Phylogenetic Analysis of Honeybees (Apis Mellifera) in Egypt Using DNA as a Molecular Genetic Marker

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ABSTRACT

The aim of this study was to detect the genetic relationship among individuals honeybee (*Apis mellifera*), the queen, males and workers. Random Amplified Polymorphic DNA (RAPD) techniques were applied using 20 oligonucleotides. Fifteen 10-mer and five 20-mer primers were used to detect genetic similarity as band sharing (BS) among individuals honeybees. The comparison, bands sharing average values were 71.95, 80.45 and 79.25 % between Queen & male, Queen & worker and male & worker, respectively. The Queen & worker showed the highest similarity, while the Queen & male casts showed less similarity. These results reveal that RAPD analysis provides a rapid and effective method for detecting the genetic variation among individuals of honeybees. The pattern of each individual could be used as marker-assisted selection during honeybee breeding programs.

Keywords: Honeybees, Phylogenetic Analysis, RAPD-PCR, genetic similarity, band sharing, marker assisted selection.

INTRODUCTION

Diverse ranges of novel molecular (DNA) markers are now available for entomological investigations. Both DNA and protein markers have revolutionized the biological sciences and have enhanced many fields of study, especially ecology. The relative of DNA markers, allozymes are cheap, often much quicker to isolate and develop, even from minute insects (aphids, thrips, parasitic wasps, etc.), and subsequently easy to use. They display single or multi-locus banding patterns of a generally easily interpretable Mendelian nature, and the statistics for their analysis are well established. DNA markers are also suitable for use with small amounts of object material like insects and can be used with dry or stored samples. Moreover, they have an expanding range of applications, many involving intra- and inter specific discriminations. Loxdale and Lushai (1998) discussed the main molecular markers commonly used and their applications including suitability for particular studies.

Electrophoretic markers are powerful molecular tools that have facilitated the study of diverse areas of biology, both pure and applied, covering questions of phylogeny, evolution, ecology and population dynamics (Loxdale & den Hollander, 1989; Smith & Wayne, 1996; Symondson & Liddell, 1996). Initially, protein markers were the main marker type employed more recently. Many of

the applications of these markers have been superseded and expanded upon using DNA-based techniques (Avise, 1994). As a consequence, greater resolution has been achieved at the population level allowing the investigation of organisms with little detectable protein variation, e.g. clonal organisms (e.g. Carvalho *et al.*, 1991). The minute quantities of DNA required also allow stored samples and specimens with small amounts of organic material to be studied. All these applications are pertinent to entomology (Hawksworth, 1994; Hoy, 1994; Crampton *et al.*, 1996; Loxdale *et al.*, 1996; Roderick, 1996; Karp *et al.*, 1998). The field of molecular biology has expanded greatly in the last ten years and many entomologists now wish to use this technology, since it is a new level of resolution for the study of insect ecological systems and taxonomy. Cook (1996) has recently provided 'A beginners' guide to molecular markers for entomologists', whilst Hoy (1994), Crampton *et al.* (1996) and Karp *et al.* (1998) provided large and detailed technical treatises on the subject of molecular markers and Loxdale (1994) and Loxdale *et al.* (1996) provided detailed entomological applications. In addition, Parker *et al.* (1998) and Haig (1998) discuss the various uses of molecular markers in population studies and in conservation biology, respectively.

The development of molecular techniques has been created new possibilities for the selection (marker-assisted selection) and genetic improvement of livestock. The discovery of the PCR had a major impact on the research of eukaryotic genomes and contributed to the development and application of various DNA markers (Marle-Koster and Nel, 2003).

Random amplified polymorphic DNA (RAPD) markers can be generated using short arbitrary primers to amplify genomic DNA, giving a genotype-specific pattern bands. RAPD analysis should lead to the saturation of the genome with markers to a level of at least one marker every 0.5 cM, without the requirement of previous genetic information and using few expensive oligonucleiotides. RAPD markers are usually generated by the amplification of random DNA segments with single short arbitrary primers.

In the present work, we used RAPD fingerprinting technology to supply the information of genetic similarity among individuals of Honeybees (*Apis mellifera*) for conservation, improvement and the use of marker-assisted selection in breeding programs.

MATERIALS AND METHODS

DNA isolation:

DNA was extracted from three different honeybee castes according to the salting out method of Sunnucks and Hales (1996) with some modifications.

The adults of insects were crushed with mortar and collected in a 1.5 ml eppendorf tube. 300 µl TENS buffer (50 mM Tris base pH 7.5, 400 mM NaCl, 20 mM EDTA, 5% SDS) and 5 µl of proteinase K (10 mg/ml) were added and incubated at 55°C for 20 min. Then, 85 µl of 5M NaCl were added and vortex for

15 sec. Centrifugation was carried out at 13,000 rpm for 5 min. Carefully, upper phase was placed in a new tube and an equal volume of cold 100% Ethanol was added. Second centrifugation was carried out for 5 min. Liquid phase was removed and the pellet was washed using 500 µl of 70% Ethanol. DNA pellet was dissolved in 30 µl 0.1X TE buffer and stored at –20 °C.

PCR Primers

Twenty primers were randomly selected and used in PCR amplifications. The nucleotide sequences, GC content and annealing temperatures for all primers are given in (Table 1).

PCR amplification and agarose gel electrophoresis:

PCR amplifications were performed following the procedure of Williams *et al.* (1993). Reactions were performed in a total volume of 25 µl consisted of 0.8 unit of Taq DNA polymerase (Fanzyme), 25 peco mol dNTPs (Di-deoxy nucleotide triphosphate), and 25 peco mol of random primer, 2.5 µl 10x reaction buffer and 40 ng of genomic DNA. The final reaction mixture was placed in a DNA thermal cycler (Eppendorf). The PCR program included an initial denaturation step at 94°C for 2 min, followed by 45 cycles (94°C for 30 sec, annealing as mentioned with each primer, 72°C for 30 sec) and final extension at 72 °C for 10 min. The samples were cooled at 4°C. The amplified DNA fragments were separated on 2.5% agarose gels and stained with ethidium bromide. The amplified patterns were visualized on a UV transilluminator and photographed using a gel documentation system (Image Analyzer).

Scoring and analysis of RAPDs

DNA bands were scored for their presence (1) or absence (0) in the RAPD profile of the honeybees among castes. The index of similarity between each two insects was calculated using the formula: $B_{ab}= 2N_{ab}/(N_a + N_b)$, where N_{ab} is the number of common fragments observed in individuals and N_a and N_b are the total number of fragments scored in a and b respectively (Lynch, 1990). The BS values (Band sharing) were calculated for each primer separately and average for all primers was per caste comparison. A dendrogram was constructed using the Average Linkage Between Groups (ALBG) as described by Sneath and Sokal (1973).

Table 1. The aveleotide sequences of the primers used, GC % and their annealing temperatures.

Primer No.	Sequence 5`- 3`	G + C content %	Annealing Tem. / Sec.
1	CAG GCC CTT CCA GCA CCC AC	70	54/30
2	GAA ACG GGT GGT GAT CGC AG	60	54/30
3	GGT GAC GCA GGG GTA ACG CC	70	54/30
4	GGA CTG GAG TGT GAT CGC AG	60	54/30
5	GGA CTG GAG TGG TGA CGC AG	65	54/30
6	AGG CCC CTG T	70	30/30
7	ATG CCC CTG T	60	30/30
8	AGC CAG CGA A	60	30/30
9	AAA GCT GCG G	60	30/30
10	ACC GCC GAA G	70	30/30
11	GGC ACT GAG G	70	42/30
12	CGC TGT CGC C	80	42/30
13	AGT CCT CGC C	70	42/30
14	TGG TGG ACC A	60	42/30
15	GAATGC GAC G	60	42/30
16	ATG ACG TTG A	40	45/30
17	CTG AGG AGT G	60	45/30
18	GGG CTA GGG T	70	45/30
19	ACC GGG AAC G	70	45/30
20	AGC AGG TGG A	60	45/30

RESULTS

All three honeybee castes were individually tested. Each produced a RAPD fingerprint presented in Figure 1. Fingerprint caste patterns 1-3 (Fig. 1) were as follows: queen, male and worker, respectively, amplified with 20 arbitrarily primers (Table 1). These primers were ten and twenty nucleotides in length and possessed 60-80% G + C content and only one primer (primer No. 16) possessed 40 % G + C content. A series of several DNA fragments (Fig. 1) were obtained from the samples under investigation. The number of fragments

generated per primer varied between one and seven fragments. All amplification products were found to be reproducible when reactions were repeated using the same reaction conditions.

The data in table 2 showed the genetic similarity estimated as band sharing (BS) for each primer between each two castes of honeybees. Band sharing values were 71.95, 80.45 and 79.25 % between Queen & male, Queen & worker and male & worker, respectively. The Queen and worker caste showed the highest similarity (80.45 %), whilst the Queen and male showed the lowest similarity (71.95 %). Two primers (13 and 14) were unable to differentiate the castes under study. Both primers showed 100 % similarity among the three castes investigated.

A phylogenetic analysis using the computer program Statistica 5 was performed for the three different castes based on 20 random amplified polymorphic DNA primers. The dendrogram as depicted in Figure 2 showed two clusters depended on the average of band sharing similarity percentages between honeybee groups (queen, male and worker).

Table 2. The similarity percentage (based on Nab/Na+Nb)* among the three genotypes studied (queen, worker and male) using 20 random primers.

Primer No.	Genotypes		
	Queen X Male	Queen X Worker	Male X Worker
1	33.0	86.0	57.0
2	67.0	100.0	67.0
3	40.0	67.0	86.0
4	100.0	67.0	67.0
5	67.0	67.0	50.0
6	83.0	92.0	92.0
7	86.0	86.0	86.0
8	50.0	50.0	100.0
9	50.0	67.0	67.0
10	67.0	40.0	86.0
11	75.0	100.0	75.0
12	67.0	100.0	67.0
13	100.0	100.0	100.0
14	100.0	100.0	100.0
15	91.0	91.0	100.0
16	83.0	100.0	83.0
17	40.0	100.0	100.0
18	60.0	89.0	73.0
19	100.0	40.0	40.0
20	80.0	67.0	89.0
Average	71.95	80.45	79.25

*N_{ab} is the number of common fragments observed in individuals
N_a and N_b are the total number of fragments scored

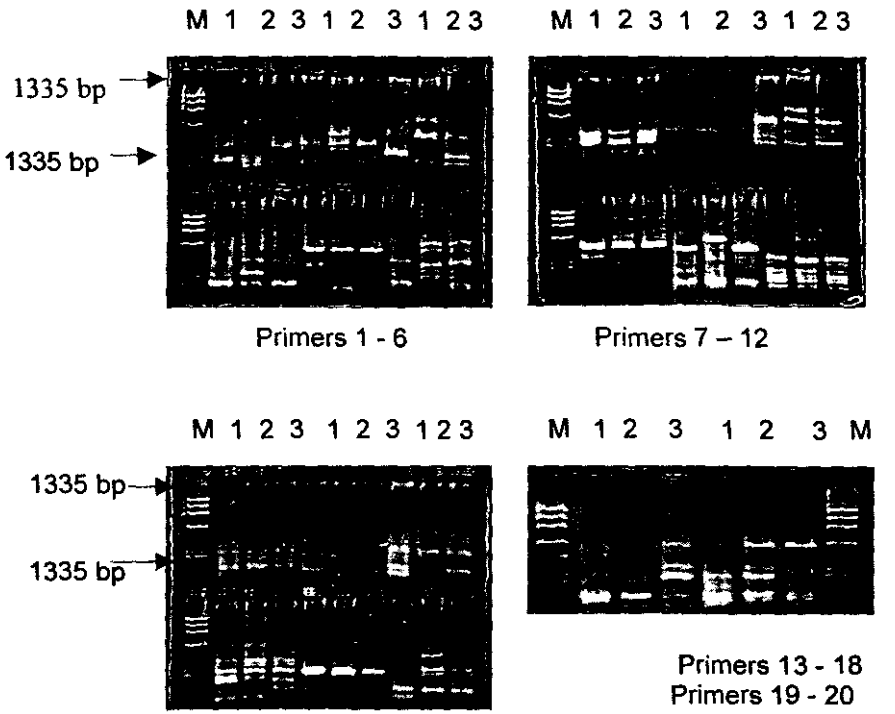

Figure 2: RAPD amplification products generated by different random primers (1-3) Lane M: DNA marker, Lane 1: queen, Lane 2: male, Lane 3: worker.

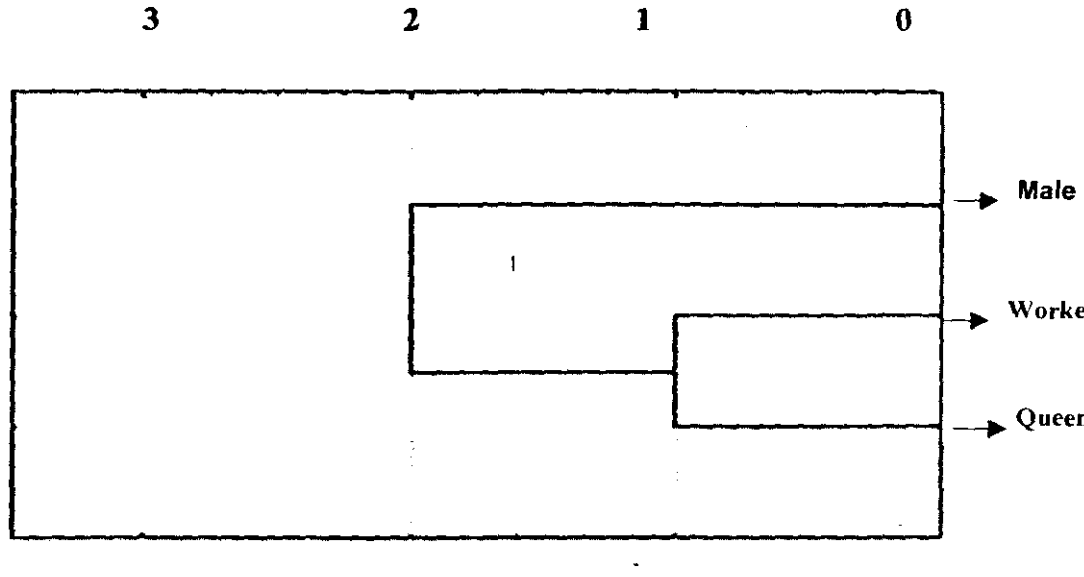

Figure 2: Dendrogram using average of similarity percentages (between groups) by of Statistica Program the honeybee (queen, male and worker) based on 20 RAPD primers.

DISCUSSION AND CONCLUSION

The sensitivity of RAPD technique played a good role in detection of these differences as observed by Hawksworth, 1994; and Haig, 1998. Thus, the RAPD profile generated for each caste can be effectively used as a supporting marker for taxonomic purposes, in relationship identification of honeybees and as marker-assisted selection during honeybee breeding programs. In taxonomy and systematic relationship, molecular markers could be a tool for honeybee's verification and in establishing the status of organism systematically and its evolution (Loxdale 1994 and Loxdale *et al.* 1996). The RAPD technique utilizes arbitrary sequences as primers with GC content > 50%. Time consuming and expensive synthesis of special primers could be avoided. A set of commercially available primers can be used for different species (Klein-Lankhorst *et al.* 1991).

Our results reflect the usefulness of such modern and fine molecular techniques. These results demonstrate the usefulness of the RAPD approach for detecting DNA similarity or polymorphisms in honeybees and establishing relationships between and among the different castes queen, male and worker. All the amplification products were found to be reproducible when reactions were repeated using the same reaction conditions.

A series of several DNA fragments (Fig. 1) were obtained from the samples under investigation. The number of fragments generated per primer varied between one and seven fragments. In general, the genetic similarity as band sharing values ranged from 71.95 to 80.45 %. This result may due to the parent selected over long periods. The genetic variation among the honeybees may be due to differences in sex, function and the evolution of each detected caste.

The data obtained showed some advantages of RAPD markers for detecting similarities as BS between and among the honeybees' different individuals. All the amplification products were found to be reproducible when reactions were repeated using the same reaction conditions. These results reflect the relationship between RAPD patterns and quantitative trait loci (QTL) of each genotype. The results are in agreement with the findings of several authors e.g. (Hoy, 1994; Zhang *et al.*, 2002 and Ali *et al.* 2003).

RAPD analysis has been used for constructing a phylogenetic tree (Fig. 2) using average linkage between groups rescaled distance cluster combine (Sneath and Sokal, 1973). This method has been used for constructing trees in other organisms: insect Karp *et al.*, 1998; chicken Ali *et al.*, 2003; farm animals Appa Rao *et al.*, 1996 and tilapia fish Baradakci and Skibinski, 1994.

In conclusion it may be emphasized that most of the RAPD primers used amplified successfully genomic DNA of the honeybee castes under study (queen, male and worker). All comparative results gave the same specific bands between the queen, male and worker. This similarity DNA fingerprint provides a rapid and effective method of detecting the genetic variation of different insect genotypes, also employ such DNA fingerprinting as marker-assisted selection during honeybee breeding programs.

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الملخص العربى

تحليل درجة القرابة الو راثي في حشرات نحل العسل في مصر باستخدام العلامات الجزينية

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قسم بحوث الأحماض النووية ، معهد بحوث الهندسة الوراثية و التكنولوجيا الحيوية ، مدينة مبارك للأبحاث العلمية ، مدينة برج العرب الجديدة ، الإسكندرية.

الهدف من هذه الدراسة التعرف علي درجة القرابة بين حشرات نحل العسل (الملكة والذكور والشغالات) ورائيا. تكنيك ال RAPD-PCR طبق باستخدام ٢٠ من البادئات منهم ١٥ بادئي طولهم ١٠ قاعدة و٥ بادئات بطول ٢٠ قاعدة استخدموا لاكتشاف التشابه الوراثي والحزم المشتركة بين حشرات نحل العسل. بمقارنة قيمة متوسطات الحزم المشتركة كانت ٧٩ و ٨٠ ٧١ % بين الملكة والذكور، الملكة و الشغالات، الذكور والشغالات على التوالي وقد ظهرت اقل نسبة تشابه بين الملكة والذكور. ونتيجة تحليل طريقة RAPD-PCR تمتاز بالسرعة والدقة لاكتشاف الاختلاف الوراثي بين حشرات نحل العسل . و هذه النتائج يمكن ان تستخدم كمعلومات مساعدة للانتخاب خلال برامج تربية حشرات نحل العسل.