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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 52 *B. dorsalis* complex with significant quarantine importance in Southeast Asia. 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* (Iwaizumi 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

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 amplication was performed in 25µl volumes: 1µl (≈ 20ng) DNA template, 15.3µl ddH_2O, 2.5µl 10 x PCR buffer, 2.5µl $MgCl_2$ (25mM), 2.5µl dNTP (2.5mM each), 0.5µl (10mM)of forward and reverse primers, and 1U Taq DNA polymerase (Takara, Dalian). PCR amplication was done with initial denaturation at 94°C for 5min, followed by 35 cycles of 95°C for 40s, 48°C for 30s and 72°C for 1min, and final extension step at 72°C for 10min. 20µl of PCR products were electrophoresed on a 1.5% agarose gel. The band corresponding to the target PCR product was excised with a sterile razor blade, 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 10µl 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. phillipiensis* and *B. occipitalis* were sequenced and deposited in GenBank under the accession numbers of AY398754 and AY398755. The new sequences were initially aligned using seqman 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 20µl. Each reaction mixture contained 10µl 10 x SYBR Green buffer (ABI Applied Biosystems, UK), 0.25µmol/L each primer, and 1µl (≈ 20ng) template DNA. The amplification protocol consists of an initial denaturation step at 50°C for 2min, followed by 45 amplification cycles at 95°C for 10s, 95°C for 15s, 60°C for 1min.

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 15s; 60°C for 20s, a linear temperature transition to 95°C within 19 min 59s (rates of 0.2°C /s), and final holding 15s 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. 5µl 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

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

Name	Sequence 5'-3'	Origin
PHIL-F[a]	TGATACCCCCTATTTACTGGA	mismatch[b]
PHIL-R	GAATAGGAAGAATAAAATTCGTAA	mismatch
OCCI-F	CCCTAAATGATTAAAAAGTCAGTTC	origin sequence
OCCI-R	GAATAGGAAGAATAAGATACCC	mismatch

[a] F, forward primer; R, reverse primer.
[b] the 4th base of the primer 3' modified.

respect to temperature (-dF/dT), for easy visualization of "melting peaks" and the Tm of the PCR products (Lyon et al., 2001), the Dissociation Curve software (version 1.0) was used in this study.

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↔A or T↔C transition and transversion was not found. The similarity of these sequences between *B. philippinensis* and *B. occipitalis* was 96.8%. The mutation sites were selected for exploring real-time PCR primers. Two primer sets (PHIL-F/PHIL-R and OCCI-F/OCCI-R) were designed specifically for *B. philippinensis* and *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.

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. In SYBR Green real-time qualitative PCR, the amplification plots of two sympatric *Bactrocera* species clearly showed that the change of fluorescence of SYBR Green I dye plotted versus cycle number (Fig. 2a and Fig. 3a), and the ΔRn values were collected at the end of each amplification cycle. In the *philippinensis*-specific SYBR Green assay, two *B. philippinensis* genomic DNA samples began to cross the threshold at the 28th and 30th cycle steps respectively, whereas the samples of *B. occipitalis* genomic DNA and no DNA template did not cross until the end of amplification (Fig. 2a). The *occipitalis*-specific SYBR Green assay 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

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 (C_T) 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 C_T 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

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Bph : TACAGTTGGAATAGACGTTGATACTCGTGCCTATTTCACTTCAGCTACAATAATTATTGCGGTACCCACA : 70
Boc : TACAGTTGGAATAGACGTTGATACTCGTGCCTATTTCACTTCAGCTACAATAATTATTACGGTACCCACA : 70

Bph : GGTATTAAAATTTTTAGTTGACTGGCTACATTACACGGTACACAATTAAACTATTCACCAGCCATATTGT : 140
Boc : GGTATTAAAATTTTCAGTTGACTAGCTACATTACACGGTACACAATTAAACTATTCACCAGCTATATTAT : 140

Bph : GAGCCCTAGGGTTTGTATTCCTATTTACAGTGGGAGGATTAACAGGAGTAGTTCTTGCTAATTCATCTGT : 210
Boc : GAGCCCTAGGATTTGTATTCCTATTTACAGTAGGAGGATTAACAGGAGTAGTTCTTGCCAATTCATCTGT : 210

Bph : AGATATTATTCTTCATGACACATATTACGTAGTAGCTCATTTCCACTATGTATTATCAATAGGAGCAGTC : 280
Boc : AGATATTATTCTTCATGATACATATTATGTAGTAGCTCATTTCCACTATGTATTATCAATAGGAGCAGTC : 280

                                  —PHIL-F ——►
Bph : TTTGCTATTATAGCAGGATTCGTTCAC[TGATACCCCCTATTTACAGGA]CTAGTATTAAACCCTAAATGAT : 350

                                                          —OCCI-F
Boc : TTTGCTATTATAGCAGGATTCGTTCACTGATACCCCCTATTTACAGGGCTAGTATTAAA[CCCTAAATGAT] : 350

Bph : TAAAAAGTCAATTTATTATCATATTTATCGGAGTAAATTTAACCTTCTTCCCACAACACTTTTTAGGATT : 420

          ——►
Boc : [TAAAAAGTCAGTTC]ATTATCATATTTATCGGAGTAAATTTAACCTTCTTCCCACAGCACTTTTTAGGGTT : 420

Bph : AGCTGGTATACCTCGACGATATTCAGACTATCCAGATGCTTACACAACATGAAATGTAGTTTCTACTATT : 490
Boc : AGCTGGTATACCTCGACGATATTCAGACTATCCAGATGCTTACACAACATGAAATGTAGTTTCTACTATT : 490

                                  ◄——— PHIL-R ———
Bph : GGTTCATCTATTTCTTTA[TTAGGAATTTTATTCTTCCTATTC]ATCATTTGAGAAAGTTTAGTAACACAAC : 560

                                  ◄——— OCCI-R ———
Boc : GGTTCATCTATTTCTTTACT[GGGAATCTTATTCTTCCTATTC]ATCATTTGAGAAAGTTTAGTAACACAAC : 560

Bph : GACAAGTAATTTACCCTATACAGCTTAGTTCTTCAATTGAATGACTCCAAAATACCCCTCCAGCTGAACA : 630
Boc : GACAAGTAATTTACCCTATACAGCTCAGTTCTTCAATTGAATGACTTCAAAATACCCCTCCAGCTGAACA : 630

Bph : CAGTTATTCAGAACTACCTCTTTTAACTAATTATCTAATATGGCAGAT : 678
Boc : CAGTTGTTCAGAACTACCTCTTTTAACTAATTATCTAATATGGCAGAT : 678
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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 accurate 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

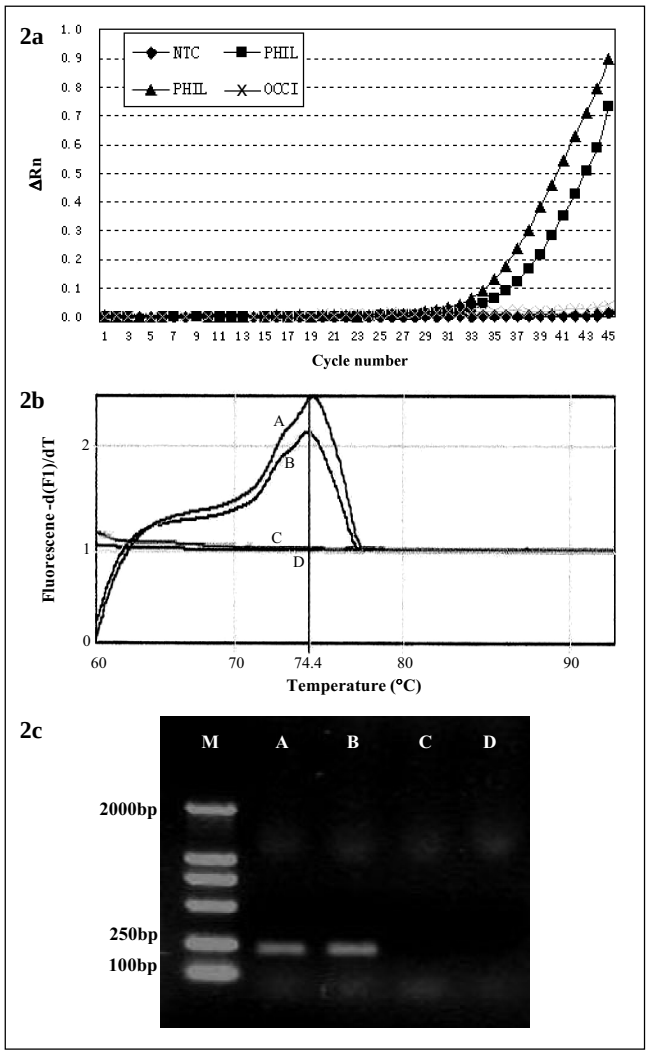

Fig. 2. Amplification of the COI gene of two symatric *Bactrocera* species genomic DNA with *B. philippinensis*–specific primer set PHIL-F/PHIL-R by SYBR Green real-time qualitative PCR using ABI Prism 7700 Sequence Detection System. Amplification plot showing the change in fluorescence of SYBR Green 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.

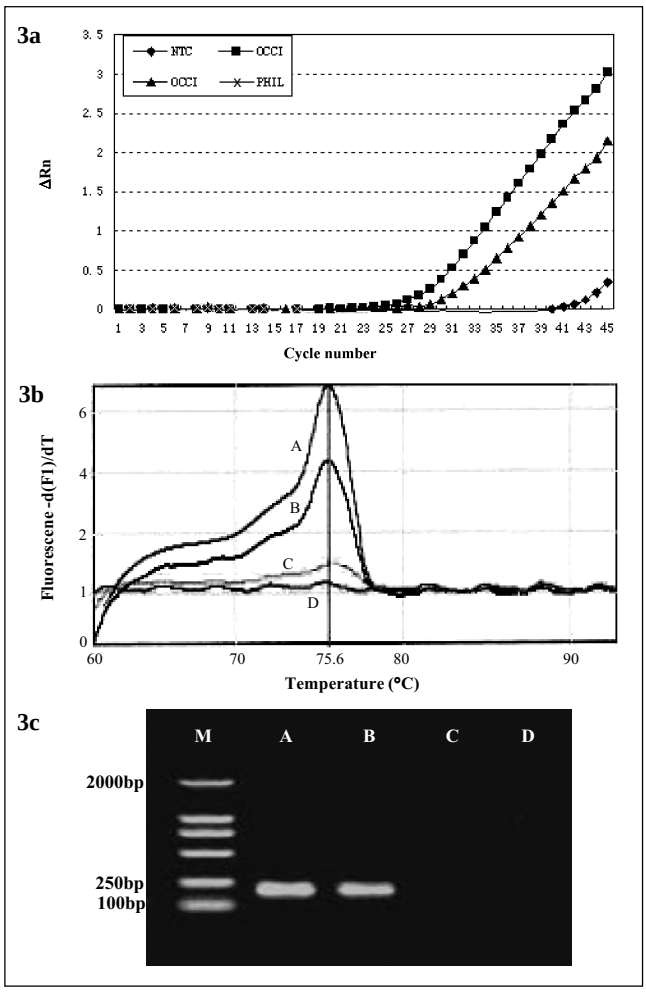

Fig. 3. Amplification of the COI gene of two sympatric species genomic DNA with *B. occipitalis*–specific primer set OCCI-F/OCCI-R by SYBR Green real-time qualitative PCR using ABI Prism 7700 Sequence Detection System. Amplification plot showing the change in fluorescence of SYBR Green I dye plotted 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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