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Genetic diversity among Bees as detected by Random Amplified Polymorphic DNA Markers

Isha Slathia, N. K. Tripathi and V. K. Gupta

1. Department of Zoology, University of Jammu, (J&K) India
2. Department of Entomology, Punjab Agriculture University, Ludhiana (PB), India

***Corresponding author**: Slathiai2015@gmail.com

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ABSTRACT

India is a land of vast physio-geographical variability, which has led to development and establishment of vast floral and faunal diversity currently existing in this sub-continent. India is declared as 3rd largest biodiversity in the world. India is regarded as the richest country in the world with respect to the economically important honey-bee species diversity. Due to their richness at species level, DNA based identification is useful from genetic point of view. Phylogeny analysis using Random Amplified Polymorphic DNA (RAPD) markers was performed for studying genetic variation in *Apis mellifera*, *Apis cerana* and *Apis dorsata* of family Apidae were investigated.

Key words: Bees, Biodiversity, Species, DNA, RAPD.

INTRODUCTION

We live in a world of insects with immense species diversity (Insects representing more than 80% of the species). Insects belong to class-Insecta of phylum Arthopoda and constitute the largest group in the animal kingdom. Among the insecta, the Hymenoptera is the 2nd largest order comprising of bees, wasps, sawflies, ants, horntails, chalcids. The name refers to the heavy wings of the insects, and is derived from the ancient Greek ὑμήν (Hymen): membrane and πτερόν (Pteron): wing, which means insects with membranous wings. The hind wings are connected to the forewings by a series of hooks called hamuli.

Hymenopterans are a diverse group of organisms and due to their richness at species level, DNA based identification is useful from genetic point of view. In earlier days morphometric methods were the important tools and being used to resolve systematic issues. The characterisation based on morphometric characters is not well suited for phylogeographical studies because they can be sensitive to environmental selection pressures, need a lot of timeand experience, and sometimes areunsuitable for identifying somehybrids. But now-a-days in the modern Hi-tech world, the field of molecular biology has been exploited greatly (Hawksworth 1994; Hoy 1994; Crampton et al. 1996; Roderick 1996; Karp et al. 1998; Kumar and Negi 2004). DNA analyses are techniques which are lately being used to understand and confirm insect (Mestriner 1969; Burns and Johnson 1971; Sylvester 1982; Berlocher 1984; Sheppard and Berlocher 1989). Misidentifications at the level of species and genera have therefore, created serious problems for researchers in the field of ecology, physiology and genetics for a comparison and evolution of their results. Thus molecular studies have therefore proved to be extremely useful for this purpose. So,

besides behavioral, morphological and cytogenetic evidence, molecular data provide strong support for phylogenetic relationships among insects.

Molecular methods have opened up a wide range of new approaches to invertebrate research, particularly with regard to molecular phylogenetic and taxonomic studies. The molecular characterization included the study of genetic diversity, genetic relatedness and phylogenetic analysis at species level. Current trends in the application ofDNA marker techniques in a diversityof insect ecological studies show that mitochondrial DNA (mtDNA), microsatellites, Random amplified polymorphic DNA (RAPD), expressed sequence tags (EST) and amplified fragment length polymorphism (AFLP) markers have contributed significantly to our understanding of the genetic basis of insects and honeybees diversity. Molecular markers are widely used in biology to address questions related to ecology, genetics and evolution. The recent characterization of genomes, completely or partially, and knowledge of the molecular basis of genetic variation have been very important sources for the development of markers and establishment of evolutionary models at the inter and intra-specific levels (Li 1997). In bees, molecular studies addressing those issues have focused on *Apis* and *Apis mellifera*.

RAPD stands for Randomly Amplified of Polymorphic DNA. It is a type of PCR reaction, but the segments of DNA that are amplified are random. The scientist performing RAPD creates several arbitrary, short primers (8-12 nucleotides), then proceeds with the PCR using a large template of genomic DNA, hoping that fragments will amplify. By resolving the resulting patterns, a semi-unique profile can be gleaned from a RAPD reaction. Randomly amplified polymorphic DNA markers (RAPD) are an important technique for genetic polymorphism and relatedness (Welsh and McClelland 1990). No knowledge of the DNA sequence for the targeted gene is required, as the primers will bind somewhere in the sequence, but it is not certain exactly where. This makes the method popular for comparing the DNA of biological systems that have not had the attention of the scientific community, or in a system in which relatively few DNA sequences are compared (It is not suitable for forming a DNA databank). Because it relies on a large, intact DNA template sequence, it has some limitations in the use of degraded DNA samples. Its resolving power is much lower than targeted, species specific DNA comparison methods, such as short tandem repeats. In recent years, RAPD has been used to characterize, and trace, the phylogeny of diverse plant and animal species. RAPD is a preliminary study for genetic polymorphism and largely been carried out, but now a days is less popular due to its poor reproducibility faint or fuzzy products and difficulty in scoring bands, which lead to inappropriate and non-authenticated inferences. In the present study RAPD analysis was used to study genetic diversity at the species level.

MATERIALS AND METHODS

Molecular analysis of honeybee samples

Molecular analysis on honeybees was performed using total DNA extracted from head and thorax region of individuals from a single location.

DNA isolation from honey bees

Reagents

2X CTAB extraction buffer: This was a 2 % solution of Cetyltrimethyl ammonium bromide (CTAB in 100mM Tris.Cl (pH 8.0) which additionally contained 20mM of Na_2EDTA (pH 8.0) and 1.4M NaCl. Immediately before use β-mercaptoethanol was added to provide a concentration of 0.1%.

TE buffer: This was a solution of 1mM Na_2.EDTA (pH 8.0) in 10mM Tris.Cl (pH 8.0)

Tris-Acetate-EDTA (TAE) 50 X buffer: This buffer was prepared by dissolving 242 g of Tris base, 57.1 ml of glacial acetic acid and 100 ml of 0.5 M Na_2EDTA (pH 8.0) in distilled water to make one litre.

Procedure

Total DNA from the head region of insect was isolated using a modified CTAB method Cubero et al. (1999). Head regions preserved in ethanol were dried on a filter paper and around 20 mg of tissue was macerated in 500 µl of 2X CTAB extraction buffer in an Eppendorf tube with the help of a hand held micro pestle. The tubes ware incubated at 65° C for 60 min with intermittent mixing of tube contents. Thereafter, 500 µl of chloroform-isoamyl alcohol (24:1) was added the contents mixed by vortexing to form an emulsion followed by centrifugation at 10000 rpm for 1 min. Using a disposable pipette tip, upper aqueous layer containing DNA was transferred to a clean Eppendorf tube. The DNA from this aqueous phase was precipitated by 500 µl of isopropanol in the presence of 50 µl of 3M sodium acetate. The precipitated DNA was collected by centrifugation at 10,000 rpm, 5 min. After removing the supernatant, the DNA pellet was washed with 70% ethanol and pellet was allowed to air dry at room temperature. The dried DNA pellet was dissolved in 100 µl of TE buffer containing DNAse free pancreatic RNAase (10µg per ml) and stored at -20°C until used. The quality of DNA isolated from insect was determined by horizontal agarose (0.7 per cent containing ethidium bromide @1µg per ml) gel electrophoresis in 1X TAE buffer at 75V for 1 hr. The DNA bands were visualized under a

UV trans- illuminator (UltraCam Gel documentation system) and recorded by photographing.

Additionally, the quality and concentration of DNA solution was determined by A_{260}/A_{280} ratio of diluted DNA solution in a UV-spectrophotometer. The concentration of DNA was estimated by using following formula:

1 A_{260} unit of double stranded DNA= 50µg/ ml

Absorbance ratio of A_{260}/A_{280} nearing 2.0 (1.9-2.1) indicate high purity DNA

Standardization of RAPD -PCR reaction

Standardization of different components for PCR reaction was done by using the varying concentration of template DNA, RAPD-PCR primers and Taq polymerase enzyme in 25µl of PCR-reaction as given below:

Components	Variable PCR conditions
Template DNA *(A.mellifera)*	0.5-2µl (15-30ng/µl) DNA extract from single insect was used in each PCR-reaction mixture for determining the amount of template DNA for optimum amplification.
Primers	2.0µl to 5.0µl of RAPD-primer (10nm solution) per reaction was studied for determining appropriate amount of primers in reaction mixture.
Taq polymerase enzyme	1-5 units of enzyme per reaction were studied for amplification of clear, distinctive bands from whitefly DNA. Taq polymerase (5U/µl) and dNTP mix (1mM each) was procured from MBI Fermentas.

Different combinations of annealing temperature (37- 40°C) and 18 primers were tested out of which 8 primers (**Table-1**) showed good amplification.

Table 1. List of RAPD-PCR Oligoprimers used for RAPD analysis.

Primer No.	Operon No.	Sequence
P1	OPC 07	GTCCCGACGA
P2	OPE 02	GGTGCGGGAA
P3	OPB 05	TGCGCCCTTC
P4	OPF 03	CCTGATCACC
P5	OPE 01	CCCAAGGTCC
P6	OPF 04	GGTGATCAGG
P7	OPH 16	TCTCAGCTGG
P8	OPH 09	TGTAGCTGGG

(A = Adenine, T = Thymine, C = Cytosine, G = Guanine. The nucleotide sequence have been given by Operon Technologies, Inc, Alameda, C,A USA and were synthesized through custom Oligonucleotide synthesis service of 'Integrated DNA Technologies, Inc", Coralville, IA, USA)

RESULT

During the present course of study, *Apis mellifera, Apis cerana* and *Apis dorsata* of family Apidae were investigated. The observations included the molecular genetic analysis of above given species was worked out.

Classification:

Kingdom - Animal
Phylum - Arthopoda
Class - Insecta
Order - Hymenoptera
Family - Apidae
Sub-family - Apinae
Tribe - Apini
Genus - *Apis*

Honey bees require an ample supply of flowers in their habitat, since this is their food source. Also need suitable places to build hives. In cooler temperate climates, the hive site must be large enough for the bees and for storage of honey to feed on during winter. The present studies on 'Molecular genetic analysis on some hymenopteran species' was carried out under laboratory conditions. Total DNA isolated from individual honeybee samples from different *Apis* species viz. *Apis mellifera, Apis cerana* and *Apis dorsata,* when analyzed on 0.7 per cent agarose gel represented a high molecular weight DNA, which was seen as a single condensed band that was free from any degradation products (Fig.1). The DNA concentration as determined by A_{260} varied around 100 ng/ µl of the 500 µl DNA solutions obtained from each insect sample. This represented a good quality of DNA preparation for subsequent molecular analysis of different *Apis* species under study.

RAPD Analysis: Resistance development in insects due to environmental pressure is known to be associated with definite changes in the insect genome /DNA. These changes may be attributed to unknown genomic rearrangements, which may be associated with over-expression of gene(s). All of these are manifestations of the genetic variability introduced into the insect by the various kinds of external pressure. In order to identify such variability in the honeybee DNA, comparative RAPD-PCR amplification of the six populations of Apis mellifera were performed using 8 different RAPD-primers.

The results show that population individuals tend to respond weakly to the RAPD-PCR profiles, so the results from RAPD needs further study (Figs 2a, b, c, d).

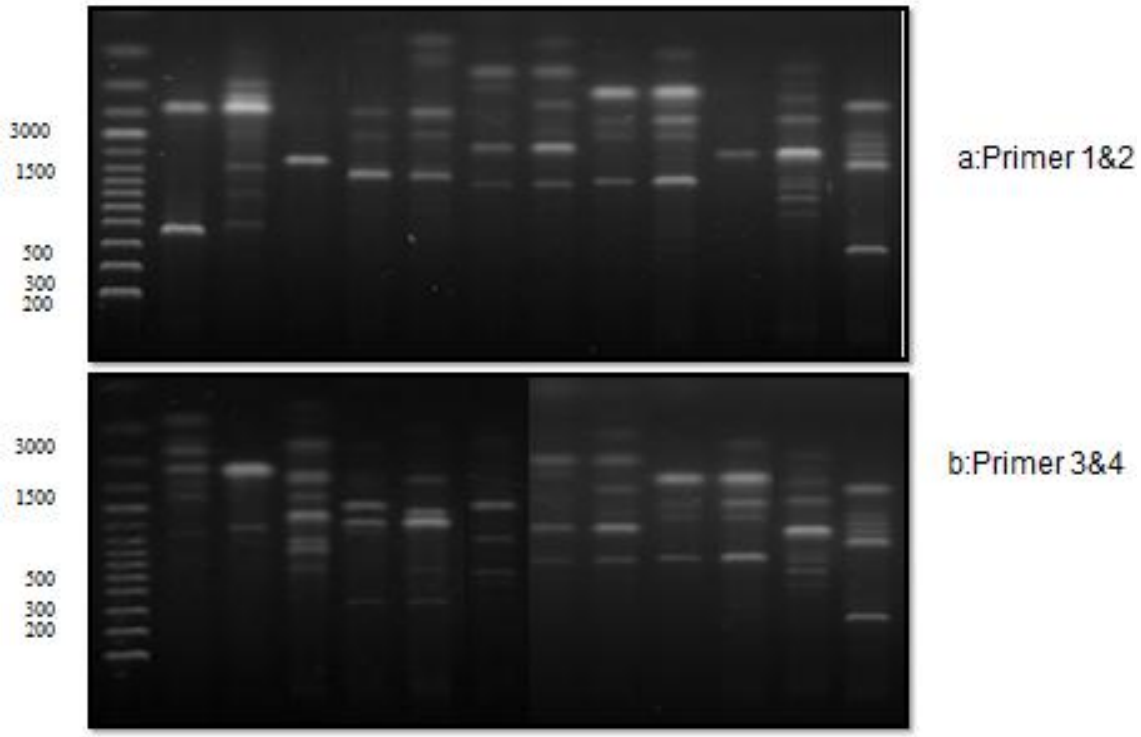

Fig 1(a&b): RAPD-PCR banding pattern showing polymorphic markers.

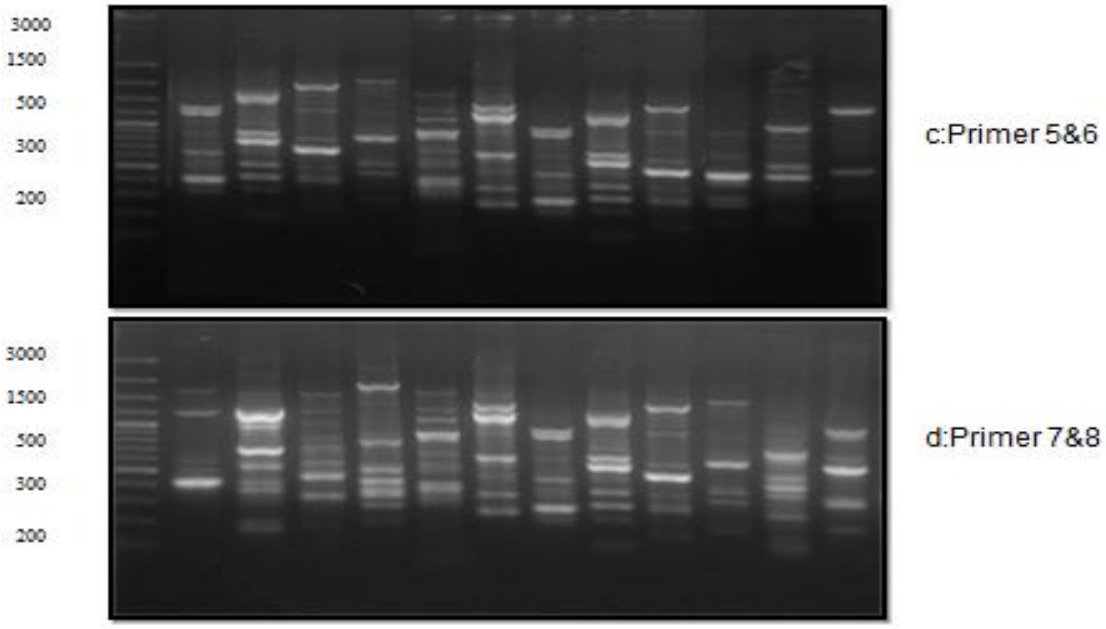

Fig 1. (c&d): RAPD-PCR banding pattern showing polymorphic markers.

DISCUSSION

India is a land of enormous physio-geographical variability, which has led to development and establishment of vast floral and faunal diversity currently existing in this sub-continent. India is declared as 3rd largest biodiversity in the world. It is therefore not surprising that India is one of the richest countries in the world with respect to the economically important honey-bee species diversity. Hussain (1939) claimed India to be the place of origin of genus *Apis*. Though Genus *Apis* is represented by a number of species and subspecies, it is represented by four major species of the genus Apis that are currently recognized to be present in India. Of these *Apis dorsata*, *Apis cerana* and *Apis* florae are indigenous whereas Apis mellifera is exotic and was for the first time introduced in India during 1960's. It was established at Nagrota in Himachal Pardesh (Atwal and Goyal 1973). In view of the vast physio-geographic diversity of the country and the evolutionary principles of natural selection, the existence of different species and subspecies of the indigenous bees can reasonly be expected. The discovery of three new species viz. *Apis andreniformis* which resembles *Apis florae*, *Apis koschenikovi* (Ruttner 1988) which is similar to *Apis cerana* and *Apis laboriosa* (Sakagami et al. 1980) the larger version of the Indian *Apis dorsata* from the Asian continent, bears testimony to the increasing diversity of the genus *Apis* and stresses upon the need to resolve honey bee systematics. Otis (1996) considered the limited research on honeybees of Asia as the main reason for not resolving the confusion. According to Crane (1993) work on the taxonomy of honeybee needs to be continued with the search for possible new species. Characteristics of recently established species should be studied further before the situation becomes more distorted by the transport of bees (Or their semen) and natural migration and movement from one region to another.

During the course of establishment of a new species in a new environment, species evolve into new species, subspecies or races which differ amongst themselves by varying degree of differences in morphological and biological characteristics. Such differences among allied species can be resolved by adopting a biosystematics approach based upon binomial system of classification. However, the other invisible biochemical and genetically changes pose problems in their identification due to lack of technical procedures. It is understood that morphological changes are reflection of a number of biochemical and genetical changes many of which though hold significance remain in apparent and unnoticeable. These are particularly more important to be identified as these represent gradual changes in the genome (Genes) leading to visible changes in morphological parameters and characteristics. The developments in the field of science and technology have today provided newer tools for systematic studies. There is therefore, a need to couple biological, behavioural, biochemical and cytological studies with the available taxonomic information in order to select more reliable discriminatory characteristics for separating insect taxa. Various scientists have depended upon the application of morphometric, ecobiology, scanning electron microscopic studies, immunoassays, electrophoresis and chromatography for analyzing minute taxonomic characters in a variety of insects [Sheppard and Berlocher 1989; Ruttner 1992; Lee et al. 2009; Ruttner 1988; Smith 1991).

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REFERENCES

Atwal AS, Goyal NP. 1973. Introduction of Apismellifera In Punjab Plains. lndian Bee J 35: 1-9.

Berlocher SH. 1984. Insect molecular systematic. Ann Rev Ento 29:403-433.

Burns JM, Johnson FM. 1971. Esterase polymorphism in butterflies Hermiragusisola: Stability in environment. Proc Nat Acad Sci USA 68:34-37.

Crampton JM, Beard CB, Louis C. 1996. The molecular biology of insect disease vectors. Chapman and Hall, London.

Crane E. 1993. Current status of research on Asian honeybees.Asian Apiculture.Wicwas Press. USA, pp 19-41.

Cubero OF, Crespo A, Fatehi J, Bridge PD. 1999. DNA extraction and PCR amplification methods suitable for fresh, herbarium-stored, lichenized, and other fungi. Pl Syst Evol 216:243–249.

Hawksworth DL. 1994. Indentification and characterization of pest organisms. CAB International, Wallingford.

Hoy MA. 1994. Insect molecular genetics. Academic Press , London. 288pp.

Hussain SW. 1939. Present position of bee keeping in India. Indian bee J., 1: 53-55.

Karp A, Isaac PG, Ingram DS. 1998. Molecular tools for screening biodiversity. Chapman and Hall, London, New York, Melbourne and Madras.

Kumar NR, Negi L. 2004. Protein profile of haemolymph from Apis species. J Bombay Nat Hist Soc (in press).

Lee, W., J. Park, J. Choi, K. Jung, B. Park, D. Kim, J. Lee, K. Ahn, W. Song, S. Kang, Y.H. Lee and S. Lee, 2009. IMGD: an integratedplatform supporting comparative genomics and phylogenetics of insectmitochondrial genomes. BMC Genomics, 10: 148. doi: 10.1186/1471-2164-10-148.

Li W. 1997. Molecular Evolution, Sinauer Associates, Inc., Sunderland, Massachusetts.

Mestriner MA. 1969. Biochemical polymorphism in bees (Apismelliferaligustica). Nature 223: 188-189.

Otis GW. 1996. Distributions of recently recognized species ofhoney bees (Hymenoptera: Apidae; Apis) in Asia. J Kansas Entomological Soc 69: 311–333.

Roderick GK. 1996. Geographic structure of insect populations: gene flow, phylogeography and their uses. Ann Rev Ento 41: 325-352.

Ruttner F. 1988. Biogeography and Taxonomy of Honeybees, SpringerVerlang, Berlin.

Ruttner F. 1988. Biogeography and Taxonomy of Honeybees, SpringerVerlang, Berlin.

Ruttner F. 1992. Naturgesichte der Honigbiene, Munchen, Ehrenwirth.

Sakagami SF, Matsumura T, Ito K. 1980. Apislaboriosa in Himalaya, the little known world largest honeybee (Hymenoptera, Apidae). Insecta Matsumurana 19: 47–78.

Sheppard WS, Berlocher SH. 1989. Allozyme variation and differentiation among four Apis species. Apidologie 20: 419-431.

Smith DR. 1991. African bees in the Americas: Insights from biogeography and genetics. Trends Ecol Evol 6: 17-21.

Sylvester HA. 1982. Electrophoretic identification of Africanized honey bees. J Apic Res 21: 93-97.

Welsh J, McClelland M. 1990. Fingerprint genomes using PCR with arbitrary primers. Nucleic Acids Res 18: 7213-7218.