

## RAPID DETECTION OF THE AFRICANIZED HONEY BEE: A TOOL FOR INDONESIAN ANIMAL QUARANTINE

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### ABSTRACT

Molecular detection methods were used to determine if Africanized Honey Bees (AHBs) are present in populations of imported *Apis mellifera* in Indonesia. The *cytochrome b* (*cyt b* gene) was amplified from mitochondrial DNA and digested with the *Bgl*II restriction enzyme (*cytb/Bgl*II). Two types of animal DNA extraction kits were used and found suitable for rapid preparation of DNA from *A. mellifera* by the Animal Quarantine facility. Results showed that all 94 colony samples from beekeepers in Java produced a 485 bp PCR product from the amplification of this gene. Two DNA fragments of 194 and 291 bp from all samples were produced after digestion with *Bgl*II. This *cytb/Bgl*II result together with the DNA sequence of *cyt b* showed that all collected samples of *A. mellifera* were the non-AHB type. Hence, this study did not detect AHB in Indonesia.

**Key words :** *Apis mellifera*, molecular detection methods, DNA, *cytochrome b*, mitochondrial genetics

### INTRODUCTION

*Apis mellifera* currently found in Australia, America, and Asia (included Indonesia) are imported from Europe, Africa and the Middle East (Ruttner 1988). The most favorable for beekeepers is the *A. mellifera ligustica* subspecies due to its tame behaviour and high honey production.

Besides *A. m. ligustica*, an aggressive African honey bee subspecies, namely *Apis m. scutellata* was imported to Brazil in the mid-1950s. Since the introduction of this African subspecies into Brazil, descendent of 'Africanized' honey bees (AHB) have spread throughout the Neotropics and into temperate North America (Schiff and

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Sheppard 1993). It inbred with the European Honey Bee (EHB = *A. m. ligustica* or *A. m. mellifera*) became a killer AHB characterized as having a deadly sting (Castro *et al.* 1994), a high wing beat frequency (Spangler 1994), sensitivity to *Varrhoo* parasitic mites (Guerra *et al.* 2000), and rapid colony multiplication. Schiff and Sheppard (1993) have surveyed 422 feral honey bee colonies from non-Africanized areas in the southern United States and it revealed that over 21% of them had mitochondrial DNA (mtDNA) derived from a European race established in North America in the 17th century, 77% of them had mtDNA common in honey bees maintained by beekeepers and about 1% exhibited African mtDNA. Further analysis revealed that the African mtDNA was derived from a North African subspecies imported to the US in the 19th century.

The AHB can be distinguished from the non-AHB bees based on the mitochondrial *cytochrome b* (*cyt b*) gene digested with *Bgl*II (*cyt b*/*Bgl*II). The AHB *cyt b* does not have a restriction site for *Bgl*II, while the non-AHB has one *Bgl*II restriction site. Therefore, the AHB type produces a single band of 495 bp, whereas the non-AHB PCR product produces a double band of 194 and 291 base pairs (bp) (Crozier *et al.* 1991). This method has been used by Australian bee keepers to detect the AHB imported to Australia. In the United States, *cyt b* has also been reported to detect the AHB (Pinto *et al.* 2003). However, currently the Indonesian Animal Quarantine Facility does not use a molecular detection method to recognize this AHB alien species. Hence, the aim of this study was to establish a rapid molecular detection method for detecting AHB status in Indonesia based on the *cyt b*/*Bgl*II. We used two DNA extraction kits designed for animal tissue for rapid DNA preparation from samples. This technique is appropriate for Animal Quarantine Officers to make a rapid and accurate decision on the presence of the AHB in imported colonies in Indonesia.

## MATERIALS AND METHODS

### *Apis mellifera* Collection

Imported *A. mellifera* were collected from apiaries located in Central Java (Pati, Kudus, Jepara) and in East Java (Jember, Malang, Kediri, Pasuruan), Indonesia. Bees were anesthetized and preserved in absolute ethanol.

### DNA extraction

Genomic DNA was extracted from the thorax of single bee using phenol–chloroform extraction and ethanol precipitation. In addition, we tested the *Genomic DNA Mini Kit* from Real Biotech Corporation (RBC) and the Genclean Column Genomic DNA Isolation Kit (GeneRay Biotech). For both kits, 20 mg of bee thorax was used for DNA extraction, and single thorax in a 1.5 ml microtube was immersed in liquid nitrogen for 15 min prior to being crushed using a grinder. Thereafter, the protocol according to the kit manufacturer was followed.

### *A. mellifera* DNA Amplification.

Part of the *cyt b* region was amplified using primers of *Cytochrome b* Forward: 5'-**tatgtactacctttgaggacaaat**atc-3' (11400); *Cytochrome b* Reverse 5'-

**attacacctcctaatttattaaggaat-3'** (11859) (Crozier *et al.* 1991). Numbers in brackets indicate the position of *A. mellifera* *cyt b* in the complete mtDNA (Accession number L06178). Cycle sequencing conditions protocol was 2 min. at 94 °C for initial denaturing, 35 cycles of 30 s at 94 °C, 30 s at 55°C and 1 min. at 72 °C for DNA elongation, followed by 10 min. for the DNA extension. DNA sequencing followed the ABI BigDye automated sequencing instruction from the supplier by using the same primers of as for DNA amplification.

### DNA Restriction Analysis

*Apis mellifera* *cyt b* PCR products were digested using *Bgl*II restriction enzyme. The digestion mixture contained the *cyt b* PCR product, dd H<sub>2</sub>O, Buffer D, *Bgl*II restriction enzyme and were incubated at 37°C overnight.

### DNA Alignment

We used CLUSTALX (Thompson *et al.* 1997) program to align the DNA sequence generated in this study with the published AHB haplotypes provided in Genbank database (<http://www.ncbi.nlm.nih.gov>; Accession Number EF016646 and EF016647).

## RESULTS AND DISCUSSIONS

### *Apis mellifera* *cyt b* Amplification and DNA Restriction Analysis

We collected samples from 94 colonies of imported *A. mellifera* from big and small apiaries in Central and East Java (data not shown). All samples from the 94 colonies produced the same size DNA band approximately 500 bp ( Figure 1). PCR products from 94 samples were digested with *Bgl*II and all produced two bands of approximately 200 and 300 bp (Figure 2).

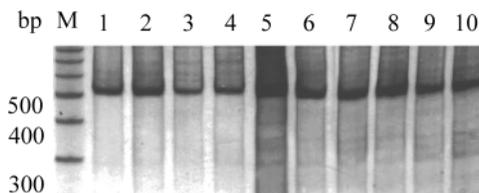


Figure 1. *Cyt b* PCR products from imported *A. mellifera* in Java, Indonesia; M = 100 bp DNA marker, 1-10 = *A. mellifera* colony sample number 1-10 from Java, Indonesia

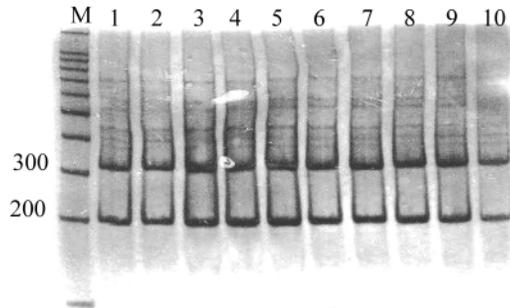


Figure 2 Cyt b/*Bgl*III restriction fragments of imported *A. mellifera* in Java, Indonesia; M = 100 bp DNA marker, 1-10 = *A. mellifera* colony sample number 1-10 from Java.

### *Apis mellifera* *cyt b* sequence data and alignment with AHB haplotypes

Since all *cyt b* PCR product of *Apis mellifera* gave the same result (Figure 1 and 2), we only sequenced one bee, i.e. *A. mellifera* from colony number 9 from Pati, Central Java (Am9PT). PCR product of Am9PT *cyt b* sequence gave a total length of 485 bp (Figure 3). The *Bgl*III sites (sequence **agatct**) digested the PCR product into two fragments of 194 and 291 bp (Figure 3) .

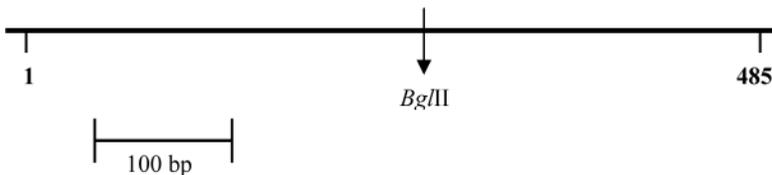


Figure 3. Restriction map of *A. mellifera* (Am9PT) *cyt b*/*Bgl*III. resulted for two DNA fragments of 194 and 291 bp

These results were in agreement with the data of Crozier (1991) from *A. mellifera* imported to Australia and data from the United States (Pinto *et al.* 2003) which have indicated that *cyt b* can be used to detect the AHB. A total of 451 colonies have been screened for the AHB characters in the USA. The United States Department of Agriculture, Animal and Plant Health Inspection Service, Plant Protection and Quarantine used trap lines and morphological identification techniques. Based on morphology, the AHB and EHB could not be distinguished by beekeepers (Montesinos 1995). Hence, this accurate molecular technique can help solve the problem of detecting the AHB. Africanized honey bees are one of the invasive alien species that are banned from entering all nations including Indonesia. Therefore, this effective system for warning about invasive alien species is needed, especially due to global trade.

### AHB and non-AHB *cyt b* Alignment

The DNA sequence alignment showed that the Am9PT *cyt b* sequenced in this study was exactly the same as that of Genbank Accession L06178; Crozier & Crozier 1993) denoted for *A. mellifera ligustica* (Figure 4). Hence, Am9PT from this

research was confirmed to be non-AHB bees. The primer amplified the partial *cyt b* gene started at nucleotide 396 of the complete *cyt b* gene sequence. The *Bgl*II site was located at nucleotide 694 based on whole sequence (Figure 4).

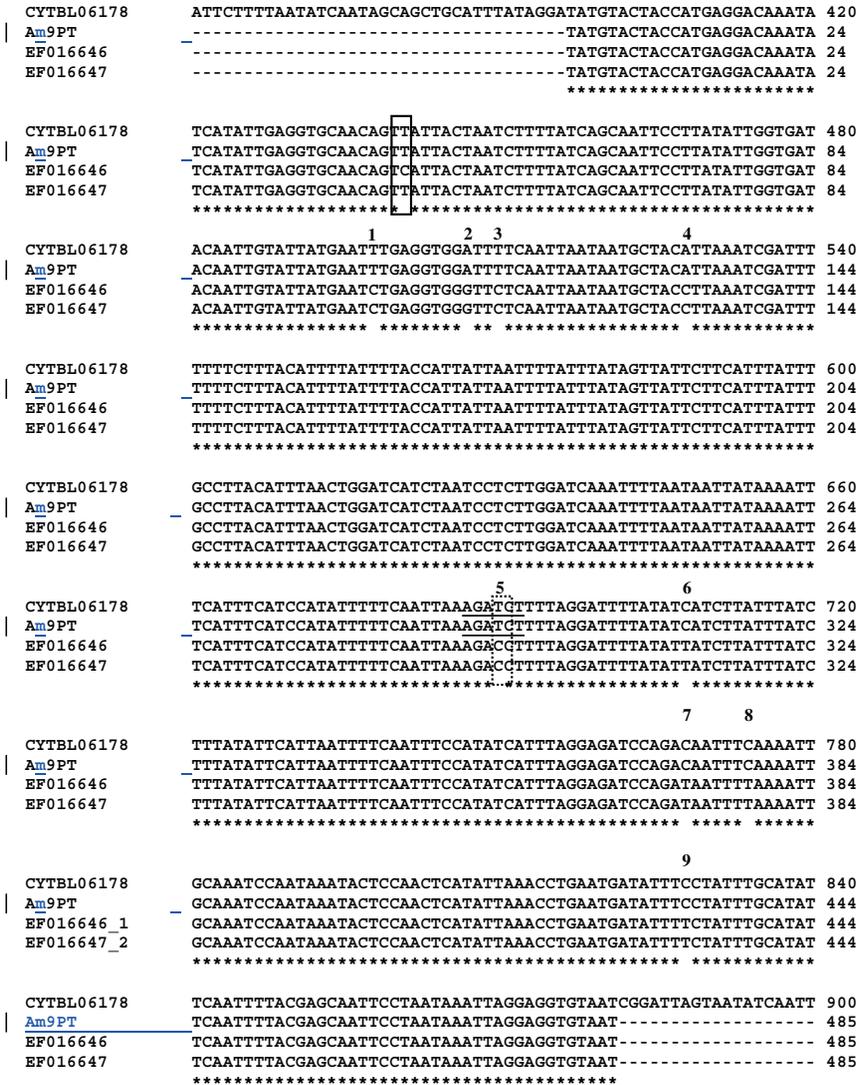


Figure 4. *A. mellifera* Am9PT from this study aligned with Genbank *cyt b* sequences CYTBL06178 (from *A. m. ligustica* Acc Num L06178), EF016646 (from AHB haplotype 1), and EF016647 (from AHB haplotype 2). \* = nucleotide homology. Underlined nucleotide = *Bgl*II sites. Solid boxed nucleotides = nucleotide differences between AHB-1 and AHB-2. Dashed boxed nucleotides = nucleotide change causing loss of *Bgl*II site in AHB type. Numbers positioned above the DNA alignment were all nucleotide differences between Am9PT, and AHB haplotype 1 and haplotype 2.

DNA alignment between Am9PT and AHB haplotype 1 and AHB haplotype 2 from GenBank (Accession numbers EF016647 and EF016647, respectively) showed nine nucleotide differences (Figure 4, see the numbers above the nucleotide alignment). The difference between the AHB and the non-AHB at base number 295 was shown in Figure 4, labelled mutation number 5. This is the position of the restriction site for *Bgl*III; it could be observed that the nucleotide “T” in CYTBL06178 and Am9PT were altered to be a “C” in both AHB haplotypes 1 and 2 (EF016646 and EF016647). This mutation removed the *Bgl*III restriction site, hence shows why a single band is seen in the AHB haplotypes. Based on homology analysis of the two AHBs, these two haplotypes also differ at nucleotide number 65 (Figure 4). The two AHB haplotypes obtained from Genbank were submitted on February 1, 2007 by Szalanski, A.L. and Mckern, J.A from the Department of Entomology, University of Arkansas, USA.

Based on the findings of this study, we suggest to carry out cooperation between the animal sections of the Agency of Agriculture Quarantine (Balai Karantina Pertanian) and the Apiaries Association in Indonesia. The cooperation could commence by performing a socialization of honey bee biology. Furthermore, the molecular detection for the AHB should be implemented at the animal quarantine in every province in Indonesia, particularly at Soekarno Hatta and Ngurah Rai airports, so that imported bees could be monitored.

## CONCLUSIONS

Molecular analysis of imported *A. mellifera* based on the *cytochrome b* gene from mitochondrial DNA digested with *Bgl*III restriction enzyme was conducted to determine whether the Africanized Honey Bee (AHB) is present in Indonesia. Out of 94 colony samples from 94 beekeepers in Java, all PCR products amplified from this gene were of the same size (485 bp). Two DNA fragments (194 and 291 bp) were produced after digested with *Bgl*III. This *cyt b*/*Bgl*III result showed that all collected samples of *A. mellifera* were of the non-AHB type. Hence, no AHB could be detected in Indonesia in this study. We found that two different DNA extraction kits could be successfully used to extract DNA for this analysis, allowing fast and effective preparation of DNA for subsequent PCR analysis.

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