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A Simple Technique Based on PCR to Distinguish Honeybee Species

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Abstract

Molecular tools such as polymerase chain reaction (PCR)-based techniques have been widely used to analyze the genetic diversity of many social insects. Since their society has a very complicated and hierarchical structure, honeybees (*Apis*) will become a good model to study the evolution of social insects. In this report, we demonstrated a simple PCR-restriction fragment length polymorphism (PCR-RFLP) to distinguish different *Apis* species. In brief, mitochondrial DNA (mtDNA) was separately isolated from workers of five major honeybee species, *Apis mellifera* (Western honeybee), *A. cerana* (Eastern honeybee), *A. dorsata* (Giant honeybee), *A. laboriosa* (Himalayan giant honeybee), and *A. florea* (Dwarf honeybee), obtained from different geographical regions. Western and Eastern honeybees, *A. dorsata* and *A. laboriosa*, and *A. florea* were collected from Japan, Nepal, and India, respectively. The PCR technique was performed to amplify a 528-bp DNA fragment of the NADH dehydrogenase subunit 4 (ND4 according to *A. mellifera*'s mtDNA sequence). Following digestion of the individual PCR products with two restriction endonucleases, *Nde*I and *Mbo*I, the digested DNA fragments were separated with agarose gel electrophoresis, stained with ethidium bromide, and visualized over UV light. The results clearly indicated that this PCR-RFLP procedure was simple but sensitive and reproducible for the identification of the genetic variability among *Apis* species. Therefore, this procedure will be useful to confirm other techniques for the identification of honeybees.

Key words: *Apis*, mtDNA, PCR-RFLP

Introduction

Many molecular techniques have been applied to study the genetic relatedness of honeybees and other living organisms. However, of these genetic methodologies, PCR-based techniques are preferably employed to investigate the biodiversity and phylogenetic relationships of honeybees, in particular when the complete sequence of mitochondria is published.[1] For example, PCR-RFLP and random amplified polymorphic DNA (RAPD) analysis are widely used to study the evolution lineage of the mitochondria of *A. mellifera*, *A. cerana*, *A. dorsata*, and *A. florea*.[2-8] Recently, De La Rua et al[9] have proven that this PCR

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amplification of mitochondrial DNA (mtDNA) with *Dra*I digestion grouped the cavity-nesting honeybee *A. cerana* from different islands in the Philippines into four different haplotypes. Similarly, Marina et al[4] found that the PCR amplification of mtDNA followed with the *Hpa*II and *Alu*I restriction could differentiate three different haplotypes of *A. mellifera* from Kenya.

In this report, we demonstrated that the PCR amplification of the *ND4* of mtDNA from five different honeybee species followed by *Mbo*I and *Nde*I double digestion analysis is simple but useful to genetically distinguish *Apis* species. This technique, therefore, is an alternative strategy or confirms other methods including a morphometric analysis[10] for the identification of honeybee species.

Materials and Methods

Samples

Honeybee workers of *Apis laboriosa, A. dorsata* and *A florea, A cerana* and *A. mellifera* were collected from Nepal, India, and Japan, respectively. Fig. 1 shows the morphology of the bee specimens. Prior to a DNA purification step, the bee samples were kept at −80℃.

mtDNA purification

Approximately 10 honeybee workers from individual colonies were used to purify mtDNA from individual species. The frozen thoracic and abdominal parts of workers were ground and the total DNA purification step was performed using DNA Extraction Kit (Stratagene®, USA). The DNA was further purified with phenol-chloroform-isoamyl alcohol, precipitated with isopropanol, and dissolved in Tris-EDTA buffer pH 7.4. For each species, the DNAs purified from each time were pooled together for PCR assay.

PCR-RFLP analysis of the ND4 coding genes

The PCR was performed with the primer sequences shown below:

primer	Sequence of primer	Annealing site
ND4-8824F	CCACGAAATTATCCCAATTAATAATATAACCTCGC	8842-8876
ND4-9369R	CATGGTTGGTTATTAAAAGCTCATGTTGATGCTCC	9335-9369

Note: *mtDNA sequence as recorded in Genbank, accession number L06178

The reaction volume was 25μl containing one unit of recombinant *Tth* polymerase (Biotools, Spain), approximately 1μg of total DNA. Generally, the reaction profile was 98℃ for 5 min for 1 cycle followed by 30 cycles of 96℃ for 30 sec, 51℃ for 1 min, 72℃ for 30 sec, and an extension at 72℃ for 4 min with a GeneAmp® PCR System 2400 thermocycler (Perkin-Elmer, USA). For RFLP analysis, 20μl aliquots of PCR products were digested with *Mbo*I and *Nde*I at 37℃ for 4 hr. The digested DNA fragments were separated on 1.5% agarose gel electro-

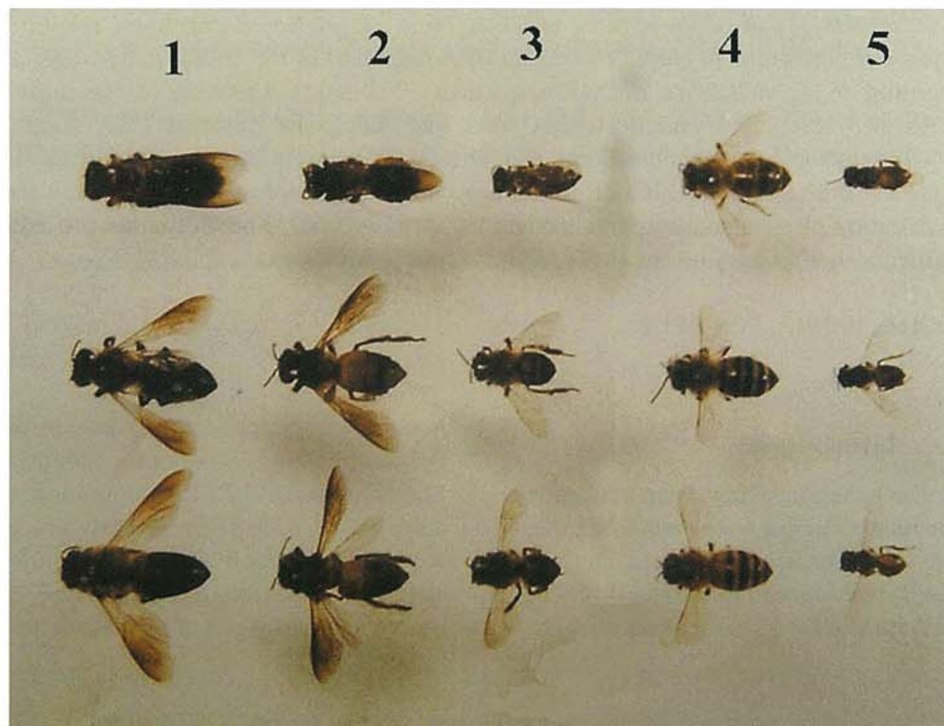

Fig. 1. The morphology of five different honeybee species: From left to right, *Apis laboriosa* (Himalayan giant honeybee, *A. dorsata* (Giant honeybee), *A. cerana* (Eastern honeybee), *A. mellifera* (Western honeybee), *A. florea* (Dwarf honeybee)

phoresis, stained with ethidium bromide, and visualized on UV light.

Results

We successfully amplified the 528-bp DNA fragment of the *ND4* of mtDNA from different *Apis*

Fig. 2a

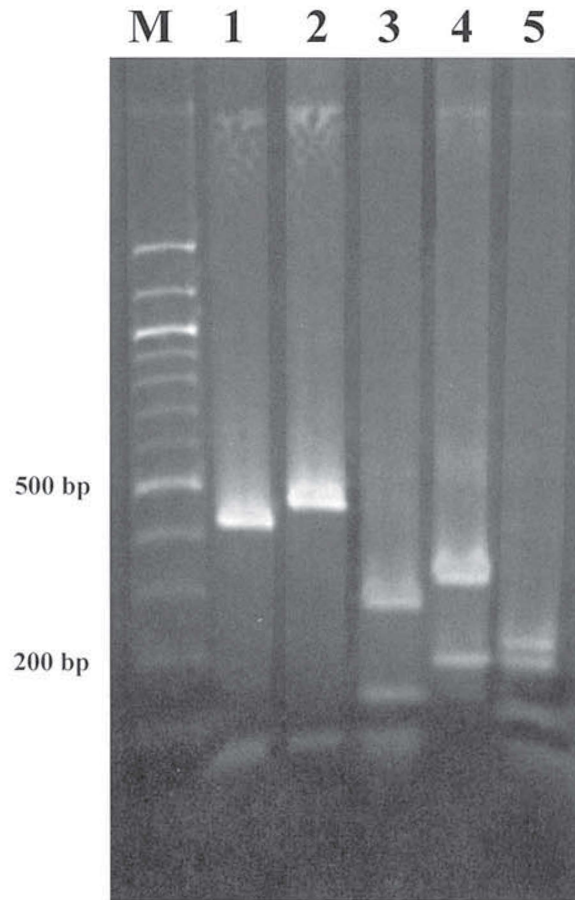

Fig. 2b

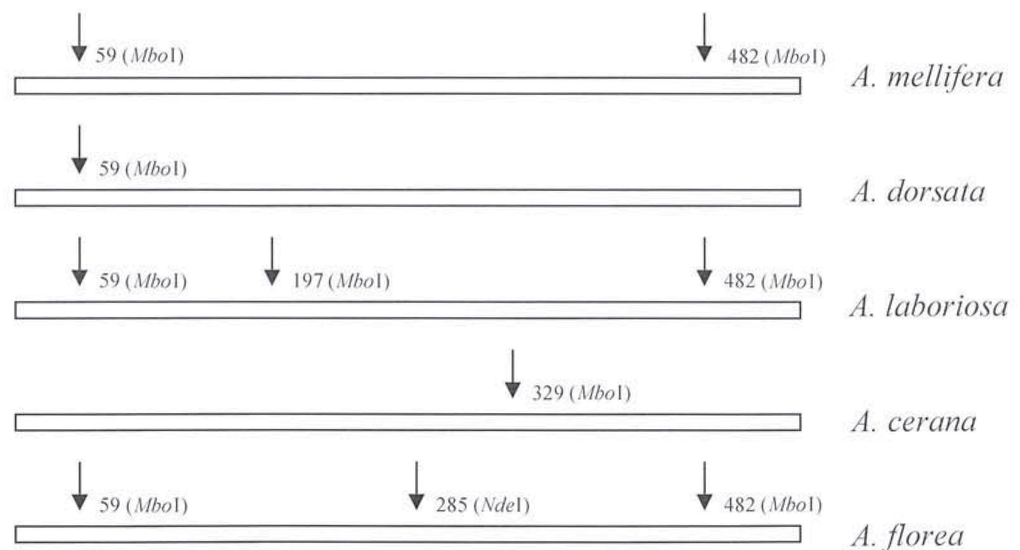

Fig. 2. PCR-RFLP analysis of mitochondrial DNA of honeybees

(a) The agarose gel electrophoresis of the *Nde*I-*Mbo*I digested amplification fragments of the *ND4* from honeybees: Lane M is 100-bp ladder; Lanes 1, 2, 3, 4 and 5 are the digested fragments from *A. mellifera*, *A. dorsata*, *A. laboriosa*, *A. cerana* and *A. florea*, respectively.

(b) The restriction maps of the 528 bases of the *ND4*

species including the rare species, *laboriosa*. Following the double endonuclease digestion of the PCR products with *Mbo*I and *Nde*I, the different restriction patterns were clearly seen with agarose gel electrophoresis analysis (Fig. 2a). The PCR-RFLP and subsequent sequence analysis (data not shown) confirmed that the digested PCR products from *A. mellifera*, *A. florea*, *A. laboriosa*, *A. dorsata*, and *A.cerana* contained three (423, 58, and 47 bp), five (226, 197, 100, 58, and 47 bp), four (285, 138, 58, and 47 bp), two (470 and 58 bp), and two fragments (328 and 200 bp), respectively. Fig. 2b shows the restriction maps of the 528 bp of the *ND4*.

Discussion

The mtDNA has been routinely used for phylogenic analysis of honeybees, because of its small size, a rapid rate of sequence divergence, and maternal inheritance. In mitochondria, the NADH dehydrogenase (NADH-Q reductase or Complex I), a large enzyme (880 kD) containing more than 34 polypeptides, is known to be involved in oxidative phosphorylation. We investigated the *ND4* rather than the intergenic region between the tRNAleu gene and the second subunit of the cytochrome oxidase gene (cytochrome oxidase II) previously reported,[6,11,12] since it is a functional gene coding an enzyme involving the electron transport system. Thus, the genetic sequence of the region must be constrained enough against evolutionary forces to maintain the enzymatic activity of its encoded protein, but still sufficiently varies from species to species.

As shown in Fig. 2, the PCR-amplification of the *ND4* of the mtDNA followed by the *Mbo*I-*Nde*I double digestion is a fast and reliable technique to distinguish honeybee species. Later, these PCR fragments were cloned and the DNA sequences were determined from each species (unpublished data). The results agreed with the restriction maps by the PCR-RFLP analysis. So, the amplification of ND4 with *Mbo*I-*Nde*I digestion is a quick and reliable method to identify honeybee species. However, in *A. florea* the addtional fragment of approximately 100 bases was not predicted, based on the sequencing data. We proposed that this unexpected DNA band from the PCR-RFLP technique resulted from the nucleotide mutations that giving unexpected restriction cleavage site(s). If the hypothesis is correct, it implies that in this *A. florea* there are at least two haplotypes in the population according to the genetic variation of the ND4. Nevertheless, genetic analysis from the larger sample of *A. florea* needs to be further investigated.

Acknowledgements

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PCR法を用いたミツバチの簡略な種判別法

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摘　要

社会性昆虫の遺伝的多様性の解析に，PCR法などの分子生物学的手法が幅広く用いられるようになってきている。社会性昆虫の中でも，ミツバチ(*Apis*属)は，複雑な階層構造をもつ社会を構成し，社会性昆虫の進化研究の適当なモデルとなりうる。本報では，様々なミツバチの種を判別するための簡略なPCR-RFLP法を開発し，その有用性を示した。ミツバチの主要な5種であるヨーロッパミツバチ(*A.mellifera*)，トウヨウミツバチ(*A.cerana*)，オオミツバチ(*A.dorsata*)，ヒマラヤオオミツバチ(*A.laboriosa*)，ヒメミツバチ(*A.florae*)の働きバチを様々な地域で得た。ヨーロッパオオミツバチ，ヒマラヤオオミツバチ，ヒメミツバチはそれぞれ日本，ネパール，インドで採集した。これら5種の働きバチからミトコンドリアDNAを分離し，ヨーロッパミツバチの配列からプライマーを作製しPCR法を用いてNADH脱水素酵素のサブユニット4(*ND4*)を増幅した。さらに，それぞれのPCR産物を2つの制限酵素*Nde*Iと*Mbo*Iで分解し，DNA断片をアガロースゲル電気泳動で分離して臭化エチジウムで染色した後，紫外線でバンドを検出し，DNA断片の長さから種判別を行なった。この開発されたPCR-RFLP法は簡略であるが，再現性があり，ミツバチ種の間の遺伝的な変異の検出に有効でることが明らかになった。したがって，この手法は他のミツバチ種の判別技術を補完するものとして有用であると考えられる。

キーワード：*Apis*, mtDNA, PCR-RFLP