REAL-TIME QUALITATIVE PCR FOR THE INSPECTION AND IDENTIFICATION OF BACTROCERA PHILIPPINENSIS AND BACTROCERA OCCIPITALIS (DIPTERA: TEPHRITIDAE) USING SYBR GREEN ASSAY

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ABSTRACT. – The Tephritidae fruit fly pests, Bactrocera philippinensis (Drew and Hancock) and B. occipitalis (Bezzi), are two sympatric species of the fifty-two B. dorsalis complex with significant quarantine importance in Asia (Drew & Hancock 1994; Norrbom et al., 1998). A 1356 base pairs of DNA fragment from the mitochondrial Cytochrome Oxidase I (COI) genes of B. philippinensis and B. occipitalis was sequenced and analyzed, respectively. The similarity of COI gene between these two species is 96.8%. Two sets of species-specific primers were designed for polymerase chain reaction (PCR). A technique of the combination of real-time qualitative PCR using SYBR Green assay with melting curve analysis is developed to detect and identify these two Bactrocera sympatric species. This assay exhibited high specificity, reliability, as well as its high speed, low cost and the possibility of automating. Thus SYBR Green real-time PCR can be used as a rapid and specific technique in species identification of quarantine pests.

KEY WORDS. – Bactrocera philippinensis, B. occipitalis, real-time PCR, identification, SYBR Green, melting curve.

INTRODUCTION

Bactrocera philippinensis (Drew and Hancock) and B. occipitalis (Bezzi) are two sympatric species of the fifty-two B. dorsalis complex with significant quarantine importance in Asia (Drew & Hancock 1994; Norrbom et al., 1998). Several morphological characters, the pattern of black bands of the abdomen and costal band of the wings, are usually used to distinguish these two sympatric species (Drew & Hancock, 1994). However these taxonomic characters are not always stable between the two species. Reproductive isolation is one of the key characters to identification of insect on species level, but it may sometimes be caused by adaptation to host plants in the genus Bactrocera fruit flies (Jamnonglik et al., 2003).

A scoring method using six characters of the males to separate those species was developed by White & Hancock (1997). Another method based on adeagal length was introduced to distinguish B. philippinensis and B. occipitalis (Iwaiizumi et al., 1997; Iwahashi 1999). These two methods are based on the characters of male adults. However, species identification of immature life stage cannot be achieved by morphological taxonomy. It usually requires to rear individuals until adult emergence (Muraji & Nakahara 2002), which is time consuming and not advantageous for fruit trade (Armstrong et al., 1997). Thus, the procedure of rapid identification of immature life stages at species level is important for quarantine services. Recently, several molecular techniques have been used to identify species in Tephritids. The AFLP technique was employed to identify and isolate species-specific markers in Ceratitis capitata and C. rosa (Kakouli-Duarte et al., 2001). PCR-RFLP analysis was introduced to discriminate species in tephritids based on using ribosomal or mitochondrial DNA (Nakahara et al., 2003; Armstrong & Cameron, 2000). However, Armstrong et al. (1997, 2000) did not analyze the fruit fly species of B. philippinensis and B. occipitalis. Four restriction enzymes have to be used to distinguish two species in Muraji and Nakahara’s result. The use of real-time PCR has not been reported in the field of tephritid fruit flies pest rapid identification for quarantine purposes.

Bactrocera philippinensis and B. occipitalis are mainly distributed in the Philippines and probably in Southeast Asia (Hardy 1974). Recently, these two species have been recorded
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in Palau [=island]. They are likely to have wide host ranges, similar to the oriental fruit fly B. dorsalis. Therefore, they are potentially serious pest species (Plant Protection Service Secretariat of the Pacific Community, 2001). For more than hundreds of times, the two sympatric species have been intercepted at quarantine inspection sites of Southern China every year. Therefore, it is important to develop rapid and precise assay to identify the species.

In this study, a real-time quantitative PCR assay using SYBR Green dye I was firstly introduced to identify these two economic Bactrocera species: B. philippinensis and B. occipitalis. Two sets of species-specific primers were designed based on mitochondrial Cytochrome Oxidase I (COI) genes. A technique of the combination of real-time PCR using SYBR Green assay with melting curve analysis was developed and successfully identified these two Bactrocera sympatric species.

MATERIAL AND METHODS

Insect species and cultivation conditions
Bactrocera philippinensis and B. occipitalis were collected and reared from imported Philippine mango (Mangifera indica) at the plant quarantine laboratory of Shenzhen Entry-Exit Inspection & Quarantine Bureau, Shenzhen, China. Voucher specimens were stored at 4°C in 100% ethanol until required for molecular analysis.

Genomic DNA preparation
Template DNA for sequencing and PCR analysis was extracted with whole body of individual adults using Genomic DNA isolation and Purification Kit (Gene valley Inc., China). DNAs were purified and stored at -20°C.

PCR amplification and sequencing
The sense primer Uea 7 and the antisense primer Uea 10 (Lunt et al. 1996) were used to both PCR amplification and sequencing. PCR amplification was performed in 25 µl volumes: 1 µl (20 ng) DNA template, 15.3 µl of PCR buffer, 2.5 µl MgCl2 (25 mM), 2.5 µl dNTP (2.5 mM each), 0.5 µl (10 mM) of forward and reverse primers, and 1 U Taq DNA polymerase (Takara, Dalian). PCR amplification was done with initial denaturation at 94°C for 5 min, followed by 35 cycles of 95°C for 40 s, 48°C for 30 s and 72°C for 1 min, and final extension step at 72°C for 10 min. 20 µl of PCR products were electrophoresed on a 1.5% agarose gel, and purified with DNA Fragment Quick Purification/Recover Kit (DingGuo Inc., China). Sequencing was performed using the ABI BigDye™ Terminator V3.0 Cycle Sequencing Ready Reaction Kit in 10 µl volume according to the manufacturer’s instructions. Sequencing was undertaken by an ABI 3100 DNA Sequencer (PE Applied Biosystems, USA) at technical centre of Shenzhen Entry-Exit Inspection & Quarantine Bureau, Shenzhen, China. Both strands of the DNA fragments were sequenced. Two 678bp lengths mitochondrial DNA fragments of COI gene of B. philippinensis and B. occipitalis were sequenced and deposited in GenBank under the accession numbers AY398754 and AY398755. The new sequences were initially aligned using segman program (DNASTAR Inc., USA) and manually adjusted as needed. Sequences alignment of COI gene of two sympatric species was performed with homologous sequences of other Bactrocera dorsalis complex species (formerly reported in GenBank) using the MegAlign software (DNASTAR Inc., USA). Two species-specific primer sets were designed manually to detect B. philippinensis and B. occipitalis. All oligonucleotide sequences (Table 1) were checked with DNAsis (version 2.5) for primer dime, and false sites, and primers were synthesized by Takara (Dalian) Corp.

Real-time qualitative PCR amplification
The ABI Prism 7700 Sequence Detection System (SDS) (PE Applied Biosystems, USA) was used for real-time PCR amplification and data collection. All reactions were carried out in a total volume of 20 µl. Each reaction mixture contained 10 µl 10 x SYBR Green buffer (ABI Applied Biosystems, UK), 0.25 µmol/L each primer, and 1 µl (= 20 ng) template DNA. The amplification protocol consists of an initial denaturation step at 50°C for 2 min, followed by 45 amplification cycles at 95°C for 10 s, 95°C for 15 s, 60°C for 1 min.

Melting curve analysis (MCA)
Melting Curve Analysis (MCA) was used to check for the presence of nonspecific products. Another Melting Allias program should be performed to analyze the melting temperature curves after a completed PCR on ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, USA). The temperature profile is 95°C for 15 s; 60°C for 20 s, a linear temperature transition to 95°C within 19 min 59 s (rates of 0.2°C / s), and final holding 15 s at 95°C.

Post-PCR agarose gels electrophoresis analysis (AGE)
After real-time SYBR Green PCR amplification and melting curve analysis, PCR products were also analyzed by agarose gels electrophoresis to ensure that products of the correct size were amplified. 5 µl of PCR product were run in a 1.5% agarose gel containing ethidium bromide and visualized over UV.

Data evaluation
In SYBR Green PCR amplification, raw data were analyzed using the SDS software (Version. 1.7) of the Sequence Detection System. A normalized report (Rn) value was obtained for each reaction tube and ΔRn, an indication of the magnitude of signal generated by the PCR, was calculated. Samples were considered positive when the change in the fluorescence of SYBR Green relative to that of ROX (ΔRn) exceeds an arbitrary threshold value (Weller et al., 2000). The first PCR cycle showing a statistically significant increase in ΔRn was defined as the threshold cycle (CT). Data were exported into a Microsoft Excel Worksheet for amplification plots analysis (ΔRn vs. cycle number).

In melting curve analysis, fluorescence vs temperature plots were converted into derivative fluorescence curves with
RESULTS

Characterization of the nucleotide

A total of 1356 aligned bases of DNA sequence was obtained from partial of the mitochondrial Cytochrome Oxidase I genes of B. philippinensis and B. occipitalis which was originated from Philippine mango fruit. The two COI gene fragment sequences were presented in Fig. 1. Similar to the other dipteran taxa, nucleotide composition of these sequences was biased for A and T, and the proportions of A+T/G+C content of B. philippinensis and B. occipitalis are 65.7/34.3 and 65.3/34.6, respectively. Among the aligned sites, 42 nucleotides substitutions occurred between B. philippinensis and B. occipitalis. All substitutions are the G↔C transition and transversion was not found. The similarity of these sequences between B. philippinensis and B. occipitalis, utilizing either the PHIL-F, PHIL-R and OCCI-R primer oligonucleotides are mismatch artificially.

SYBR Green real-time qualitative PCR

SYBR Green chemistry assay was developed for detecting B. philippinensis and B. occipitalis, utilizing either the philippinensis-specific primer set PHIL-F/PHIL-R or the occipitalis-specific primer pair OCCI-F/OCCI-R were designed specifically for B. occipitalis and the amplicons were 224bp and 186bp, respectively. Primer design incorporated differences at the first and the fourth nucleotide position at the 3’ end of the oligonucleotide. In order to use this strategy, the fourth nucleotide at 3’ end of PHIL-F, PHIL-R and OCCI-R primer oligonucleotides are mismatched artificially.

Table 1. Oligonucleotide primers used for real-time polymerase chain reaction (PCR) amplifications

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5'-3'</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHIL-F</td>
<td>TGGATCCCCCCTATTATCTGGA</td>
<td>mismatch</td>
</tr>
<tr>
<td>PHIL-R</td>
<td>GAATAGGAGAAATATAATTCTGA</td>
<td>mismatch</td>
</tr>
<tr>
<td>OCCI-F</td>
<td>CCCTAAAAATGATTTAAAAAGTCTGTT</td>
<td>origin sequence</td>
</tr>
<tr>
<td>OCCI-R</td>
<td>GAATAGGAGAAATAGATACCC</td>
<td>mismatch</td>
</tr>
</tbody>
</table>

* F: forward primer; R: reverse primer.
* b the 4th base of the primer 3’ modified.

SYBR Green real-time qualitative PCR exhibited similar results to the philippinensis-specific SYBR Green assay. The C_T values of two B. occipitalis genomic DNA samples were at the 25th and 27th cycle steps, respectively (Fig. 3a).

PCR products melting curves and gel electrophoresis analysis

The real-time PCR product melting curves analysis showed that single large product peak was present at two independent samples with their species-specific primer sets (Fig. 2b, and Fig. 3b), whereas the negative and no DNA control showed either no product peak (Fig. 2b) or several broad peaks with substantially lower melting temperatures (Fig. 3b). The two specific products have sharp melting curves at 74.4°C for B. philippinensis and at 75.6°C for B. occipitalis. The PCR products were corresponded well with the amplicons of expected size 186bp for B. philippinensis and 224bp for B. occipitalis after 1.5% agarose gel electrophoresis (Fig. 2c and Fig. 3c).

DISCUSSION

Insect mitochondrial Cytochrome Oxidase I (COI) gene is a popular molecular marker for evolutionary studies (Zhang & Hewitt, 1996). The region amplified with primer pairs UEA7 and UEA10 is more variable and suitable for lower-level analysis, i.e., intraspecific variation, and the phylogenetics of closely related species (Lunt et al., 1996). The similarity between B. philippinensis and B. occipitalis is 96.8% within this COI gene. The genetic variability of COI gene between B. philippinensis and the other Bactrocera species, B. dorsalis, B. papayae, B. carambolae, B. latifrons and B. correcta, is 84.5%-98.7%, and B. occipitalis is 84.2%-97.3% (data not shown).

Species-specific primers set is the most important factor for successful amplification in SYBR Green real-time PCR. In this study two Bactrocera species-specific primer pairs are obtained based on COI gene. The primer sets can specifically discriminate B. philippinensis from B. occipitalis using SYBR Green real-time PCR (Fig. 2a and Fig. 3a). And two expected amplified 224bp and 186bp specific targets were clearly showed after analyzing the PCR products on an agarose gel (Fig. 2c and Fig. 3c). The individuals of B. philippinensis and B. occipitalis from different populations and several other Bactrocera taxa, such as B. dorsalis, B. papayae, B. carambolae, B. latifrons and B. correcta, are applied to test the specificity of the primer sets. And the results show they
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are only perfectly species-specific to those two sympatric fruit flies, although they are originated from different populations (data not shown). Even then more specimens from different localities should be considered to check the specificity of the two primer sets further because the variability of COI gene within B. philippinensis and B. occipitalis are still unknown.

In SYBR Green real-time PCR, fluorescence is monitored once for each cycle after product extension (Ririe et al., 1997). The initial template concentration is the key factor to Cycle threshold (Ct) value of amplification plot. An increase of DNA concentration of 10-fold corresponded to a decrease in Ct values of 2-3 cycles (unpublished data). However we only consider the technique of SYBR Green I assay real-time PCR to discriminate insect species qualitatively. Therefore, the template concentration is less important in qualitative real-time PCR. The variety of Ct value data in Fig.2a and Fig.3a does not show the specificity and efficiency of amplification.

PCR products can be distinguished by their melting curves respectively. The melting curve of a product can be obtained during PCR by monitoring the fluorescence of dsDNA dyes as the temperature passes through the product denaturation temperature. The melting curve is dependent on G+C content, length, and sequence of the target fragment (Ririe et al., 1997). In this study, the difference of G+C content between B. philippinensis (34.6%) and B. occipitalis (35.0%) is not significant, and the length of the target DNA fragment is 224bp and 186bp, respectively. However, the melting temperatures of the two targets have 1.2°C difference, and the PCR products can be used easily to distinguish B. philippinensis and B. occipitalis.

Fifty-two species are recorded in the B. dorsalis complex in Asia, and both B. philippinensis and B. occipitalis are

Fig. 1. DNA alignment of the portions COI gene for B. philippinensis and B. occipitalis. Nucleotidae differences between the two species are highlighted by shaded boxes. The location of PCR primers is depicted by open boxes. Bph : B. philippinensis; Boc: B. occipitalis.
considered economic importance among eight species within the complex (Drew & Hancock, 1994). It is important to accurately distinguish those species, which may seriously affect fruit trade worldwide, from low-risk species (Armstrong et al., 1997). The identification of Tephritidae fruit flies in quarantine practices is commonly based on their adult morphology (White & Elson-Harris, 1992). However, it is difficult to apply this traditional method in discrimination among several species of B. dorsalis complex, and it is also not satisfied for quarantine requirements of accurate and rapid identification. In order to resolve this problem, some molecular techniques, such as PCR-RFLP and AFLP, have been applied to discriminate fruit fly species based on different nucleotidae sequence markers (Armstrong et al., 1997, 2000; Muraji & Nakahara 2002; Nakahara et al., 2002). However, the results of these two methods are too complex to recognize because too many sites of restriction fragment are same at different species. Thus the molecular techniques referred above have not been widely applied in the practice of quarantine.

Compare with the techniques for species identification listed above, the advantage of SYBR Green I assay chemistry is that it could be used with any set of primers for any target and no probe is required, thus reducing assay setup and

![Fig. 2. Amplification of the COI gene of two sympatric Bactrocera species genomic DNA with B. philippinensis-specific primer set PHIL-F/PHIL-R by SYBR Green real-time qualitative PCR using ABI Prism 7700 Sequence Detection System. Amplification plot showing the change in fluorescence of SYBR Green I dye plotted versus cycle number (a). Two independent assay were analyzed using duplicated B. philippinensis DNA sample, NTC: No DNA Template, phil: B. philippinensis genomic DNA, occi: B. occipitalis genomic DNA. The melting curves (a graph showing the rate of change in fluorescence over time as a function of temperature for each sample) are shown for different amplification products (b). The same PCR products were subsequently analyzed on an agarose and clearly showed the amplified 224bp specific target (c). M: DL 2000 Marker, A and B: B. philippinensis genomic DNA sample, C: B. occipitalis genomic DNA sample, D: No DNA Template control.]

![Fig. 3. Amplification of the COI gene of two sympatric species genomic DNA with B. occipitalis-specific primer set OCCI-F/OCCI-R by SYBR Green real-time qualitative PCR using ABI Prism 7700 Sequence Detection System. Amplification plot showing the change in fluorescence of SYBR Green I dye plotted versus cycle number (a). Two independent assay were analyzed using duplicated B. occipitalis DNA sample, NTC: No DNA Template, occi: B. occipitalis genomic DNA, phil: B. philippinensis genomic DNA. The melting curves (a graph showing the rate of change in fluorescence over time as a function of temperature for each sample) are shown for different amplification products (b). The same PCR products were subsequently analyzed on an agarose and clearly showed the amplified 186bp specific target (c). M: DL 2000 Marker, A and B: B. occipitalis genomic DNA sample, C: B. philippinensis genomic DNA sample, D: No DNA Template control.]
running costs (Giulietti et al., 2001). The melting curve profile provide a good ideal to dissolve the disadvantage of SYBR Green dye to detect all dsDNA, including primer dimers and other undesired products. A single large product peak (Fig. 2b and Fig. 3b) was present with the specific PCR product, whereas the negative control shows no peak or several broad peaks with substantially lower melting temperatures. Therefore the melting curve analysis can successfully replace post PCR agarose gel analysis and eliminate the need of this further time consuming step. The other advantage of SYBR Green real-time PCR is the reliability, in which only the suitable molecular marker and species-specific primers were needed.

The technique of SYBR Green real-time qualitative PCR combining melting curve analysis is a new method to discriminate the sympatric species, which overcomes the strict of taxonomic characters and experiences by morphological species identification. It is very convenient for rapid quarantine practices, and suitable for detection the immature life stages of fruit fly, such as larvae, pupae and eggs. Given the specificity and reliability of the assay, combined with high speed, low cost and the possibility of automating, SYBR Green real-time PCR can be used as a rapid technique for pest species identification in plant quarantine.

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LITERATURE CITED


