Full Length Research Paper

Genetic variability of European honey bee, *Apis mellifera* in mid hills, plains and tarai region of India

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To observe the genetic variability in European honey bee, *A. mellifera*, PCR was run separately with five primers and analysis of the banding pattern was worked out to investigate the molecular profile of honey bee genotypes collected from different locations having random amplified polymorphic DNA (RAPD) primers. All the five primer screened, amplified the product in between the range of 100 to 1300 bp and 49 scorable markers bands were generated through polymerase chain reaction (PCR), of which 38 (77.55%) were polymorphic and 11 (22.44%) were monomorphic bands identified. Based on the estimated genetic similarity matrix, the highest genetic similarity value (0.861) was noticed between the mid hill region, Jeolikote and tarai region, Pantnagar and lowest genetic similarity value (0.375) was observed between Haldwani and Rajasthan. The major gene cluster consisted of eight European honey bee, *A. mellifera* accessions from Haldwani, Almora, Do-gaun, Jeolikote, Fatehpur, Ramnagar, Aligarh, Pantnagar, while the minor gene cluster comprised single accession from Rajasthan.

Key words: Genetic variability, *Apis mellifera*, RAPD primers, DNA extraction, polymerase chain reaction (PCR).

INTRODUCTION

European honeybee (*Apis mellifera* L.) has been object of numerous studies and extensively used from different points of view like genetic, morphometrical and molecular studies (Ruttner, 1988). The need to conserve the genetic diversity of domesticated plants is well docu-mented (Rogers, 2004). More recently, Scherf (2000) drew attention to the loss of genetic diversity in livestock world-wide, focusing on domesticated mammals and birds. Despite the widespread consensus that preserving the genetic diversity of domesticated species may prove valuable to humanity, there have been few efforts to preserve the genetic diversity of beneficial arthropod species. While several arthropod species are cultured by humans, *A. mellifera* L., the western honey bee, is the most economically important beneficial insect. *A. mellifera* is very well adapted to life in tropical conditions and have desirable characteristics for beekeeping

(Delaplane and Mayer, 2000). Insects comprises the largest species composition in the entire animal kingdom and posses a vast undiscovered genetic diversity and gene pool that can be better explored using molecular marker techniques. Insect population, even within a species varies, in their behavior and morphology that attributes to their complex interaction with the environment (Dempster and McLean, 1999).

In insects, DNA markers are used to provide raw information, based on which an ecologist make estimates of genetic diversity and gene flow between species (Speight et al., 2005). The greater level of polymorphism could be obtained by using DNA markers than by using protein markers (Richardson et al., 1986). Out of four species of honey bees namely, *A. mellifera*, *A. cerana*, *A. florea* and *A. dorsata* that are found in India, the former is the only one which is of great interest for commercial

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Table 1. Brood sample collected from different locality for the determination of genetic variability.

Place	Number of brood sample collected
Haldwani (tarai)	10 (240)*
Almora (mid hill)	10 (25)*
Do Gaon (mid hill)	10 (150)*
Jyolikot (mid hill)	10 (140)*
Fatehpur (plain)	10 (100)*
Ramnagar (tarai)	10 (275)*
Aligarh (plain)	10 (230)*
Rajasthan (plain)	10 (200)*
Pantnagar (tarai)	10 (125)*

*Value shown in parentheses represents the total number of honey bee colonies with the beekeeper.

beekeeping because it can be maintained in hives. Therefore, knowledge of genetic diversity levels and population subdivisions of *A. mellifera* in India is important for understanding distribution patterns and colonization of this species. However, its conservation is also important from a biodiversity perspective, where a priority is laid on preserving the endemic races of honey bees in this region.

India is rich in biodiversity due to varied climatic and topographical conditions. It has great potential to facilitate pollination services in all the crops. Its different states have special importance of pollinators' services due to cash crop farming in hills, agri-export zones and limited cultivable area. Nevertheless, there are magnificent infor-mation missing gaps regarding various aspects of polli-nation and pollinators of the different states. Considering its different states, it is evident from the literature that meager information is available on the genetic diversity of insect pollinators.

MATERIALS AND METHODS

To observe the genetic variability of the European honeybee, *A. mellifera* samples were collected from 8 beekeepers (Table 1) at mid hills, plains and tarai region of India. Honeybee brood samples (n = 10) per colony were collected and stored in a refrigerator at -20°C and DNA was extracted for further processing. The genetic variability of European honeybee, *A. mellifera* was assessed at IPM Laboratory, Department of Plant Pathology, College of Agriculture, G. B. Pant University of Agriculture and Technology, Pantnagar, U. S. Nagar.

DNA isolation protocol

The DNA was extracted from the honeybee (*A. mellifera*) broods. For this purpose, lyophylization of the honeybee broods was done for the easy, rapid grinding of broods and also to minimize the number of organic solvent extractions according to the Hall (1986) protocol. The following steps were used in the DNA extraction of honey bees: bee brood was lyophilized, and ground with liquid nitrogen solution with the help of mortar and pestle. The powder of

bee brood collected from each location was transferred to a microcentrifuge tube using a stiff paper (for parallel preparations, the mortar and pestle are cleaned each time with a dry cloth). The powder was then suspended in 100 mM Tris-HCL, pH 8.5, 250 mM NaCl, 25 mM EDTA, 1% sodium dodecyl sulfate (SDS), 2 mg/ml RNase A (100 µl/10 mg of material) by stirring with a pipette tip and left for 10 min at 37°C. Now 0.8 ml of phenol (equilibrated with 0.1 M Tris-HCL, pH 8.0) was added to it and the tubes were shaken for 2 min for homogeneous suspension. Then, 0.4 ml of chloroform was added and the tubes were shaken for 1 min and centrifuged for 5 min at 13000 rpm (15000 x g). The upper aqueous phase was taken off, re-extracted with 1 ml of chloroform and again centrifuged. Now the supernatant aqueous phase was transferred to a sterile microcentrifuge tube and 0.6 ml of isopropanol was added, and the tube were held in a horizontal position (to maximize the area between liquids) and shaken for 1 min. DNA precipitates as a visible aggregate, and the liquid can be removed with a micropipette. Alternatively, DNA can be recovered by centrifugation for 1 min at 5000 rpm.

The DNA precipitate was rinsed twice (without centrifuge) with 70% ethanol. The tubes were spun for a few seconds, and residual liquid is removed; then the DNA is dried and re-suspended in 10 mM Tris-HCL, pH 8.0, 0.1 mM EDTA. Extracted DNA samples were store at -20°C.

DNA purification

2 µl RNAse A was added to the eppendorf tube containing 200 µl of extracted DNA and then incubated for 3 h at 37°C in a water bath. The DNA was further extracted with equal volume of Phenol: Chloroform: Isoamyl alcohol (25: 24: 1, v/v) and centrifuged at 10,000 rpm for 12 min at 10°C. Supernatant was taken into a fresh eppendorf tube. 0.6 m^3 ice cold isopropanol and 0.1 m^3 of ice cold sodium acetate (3 M) were added and the mixture was kept at -20°C for at least 2 h. The mixture was then centrifuged at 10,000 rpm at 10°C for 12 min. Supernatant was removed using a micropipette and pellet was washed with 70% ethanol and dried completely. The DNA pellet was re-dissolved in minimum amount of TE buffer.

DNA concentration, quality and integrity determination

The quantification of genomic DNA was done by taking the absorbance on Genesys UV spectrophotometer. The optical density was measured at 260 and 280 nm. The concentration of the DNA in the sample was related to optical density by the following formula:

Table 2. RAPD PCR amplification.

Ingredient	Stock	Volume added (µl)
PCR Buffer (Invitrogen)	10X	2.0
MgCl2 (Invitrogen)	50 mM	0.8
dNTP Mix (Fermantas)	10 mM	2.0
Primer	10 µM	1.5
Taq DNA polymerase (Invitrogen)	5 U/µl	0.3
Diluted DNA sample	-	1.2
Nuclease free water (Promega)	-	12.2
	Total	20.0

$$\text{Conc. of DNA } (\mu g/ml) = \frac{OD_{260} \times 50 \times \text{Dilution factor}}{1000}$$

The ratio of $OD_{260/280}$ was an indication of the amount of RNA or protein contamination in the preparation. A value of 1.8 is optimum for best DNA preparation. A value of the ratio below 1.8 indicated the presence of protein in the preparation and a value above 1.8 indicated that the sample has RNA contamination.

Polymerase chain reaction (PCR)

PCR amplification was performed with primers, obtained from Eurofins Genomics India Pvt. Ltd., Bangalore. PCR conditions were standardized separately for RAPD in two steps: 1) By taking all the primers and keeping their concentration constant to find out as to which primer gave results; 2) taking template DNA concentration as 20, 40, 60 ng and primer concentration as 60, 80 and 100 ng to standardize the template DNA and primer concentration; for PCR amplifications, a total of 20 µl m³ containing the following components were used (Table 2).

Sets of 10 oligo-nucleotide primers were employed for RAPD PCR amplification. The details of primers are given in Table 5. A master mix (minus template DNA) was prepared to reduce pipetting error. The master mix was then distributed in each tube (18.8 µl each) and finally 1.2 µl of template DNA was added in each tube. The mixture was gently mixed and centrifuged at 5000 rpm for 10 s. The PCR amplification was achieved in M. J. Research Thermocycler (PTC 200). The amplification reaction was pre-denatured at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 37°C for 1.30 min and polymerization at 72°C for 1 min. The last cycle of final extension was carried out at 72°C for 2 min.

Analysis of amplification products and procedure of agarose gel electrophoresis

After the completion of PCR cycles, 15 µl of the PCR product was analyzed on 1.3% agarose gel by electrophoresis. Horizontal gel electrophoresis unit was used for fractionating RAPD markers on agarose gel. Agarose gel (1.3%) was prepared by dissolving appropriate amount of agarose in 0.5X TBE buffer. For each well, DNA loading dye and DNA samples were mixed in 1:6 ratio and loaded with a micropipette. Electrophoresis was done at 70 V for 3 h in 0.5X TBE electrophoresis buffer. The gel was then stained in ethidium bromide solution. After de-staining in de-ionized water, the gel image was viewed in U. V. transilluminator and stored in gel documentation system.

Molecular markers data analysis

DNA fingerprints were scored for the presence (1) or absence (0) of bands of various molecular weight sizes in the form of binary matrix. Data were analyzed to obtain Jaccard's similarity coefficients among the isolates by using NTSYS-pc (version 2.11s; Rohlf, 2002). The SIMQUAL program was used to calculate the Jaccard's similarity coefficients. All the gels were scored twice manually and independently. Band presence was indicated by 1 and its absence by 0. All unique bands were also scored and included in the analysis. Presence or absence of unique, shared and polymorphic bands was used to generate similarity coefficients. The similarity coefficients were then used to construct a dendrogram manually by unweighted pair group method using arithmetical averages (UPGMA). The analysis work was based on Jaccard's similarity coefficient given as:

$$\text{Similarity coefficient} = \frac{\text{Number of polymorphic bands}}{\text{Total number of band}}$$

Based on the similarity coefficient, separate dendogram for RAPD were prepared.

RESULTS AND DISCUSSION

The results obtained from the present investigation are presented under the following subheads:

DNA fingerprinting of European honey bee, *Apis mellifera* L.

The PCR was run separately for the five primers and the amplified products were separated in agarose gel and viewed in the gel doc system and photograph was saved. The analysis of the banding pattern revealed the molecular profile of honey bee genotypes collected from different places with different RAPD primers which are given in Tables 3 and 4.

Amplification profile of RAPD primers

Primer 1 (501)

On agarose gel, this primer showed amplification with all

Table 3. Summary of RAPD amplified products.

Parameter	Value
Total number of primers tested	72
Number of polymorphic primers	05
Total number of monomorphic primers	67
Total number of bands region amplified	49
Size range of amplified products (bp)	100 to 1300
Average number of bands per primer	10
Total number of unique bands identified	3
Total number of polymorphic bands identified	38
Total number of monomorphic bands identified	11
Percentage of all bands that were polymorphic	77.55%

Table 4. Amplified products and polymorphism obtained with RAPD primers.

Primer	Number of amplified products	Polymorphic band (numbers)	Monomorphic band (numbers)	Percent (%) polymorphism
501	10	8	2	80
502	9	6	3	66.66
503	11	9	2	81.81
504	10	9	1	90
505	9	6	3	66.66

Table 5. Base sequence of primers used in gene expression analysis.

Oligonucleotide code	Base sequence (5'-3')	Yield (µg)	Yield (n/mol)	Volume for 100 (pmol/µl)	Temperature (°C)	MW (g/mol)
501	CGGTTAGACG	473	154.1	1541	32.0	3068
502	CTTACGTCAC	400	134.9	1349	30.0	2962
503	CCCAACACAC	387	132.3	1323	32.0	2925
504	AAACCAGGCG	383	125.8	1258	32.0	3046
505	GGTTTGGAGG	469	149.3	1493	32.0	3139

nine samples of European honey bee, *A. mellifera* (Figure 1). This primer amplified the products in the range of 100 to 1200 bp. Loci amplified by this primer in respective genotypes were 80% polymorphic.

Primer 2 (502)

This primer amplified nine scorable RAPD loci on agarose gel with all nine genotypes, out of which six were polymorphic and three were monomorphic at 450 and 550 bp, respectively (Figure 2). Amplified product size ranged from 450 to 1200 bp. The loci amplified by this primer were 66.66% polymorphic.

Primer 3 (503)

On agarose gel, this primer revealed 11 scorable ampli-

fied RAPD loci in nine genotypes of *A. mellifera* (Figure 3). The amplified products were in the range of 100 bp to more than 1250 bp.

Out of 11 amplified products by this primer, nine loci were observed to be polymorphic while 2 loci were found monomorphic at approximately 400 and 500 bp location, respectively. Three bands at the positions 850, 1200 and 1250 bp were observed unique to genotype collected from Haldwani province.

Primer 4 (504)

This primer amplified 10 scorable RAPD loci on agarose gel with all nine genotypes, out of which nine were polymorphic and one was monomorphic at around 200 bp (Figure 4). Amplified product size ranged from 200 to 1200 bp. The loci amplified by this primer were 90%
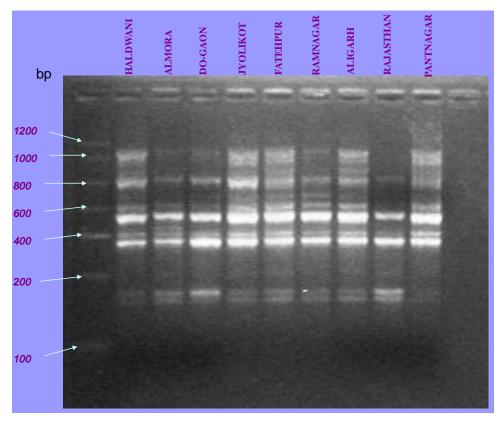
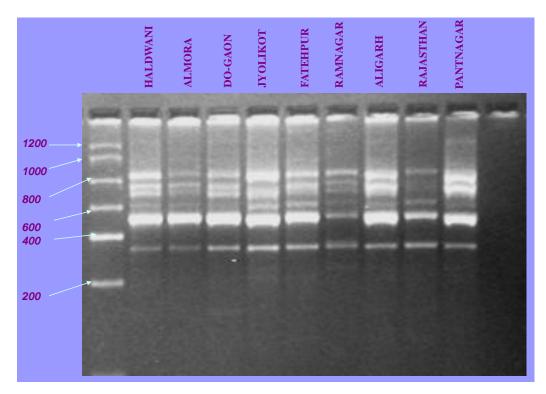**Figure 1.** RAPD profile of European honey bee, *A. mellifera* genotypes generated by the primer 501 on agarose gel.

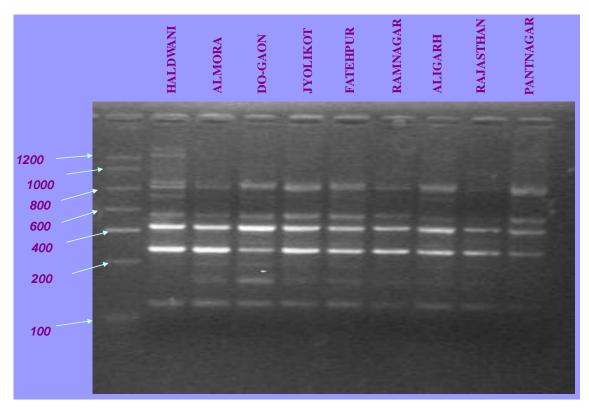**Figure 2.** RAPD profile of European honey bee, *A. mellifera* genotypes generated by the primer 502 on agarose gel.

Figure 3. RAPD profile of European honey bee, *A. mellifera* genotypes generated by the primer 503 on agarose gel.
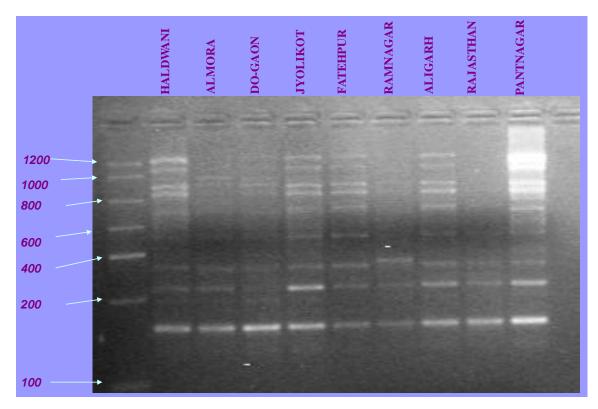

Figure 4. RAPD profile of European honey bee, *A. mellifera* genotypes generated by the primer 504 on agarose gel.
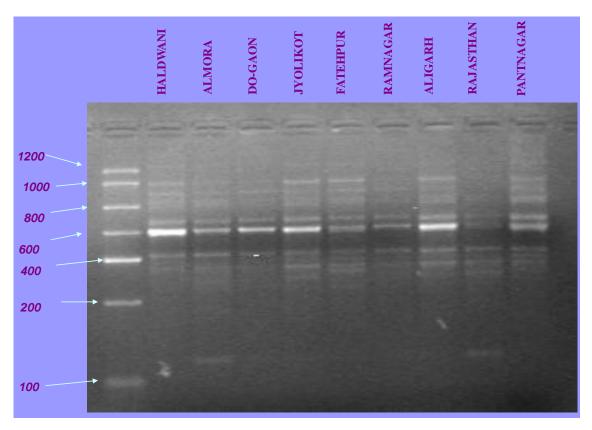
Figure 5. RAPD profile of European honey bee, *A. mellifera* genotypes generated by the primer 505 on agarose gel.

polymorphic.

Primer 5 (505)

On agarose gel, this primer showed amplification with all nine samples of *A. mellifera* (Figure 5). This primer amplified nine scorable RAPD loci in nine genotypes, out of which six were polymorphic while three bands were found monomorphic at the positions 500, 600 and 650 bp, respectively. Amplified product size ranged from 400 to 1200 bp. The loci amplified by this primer were 94.11% polymorphic. In the present study, our results show that all the five primer screened, amplified the product in between the range of 100 to 1300 bp while Hall et al. (1998) screened 700 primers, of which five were found to reveal useful distinguishable differences and the bands generated in between 0.73 to 1.75 kb. In the Africanized populations, the alleles were most frequently 105 to 108 bp in size, although some were 116 to 122 pb. This contrasts with 100 to 103 bp for most alleles in the African population, and the absence of alleles greater than 114 bp in the African population. Alleles of greater size are characteristic of European honey bee races, particularly of the Mediterranean races (Estoup et al., 1995). Raffaele et al. (2007) reported that number of

alleles and expected heterozygosity per locus A107 was highly polymorphic with a total of 22 alleles detected (size range from 105 to 136 bp) and had the highest *Ho* values. Only five alleles were scored for locus A28 (130 to 140 bp) and loci A28 and A88 showed low hete-rozygosity values, in particular for the *A. mellifera ligustica* groups. In another study, Hunt and Page (1992) used the polymorphism and segregation of RAPD markers. The primers generated 1018 scorable marker-bands (an average of 7.7 per primer), of which 409 (40%) were polymorphic, whereas in the present study, similar types of results was observed, 49 scorable markers bands generated (an average of 10 per primers), of which 38 (77.55%) were polymorphic and 11 (22.44%) were monomorphic bands identified.

Polymorphisms generated with RAPD primers result from success or failure of the primer to bind, creating the presence or absence of a particular amplified band, and from insertions or deletions that change the length of the amplified region. Most RAPD markers are of the first type and are dominant in expression, whereby a diploid with two copies of a RAPD marker cannot be distinguished from those containing one copy (Tingey et al., 1992; Williams et al., 1990). A small proportion of the screened primers were found to be useful. Thus, RAPD as a source of diagnostic genetic markers in honey bees has

Table 6. Similarity coefficient between genotypes of *A. mellifera* collected from different locations.

Genotype	Haldwani	Almora	Do-goun	Jeolikote	Fatehpur	Ramnagar	Aligarh	Rajasthan	Pantnagar
geno1	1								
geno2	0.561	1							
geno3	0.513	0.656	1						
geno4	0.725	0.537	0.45	1					
geno5	0.667	0.568	0.524	0.805	1				
geno6	0.556	0.667	0.667	0.571	0.488	1			
geno7	0.732	0.585	0.463	0.842	0.810	0.500	1		
geno8	0.375	0.581	0.467	0.459	0.463	0.464	0.514	1	
geno9	0.700	0.476	0.462	0.861	0.738	0.500	0.769	0.395	1

not been as successful, in our hands, as RFLPs. Specific RAPD markers have been found more readily in other organisms, particularly when the characterization has been done at a higher taxonomic level, for example, as species-specific markers (Kambhampati et al., 1992) or for populations of known genetic origin, such as cultivars or strains (Guthrie et al., 1992). Sirikul et al. (2008) also reported similar type of work with *Trigona pagdeni.* The presence or absence of 51 TE-AFLP bands was scored for each individual. Eight bands (16%) were fixed. The remaining 43 bands, each of which were present in at least one individual and absent in at least one individual, were considered polymorphic. Thirty five of these bands (69%) had inferred allele frequencies in the range of 5 to 95%.

Relationships among nine genotypes of *A. mellifera* using RAPD markers

Based on the estimated genetic similarity matrix (Table 6), the highest genetic similarity value (0.861) was noticed between the pair Jeolikote and Pantnagar followed by (0.842) between the pair, Jeolikote and Aligarh, (0.810) between Fatehpur and Aligarh, (0.805) between Jeolikote and Fatehpur, (0.769) between Aligarh and Pantnagar, (0.738) between Fatehpur and Pantnagar, (0.732) between Haldwani and Aligarh accession, (0.725) between Haldwani and Jeolikote, (0.7) between Haldwani and Pantnagar, (0.667) between Haldwani and Fatehpur also same between Almora and Ramnagar and also among Do-gaun and Ramnagar, (0.656) between Almora and Do-gaun accessions, (0.585) between Almora and Aligarh and (0.581) between Almora and Rajasthan. The lowest genetic similarity value (0.375) was observed between Haldwani and Rajasthan followed by (0.395) between Rajasthan and Pantnagar, and (0.45) between Do-gaun and Jeolikote. Unbiased estimates of gene diversity (Nei, 1987) were higher in the Africanized population (0.937) than the respective estimates for the Johannesburg (0.898), Forli (0.810) and Avignon (0.377) populations.

Pilar et al. (2002) reported that *A. mellifera* gene diversity varied between 0:589 ± 0:247 (Mallorca) and 0:349 ± 0:331 (Formentera). Sirikul et al. (2008) revealed significant genetic differentiation among the four populations of *Trigona pagdeni* (φPT = 0.18, P = 0.01). They detected differentiation (φPT = 0.13, P = 0.001) between samples collected north and south of the Kra ecotone, a biogeographical zone of transition between seasonal evergreen and mixed moist deciduous forests. However, the greatest differentiation was detected between samples from the northeast and the other locations combined (φPT = 0.21, P = 0.001).

Analysis of gene cluster formed

The dendogram constructed from RAPD marker analysis in honey bee genotypes revealed that there were two major gene clusters formed breaking at 0.46 Jaccard's coefficient of similarity (Figure 6). The major gene cluster consisted of eight *A. mellifera* accessions from Haldwani, Almora, Do-gaun, Jeolikote, Fatehpur, Ramnagar, Aligarh, Pantnagar, while the minor gene cluster comprised single accession from Rajasthan. The major gene cluster within accessions from Jeolikote and Pantnagar were not further separated indicating the high level of genetic similarity between the two (> 86%). The secondary gene cluster was formed within the major gene cluster around 0.5 Jaccard's coefficient of similarity. The secondary gene cluster divided the major gene cluster into two subgroups. One of it consisted of five accessions from Haldwani, Jeolikote, Pantnagar, Fatehpur and Aligarh and the latter consisted of three accessions of honey bee from Almora, Do-gaun and Ramnagar. According to Pilar et al. (2002), in the genetic differentiation test, the honeybee populations of the Balearic Islands clustered into two groups: Gimnesias (Mallorca and Menorca) and Pitiusas (Ibiza and Formentera), which agrees with the biogeography postulated for this archipelago. As is the case with PCR reactions, the results of RAPD analyses must be carefully interpreted, especially when the nature of the amplified
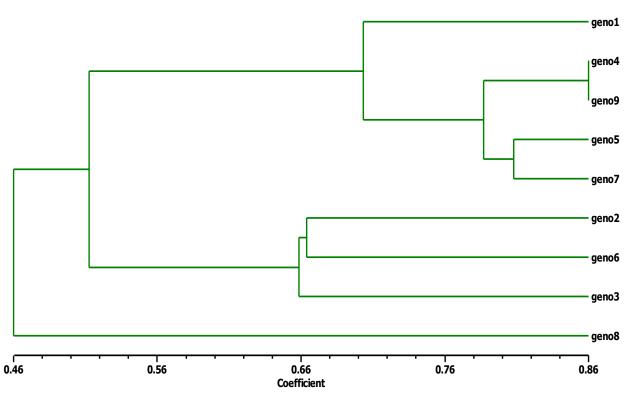
Figure 6. Dendrogram of genetic distance of 9 genotypes of European honey bee, *Apis mellifera* constructed from RAPD markers.

sequence is not known. RAPD analyses are particularly sensitive to the concentrations of DNA, magnesium, primers and Taq DNA polymerase.

The generation of many bands results in artifacts during the amplification reaction (Anglani et al., 1990; Nagamine et al., 1989).

Conclusion

In the present study, genetic variability in European honey bee, *A. mellifera*, has been worked out by using five RAPD primers. Based on the estimated genetic similarity matrix, the highest genetic similarity value (0.861) was noticed between the pair Jeolikote and Pantnagar while lowest genetic similarity value (0.375) was observed between Haldwani and Rajasthan. The major gene cluster consisted of eight European honey bee (*A. mellifera)* accessions from Haldwani, Almora, Dogaun, Jeolikote, Fatehpur, Ramnagar, Aligarh, Pantnagar, while the minor gene cluster comprised single accession from Rajasthan.

A small proportion of the screened primers was found to be useful. The RAPD markers reported here are specific to groups of honey bee species and their representation in populations coincides with what had been found with RAPD markers. Nevertheless, RAPD markers can also provide an efficient assay for polymorphism

which should allow rapid identification and isolation of chromosome specific DNA fragments. These RAPD markers are expected to be useful in distinguishing African and European bees and following interactions between the two. India is one, among 12- mega diversity countries and is also one of the countries whose insect fauna is very poorly known and documented.

A sound insect biodiversity knowledge base is prerequisite for effective crop cultivation, conservation, environmental assessment, ecological research management and sustainable use of biological resources. In the current millennium, India will become an economic power because of its biodiversity.

Many a time, molecular marker data help to distinguish between different species, where there is no other comprehensive way available to do so. Thus, DNA markers will be valuable in the continued elucidation of African-European honeybee interactions and population dynamics.

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