による識別の正確性が確認できなかったが，基本的にバンドパターンと産卵管長/CuA1 脈長による識別結果は一致した。

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