

Biochemical and molecular characterization for three subspecies of honey bee worker, *Apis mellifera* L. (Hymenoptera: Apidae) in Egypt

Saadya M. El-Bermawy, Khalafalla S. Ahmed, Heba Z. Al-Gohary and Abeer M. Bayomy

Biological and Geological Sciences Department, Faculty of Education, Ain Shams University, Roxy, Heliopolis, P.C.11341, Cairo, Egypt

ABSTRACT

Protein, isozymes and RAPD-PCR were conducted to assess the biochemical and molecular characterization for three subspecies of honey bee worker, *Apis mellifera* L. Low polymorphism percentages were recorded in different protein patterns ranging from 18 to 42%. Eighteen, five and four unique bands distinguished the Egyptian, the Italian and the Carniolan subspecies, respectively. The isozyme systems recorded low polymorphism percentages, except peroxidase (67%). High levels of polymorphism were observed in RAPD-PCR profiles. 28 unique bands were identified out of 39 polymorphic ones; fourteen in the Egyptian subspecies, six in the Italian subspecies and eight in the Carniolan subspecies. These unique bands were considered as molecular markers for these subspecies. The dendrogram separated the Egyptian subspecies from the other two subspecies with a highly genetic distance of 0.25. The Italian and Carniolan subspecies were grouped into one main cluster with a genetic distance of 0.01 between them.

Key words: *Apis mellifera*, protein, isozymes, RAPD, PCR, polymorphism

INTRODUCTION

The original distribution range of honey bee, *Apis mellifera* L. included Africa, Europe (except the northern part) and the Near East. Within this large area the species was differentiated into several evolutionary branches. Up to 29 subspecies have been distinguished on the basis of morphological traits (Sheppard and Meixner, 2003) and these were originally grouped into five evolutionary lineages: (M) from northern and western Europe and northern Africa, (A) from southern and central Africa, (C) from the northern Mediterranean region and eastern Europe, (O) from the eastern Mediterranean and the Near and Middle East region, and (Y) from the east African country of Ethiopia (Ruttner, 1988; Hall and Smith, 1991; Garnery *et al.*, 1992; Arias and Sheppard, 1996; Franck *et al.*, 2000, 2001). Based on various phylogenetic parameters the speciation event that produced *A. mellifera* has been estimated to have occurred between 0.7 to 1.3 million years ago (Ruttner, 1988; Cornuet and Garnery, 1991; Arias and Sheppard, 1996). The taxonomy of honey bee has been in chaos for the last many decades. Morphometric studies have grouped the available subspecies into four lineages (Ruttner, 1988, 1992; Sheppard *et al.*, 1997). As the morphometric studies are challenged continuously molecular level study was begins recently with the help of allozymes (Nunamaker and Wilson, 1982; Badino *et al.*, 1988), nuclear DNA (Hall, 1990; Tarès *et al.*, 1993), mitochondrial DNA (mtDNA) (Moritz *et al.*, 1986; Smith *et al.*, 1989, 1991; Hunt and Page, 1992; Garnery *et al.*, 1993; Oldroyd *et al.*, 1995; Arias and Sheppard, 1996; Pedersen, 1996; De la Rúa *et al.*, 2000) and microsatellites (Estoup *et al.*, 1993; Garnery *et al.*, 1998).

Protein and enzyme polymorphism in honeybees proved to be useful in developmental studies, population genetics and in classification (Hamrick, 1989). Different methods of DNA analyses have been fragmentarily applied to clarify the race standard of the local honey bee (Ivanova *et al.*, 1998; Ivanova and Bouga, 2009; Ivanova *et al.*, 2010). RAPDs are able to produce multiple bands fragments. These fragments are usually generated from different regions of the genome and hence multiple loci may be examined very quickly (Edwards 1998).

In the present study, experiments were conducted to characterize three subspecies of honey bee workers, *Apis mellifera* L.; *A.m. lamarckii* (Egyptian), *A.m. ligustica* (Italian) and *A.m. carnica* (Carniolan), using biochemical (protein and isozymes) and molecular (RAPD-PCR) markers. And to illustrate the relationship among them.

MATERIAL AND METHODS

Material:

The three subspecies of honey bee worker, *Apis mellifera* L.; *A.m. lamarckii* (Egyptian), *A.m. ligustica* (Italian) and *A.m. carnica* (Carniolan), were supplied by Entomology Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Methods:

a- Protein analyses:

SDS-polyacrylamide gel electrophoresis was performed in 12% acrylamide slab gels following the system of Laemmli (1970). Native proteins, protein bound carbohydrates (glycoproteins) and protein bound lipids (lipoproteins) of the six organs; head, sting, haemolymph, honey stomach, mid gut and pitrophic membrane of the three subspecies of the honey bee worker, *A. mellifera* were separated using non-denaturing discontinuous polyacrylamide gel electrophoresis (PAGE) according to the method of Davisb (1964).

b- Isozymes analyses:

The Isozymes used were: α - and β -esterases (Est.), Acid phosphatase (Acph.), alcohol dehydrogenase (Adh.), aldehyde oxidase (Ao.), Malic enzyme (Me) malate dehydrogenase (Mdh) and peroxidase (Px). Isozymes were separated in 10 % Native-polyacrylamide gel electrophoresis as described by Stegemann *et al.* (1985). In gels staining, protocols of Scandalios (1964) were used for α and β -Est.; Wendel and Weeden (1989) for Ao and Acph; Weeden and Wendel (1990) for Adh; Jonathan and Wendell, (1990) for Mal and Mdh; Heldt, W.H., (1997) for Px. After bands appearance, gels were washed two or three times with tap water; fixed in ethanol: 20% glacial acetic acid (9:11 v/v) for 24 hours; and photographed.

c- Molecular analysis

Five primers of the PCR-based technique; RAPD were used. The DNA was extracted according to the method of Sambrook *et al.* (1989). For RAPD analysis, five 10-mer random DNA oligonucleotide primers (Operon Technologies, Inc, USA) of arbitrary sequences were independently used in PCR reactions as described by Williams *et al.* (1990). Codes and sequences of these primers were listed in Table (1). Amplifications were performed in 50 μ l reaction volume containing: 0.2 mM dNTPs, 1.5 mM MgCl₂, 5.0 μ l 10X buffer, 0.2 μ l Primer, 3.0 μ l template DNA (50 ng/ μ l) and 0.3 μ l Taq DNA polymerase (5U/ μ l). Each of the reaction mixtures was overlaid with a drop of light mineral oil per sample. Amplifications were carried out in Perkin Elmer thermocycler.

Table 1: List of primers and their nucleotide sequences.

Primer code	Sequences
OP- A18	5' AGG TGA CCG T 3'
OP-C05	5' GAT GAC CGC C 3'
OP-C13	5' TGG TGG ACC A 3'
OP-D13	5' GGG GTG ACG A 3'
OP-I17	5' TGG TGG ACC A 3'

The optimal conditions for PCR amplification were as follows: an initial 4 minutes denaturation step at 94 °C followed by 37 cycles of 1 minute at 94 °C, 1 minute at 37 °C and 2 minutes at 72 °C, with a final extension step at 72 °C for 8 minutes. A volume of 15 µl of the RAPD products were electrophoresed in 1.2 % agarose gel and run was performed at 100 V for about 60 minutes in Pharmacia submarine (20cm x 20cm). The bands were visualized on UV trans-illuminator and photographed by gel documentation system. Bands of RAPD technique was visualized on UV- transilluminator and photographed by Gel documentation system (Biometra Bio Doc Analyze 2000).

d- Data analysis

Differences in bands intensity among profiles of the different samples were not considered. The binary data generated is used to estimate levels of polymorphism by dividing the polymorphic bands by the total number of scored bands. Then the presence or absence of each protein, isozyme and RAPD band was treated as a binary character in a data matrix (coded 1 and 0, respectively) to calculate genetic similarity and to construct dendrogram tree among the studied three subspecies. Genetic distance was calculated by the following formula (Genetic distance = 1- similarity coefficient) according to Nei and Li (1979) as implemented in the computer program SPSS-11.

RESULTS AND DISCUSSION

In the present study, SDS-protein, native protein, glycoprotein, lipoprotein, isozymes and randomly amplified polymorphic DNA based polymerase chain reaction (RAPD-PCR) analyses are conducted to detect the biochemical and molecular polymorphism among three different subspecies of honey bee worker, *A. mellifera*. Schiff and Sheppard (1995) used mitochondrial DNA (mtDNA) and allozyme variation to characterize 142 breeder queen colonies from 22 apiaries in the southeastern United States.

The overall results of the utilized four protein systems for identifying samples of the six organs; head, sting, heamolymph, honey stomach, mid gut and pritrophic membrane of the three subspecies of honey bee worker, *Apis Mellifera* L. were pooled together in Figure (1) and Table (2). 128 bands were recorded in the four- protein systems, the highest number (57) was in native pattern with highest polymorphism percentage (42%). Eighty monomorphic and fourty eight polymorphic bands were scored. 27 uinique bands could be considered as protein (biochemical) markers to discriminate among the three subspecies of the honey bee worker. Ivanova *et al.*, (2000) investigated two thousand specimens of *A. mellifera* from three domesticated populations in Bulgaria by different means of gel electrophoresis, and found specific larval protein bands which discriminated among the larval, pupal and the adult stages.

Table 2: Pooled data of the protein patterns used for identifying samples of the six organs; head, sting, haemolymph, honey stomach, mid gut and pritrophic membrane of the three subspecies of honey bee worker, *Apis mellifera* L. as well as the percentage of polymorphism detected by each pattern.

Protein system	Monomorphic band	Polymorphic band		Total	Polymorphism (%)
		Unique	Shared		
SDS	30	6	11	47	36
Native	33	14	10	57	42
Glycoprotein	8	5	0	13	38
Lipoprotein	9	2	0	11	18
Bands total	80	27	21	128	37.5

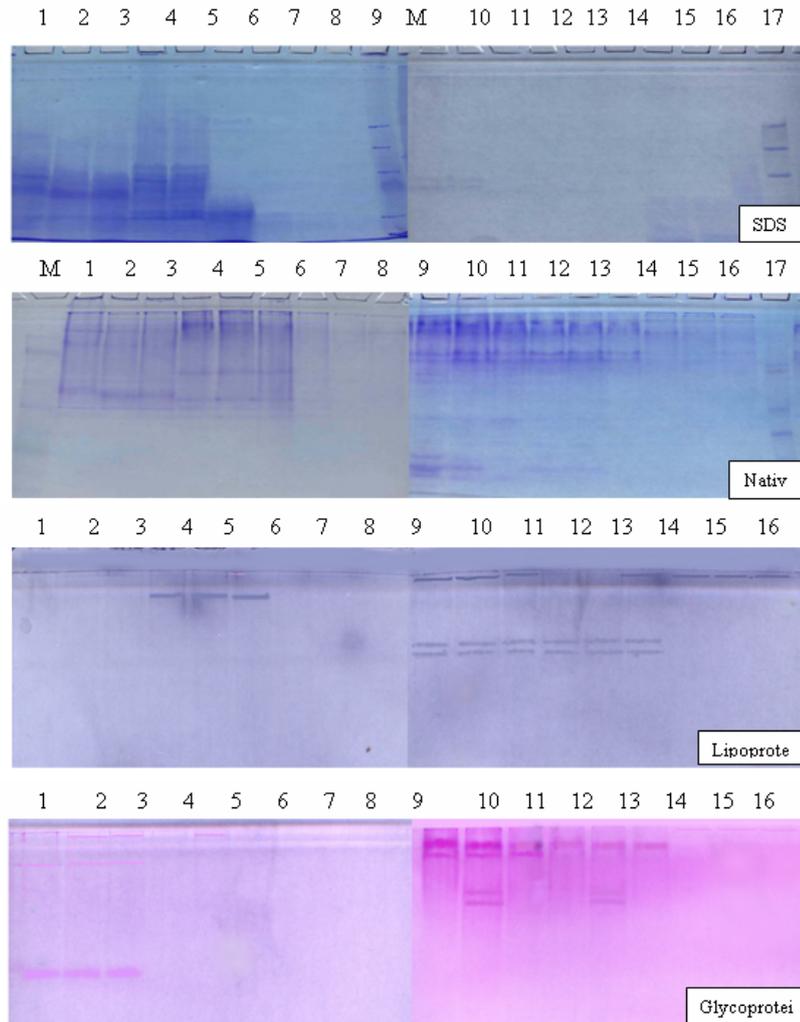


Fig.1: The produced protein profiles of three subspecies of *Apis mellifera* L. using SDS-PAGE, native, lipoprotein and glycoprotein techniques. E=Egyptian, I=Italian and C=Carniolan.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
E	I	C	E	I	C	E	I	C	E	I	C	E	I	C	E	I	C
Head			Haemolymph			Honey stomach			Mid gut			Pritrophic mem.			Sting		

Eighteen unique bands characterized the Egyptian subspecies; four in SDS pattern, twelve in native pattern and one in each of glycoprotein and lipoprotein patterns. On the other hand, five and four unique bands distinguished the Italian and the Carniolan subspecies, respectively.

These results were in agreement with Zalut *et al.*, (2002) who reported bands that discriminated between the venom of the Egyptian honeybee and Carniolan bees. In general, low polymorphism percentages were recorded in different protein patterns

ranging from 18 to 42% as shown in Table (2). This agreed with Yu and Jian-ke (2009) who detected significant differences in the proteome profile between the high royal jelly producing bees and the native Italian bees. Also, Vagner and Da Cruz-Landim (2004) used the electrophoretic pattern of proteins to establish a correlation with the metamorphosis events, and they recorded greatest variation in protein electrophoretic bands during prepupal stage and the smallest variation during the brown eyed pupae stage.

Application of isozyme techniques has an important potential to provide a precise tool to study the relationships among insect species. Ivanova *et al.*, (2011) studied the genetic variability of honey bee populations of three subspecies selectively reared in Poland (*A. m. carnica* and *A. m. caucasica*) and Bulgaria (*A. m. macedonica* – type *rodopica*) using isoenzyme analysis of six enzyme systems (Mdh-1, Me, Est-3, Alp, Pgm and Hk). In the present study, eight isozymes polymorphisms were used to establish the biochemical genetic profile of Egyptian, Italian and Carniolan subspecies of the honey bee workers, *A. mellifera*. The results of the utilized eight-isozymes systems for identifying samples of the six organs; head, sting, hemolymph, honey stomach, mid gut and peritrophic membrane of the three subspecies of honey bee worker, *A. mellifera* are pooled together in Table (3) and as shown in Figure (2-a,b). 119 bands were recorded in the eight-isozymes systems, the highest number was 27 bands in β -*est* pattern, while the lowest was seven in *malic* enzyme which did not detect any polymorphism percentage. Eighty-one monomorphic and thirty-eight polymorphic bands were scored in the eight-isozymes systems. 21 unique bands could be considered as isozymes (biochemical) markers to discriminate among the three subspecies of the honey bee worker. Seventeen unique bands characterized the Egyptian subspecies; six in β -esterase pattern, four in *Px* pattern, two in each of *Alo* and α -esterase and one in each of *Acph*, *Adh* and *Mdh* patterns. On other hand, four unique bands distinguished the Carniolan subspecies; as one in each of *Acph*, *Adh*, α - and β -esterase patterns. The Italian subspecies did not record any marker in eight-isozyme systems. Also, as in protein patterns, the isozymes systems recorded low polymorphism percentages, except peroxidase (67%), as shown in Table (3).

Table 3: Pooled data of the isozymes patterns used for identifying samples of the six organs; head, sting, hemolymph, honey stomach, mid gut and peritrophic membrane of the three subspecies of honey bee worker, *Apis mellifera* L. as well as the percentage of polymorphism detected by each pattern.

Isozyme system	Monomorphic band	Polymorphic band		Total	Polymorphism (%)
		Unique	Shared		
<i>Acph</i>	7	2	0	9	22
<i>Adh</i>	18	2	3	23	22
<i>Alo</i>	9	2	2	13	31
α - <i>Est</i>	12	3	5	20	40
β - <i>Est</i>	15	7	5	27	44
<i>Malic</i>	7	0	0	7	0
<i>Mdh</i>	10	1	0	11	10
<i>Px</i>	3	4	2	9	67
Bands total	81	21	17	119	32

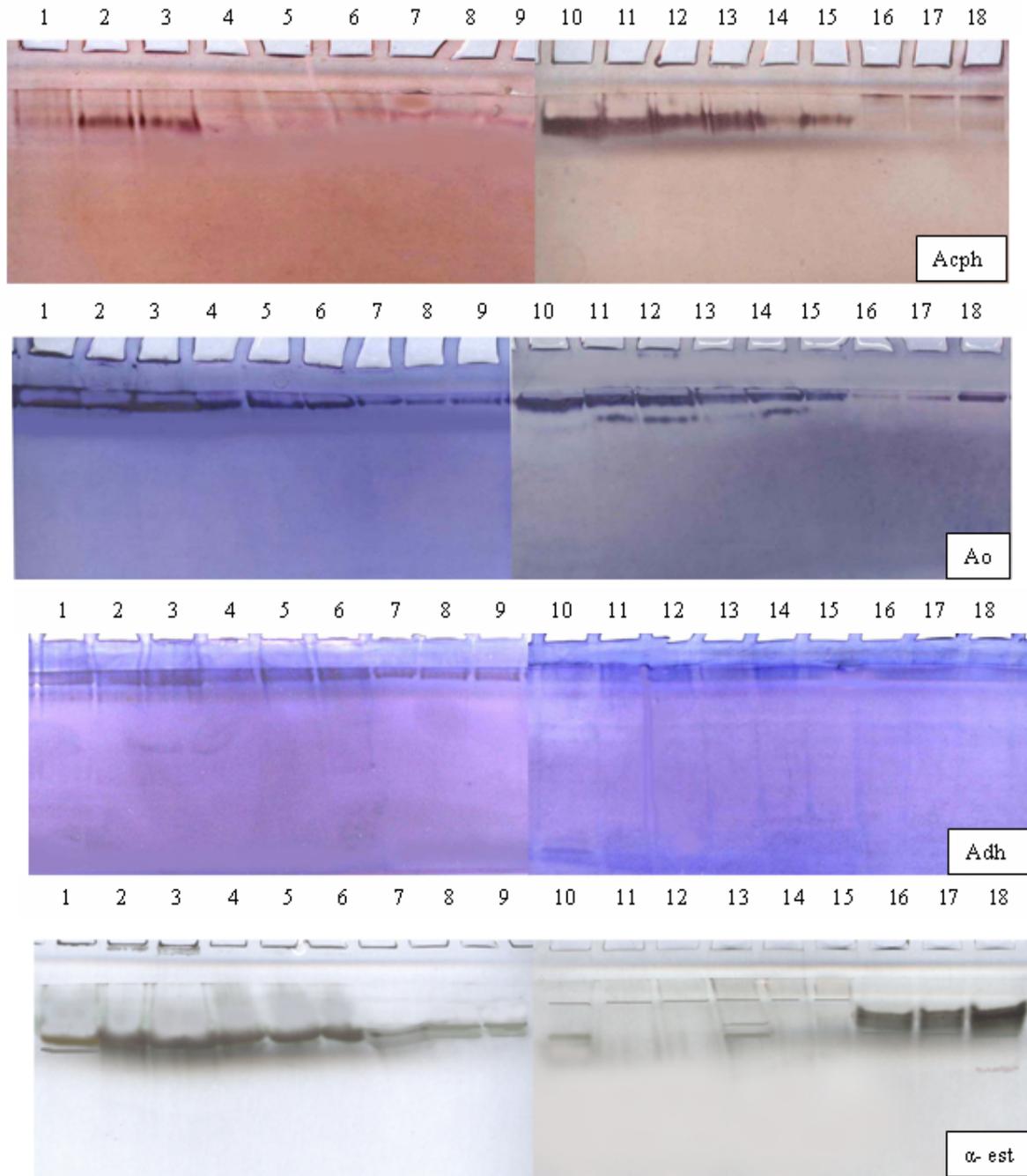


Fig. 2-a: The produced zymograms of three subspecies of *Apis mellifera* L.by using isozyme techniques. acid phosphatase (Acph.), aldehyde oxidase (Ao.), alcohol dehydrogenase (Adh.) and α -esterase (α - Est.). E=Egyptian, I=Italian and C=Craniolan.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
E	I	C	E	I	C	E	I	C	E	I	C	E	I	C	E	I	C
Head			Haemolymph			Honey stomach			Mid gut			Pritrophic mem.			Sting		

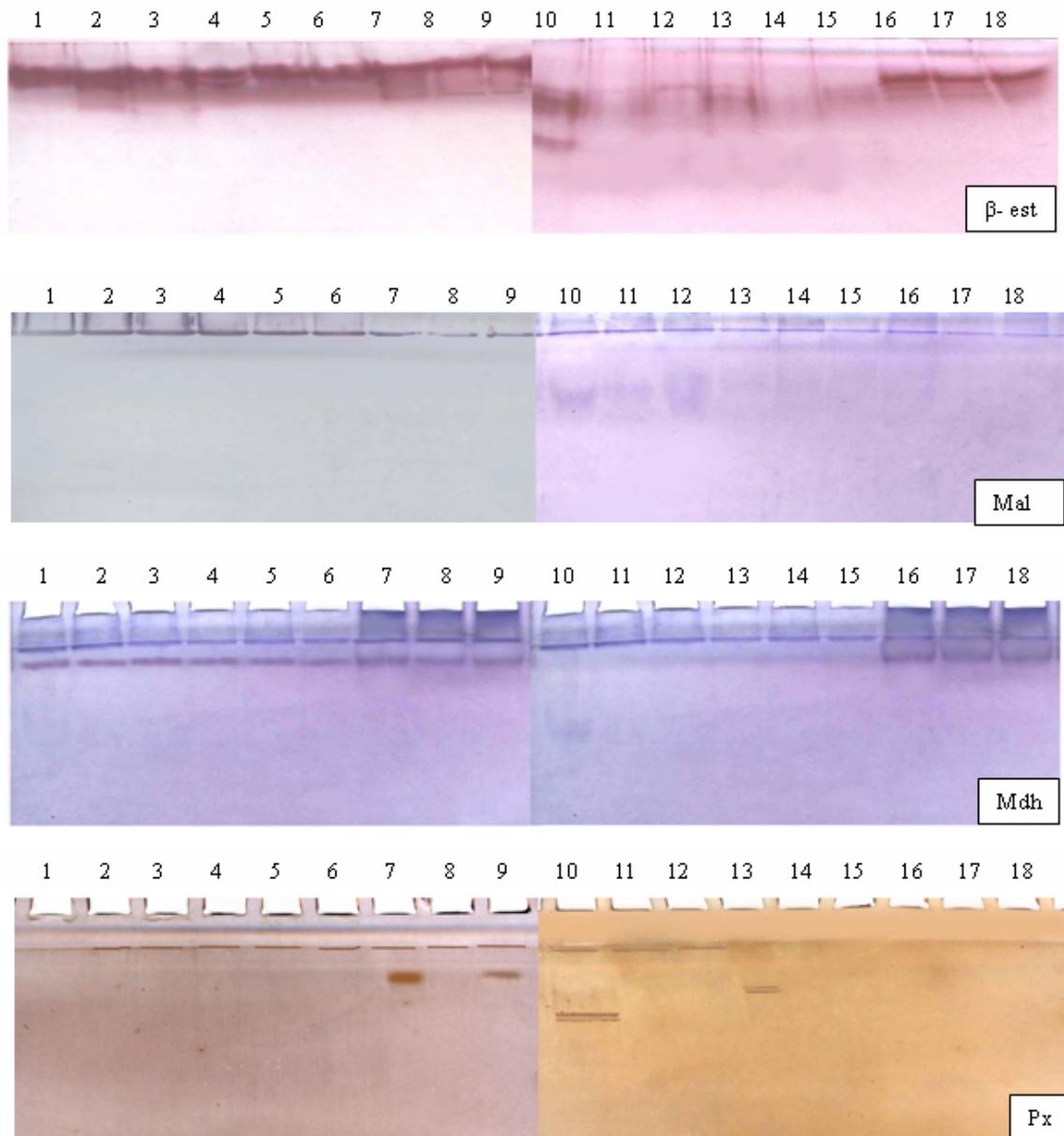


Fig. 2-b: The produced zymograms of three subspecies of *Apis mellifera* L. by using isozyme technique. B. β -esterase (β - Est.), Malic enzyme (Mal), malate dehydrogenase (Mdh.) and Peroxidase (Px). E=Egyptian, I=Italian and C=Craniolan.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
E	I	C	E	I	C	E	I	C	E	I	C	E	I	C	E	I	C
Head			Haemolymph			Honey stomach			Mid gut			Pritrophic mem.			Sting		

Also, Tong *et al.*, (2002) indicated that the four subspecies of *A. mellifera* showed the same esterase zymogram. Schiff and Sheppard (1995) found that of the 6 enzymes known to be polymorphic in adult honey bees, only malate dehydrogenase was polymorphic in the commercial colonies of their study. It is obvious that no one of the protein or isozyme indices used above can stand alone to provide sufficient polymorphic profile to distinguish among the three subspecies of the honey bee worker, *A. Mellifera*. Therefore, combined class patterns seem to solve this problem as they offer higher resolution to characterize each subspecies and assign it to a unique class pattern.

These results were similar to that of Tendero *et al.*, (2007) who detected low genetic variations in studied isozymes within population of European honeybee, *Apis*

mellifera carnica P., and mentioned that could be due to non-random mating in honeybees.

RAPD is a very important part of the combination of methods planned for studying the discrimination of honey bee populations (Magnus *et al.*, 2011). This reveals polymorphisms which are useful as genetic and taxonomic markers based on RAPD-PCR to determine the phonetic relationship at intraspecific level (Bardakci, 2001). Five 10-mer arbitrary oligonucleotide primers of RAPD-PCR; OP-A18, C05, C13, D13 and I17 as in Figure (3) and Table (1) were used to discriminate among three subspecies; Egyptian, Italian and Carniolan, of the honey bee worker, *Apis mellifera* L. Suazo *et al.*, (1998) used nuclear genetic markers in the form of random amplified polymorphic DNA (RAPD) to distinguish African and European honey bees (*A. mellifera*).

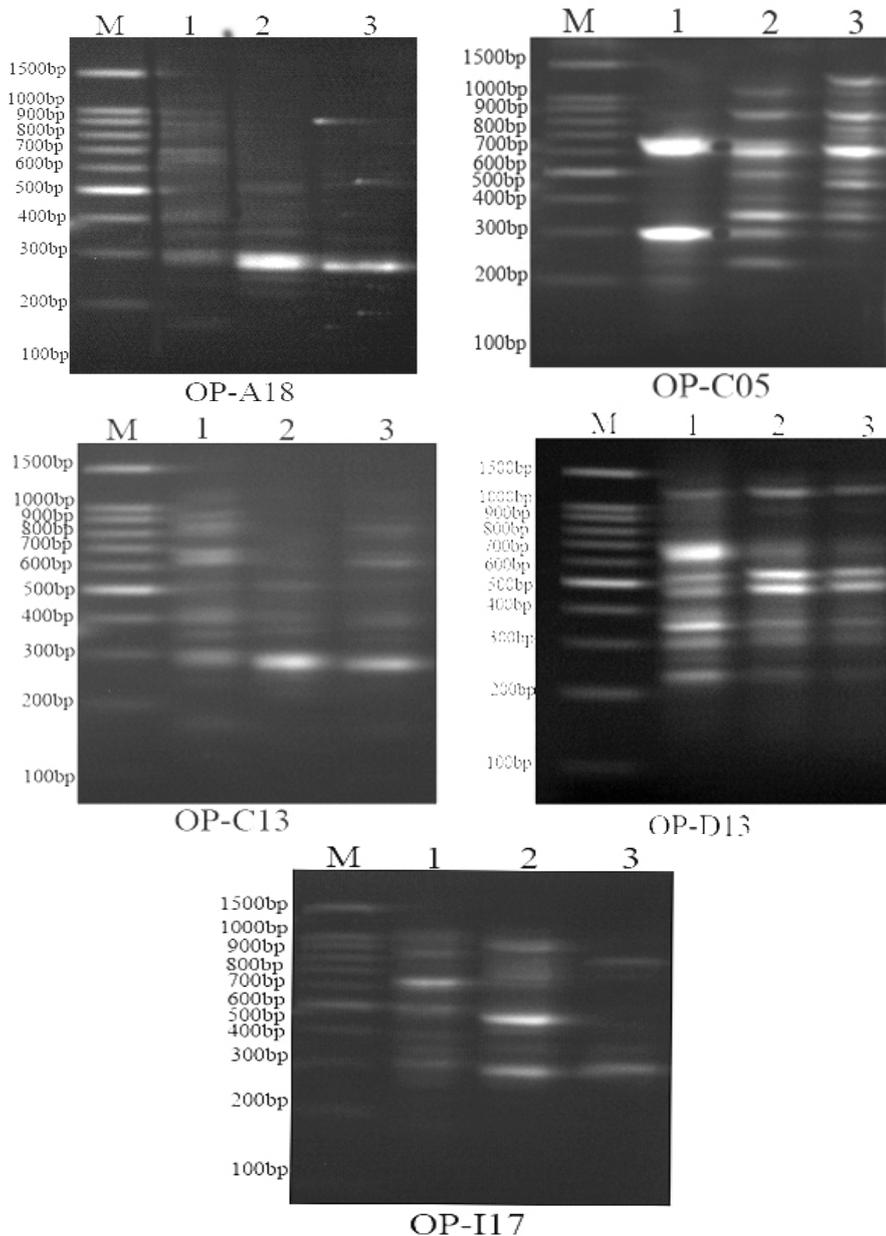


Fig. 3: RAPD profiles of three subspecies of *Apis mellifera* L. generated with the random primers OP A18, C05, C13, D13 and I17. M = marker. 1=Egyptian, 2=Italian and 3=Carniolan.

A maximum of 13, 13, 8, 7 and 8 DNA bands were scored in the RAPD profiles generated by the primers OPA18, C05, C13, D13 and I17, respectively (Table 4). No monomorphic bands were detected in primers OP A18 and I17. A sum of 39 polymorphic bands were generated by these primers in the samples under study. In the present study, a total of 28 unique bands were identified out of the polymorphic ones; fourteen in the Egyptian subspecies, six in the Italian subspecies and eight in the Carniolan subspecies. These unique bands were discriminated among the three subspecies and could be used as molecular markers for these subspecies. High levels of polymorphism were observed in RAPD-PCR profiles and ranged between 29 to 100% as shown in Table (4). The previous results were in accordance with Suazo *et al.*, (1998) who reported RAPD markers that were specific to groups of honey bee subspecies and expected to be useful in distinguishing African and European bees. Advantages of analyzing genetic polymorphisms at DNA level are many fold (Cavalli-Sforza, 1998). DNA contains more information than protein sequences do as well as many genetic polymorphisms.

Table 4: Number and types of the amplified DNA bands as well as the percentage of the total polymorphism generated by five primers in the three subspecies of honey bee worker, *Apis mellifera* L.

Primer code	Monomorphic bands	Polymorphic bands		Total bands	Polymorphism %
		Shared	Unique		
OP-A18	0	2	11	13	100
OP-C05	2	4	7	13	85
OP-C13	3	3	2	8	63
OP-D13	5	1	1	7	29
OP-I17	0	1	7	8	100
Total	10	11	28	49	75.4

Genetic similarity matrix based on protein, isozymes and RAPD.-PCR data among the three subspecies of the honey bee worker, *A. mellifera* is presented in Table (5). The highest similarity value (0.854) was recorded between the Italian and Carniolan subspecies, meanwhile the lowest genetic similarity coefficient (0.779) was observed between the Egyptian and Carniolan subspecies. The dendrogram illustrated in Figure (4) separated the Egyptian subspecies from the other two subspecies with a highly genetic distance of 0.25 between them. The Italian and Carniolan subspecies were grouped into one main cluster with a genetic distance of 0.01 between them. This agreed with Francoya *et al.*, (2006) revealed the variance between Africanized bees and honey bees of European origin through detection of significant differences between commercial USA Italian bees, German Carniolan bees and Africanized honey bees based on multivariate analysis. In conclusion, biochemical (protein and isozymes) and molecular (RAPD-PCR) markers revealed the intraspecific variations and genetic relationships of honey bee worker subspecies and separated the Italian and Carniolan bees of three subspecies into one group.

Table 5: Similarity matrix of the three subspecies of the honey bee worker, *Apis mellifera* L. based on the pooled results derived from protein, isozymes and RAPD-PCR analyses.

Subspecies	Egyptian	Italian	Craniolan
Egyptian	1.00	-	-
Italian	0.824	1.00	-
Craniolan	0.779	0.854	1.00

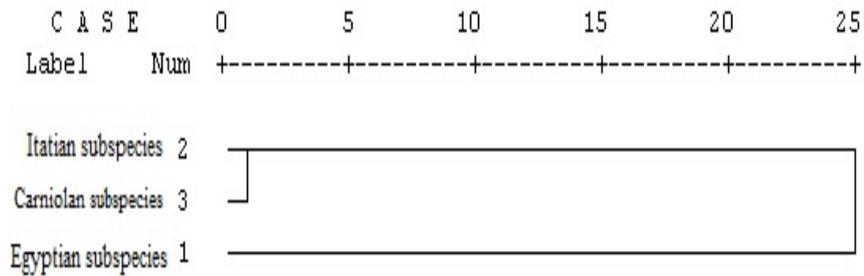


Fig. 4: Dendrogram demonstrates the relationship among the three subspecies of the honey bee worker, *Apis mellifera* L. based on the pooled results derived from protein, isozymes and RAPD-PCR analyses.

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ARABIC SUMMARY

التوصيف البيوكيميائي والجزئي لثلاثة نويغات من شغالات نحل العسل/أبيس ميليفيرا (رتبة: غشائية الأجنحة- فصيلة أبيدي) في مصر"

سعدية محمد البرماوي- خلف الله صابر أحمد- هبة زكريا عبد السميع – عبير مصطفى بيومي
قسم العلوم البيولوجية والجيولوجية- كلية التربية- جامعة عين شمس

استخدمت تقنيات التفريد الكهربى للبروتينات والمشابهاة الإنزيمية وسلسلة تفاعلات إنزيم البلمرة لقطع الدنا المكثرة عشوائياً لتوصيف ثلاثة نويغات من شغالات نحل العسل أبيس ميليفيرا (رتبة: غشائية الأجنحة- فصيلة أبيدي " بيوكيماوياً وجزئياً. وقد سجلت نتائج التفريد الكهربى للبروتينات نسبة منخفضة من التباين تراوحت ما بين 18 الى 42%. وسجلت الأنواع الثلاثة المصري والإيطالي والكرينولي عدد 18 و5 و4 حزم متفردة على الترتيب. كذلك أظهر التفريد الكهربى للمشابهاة الإنزيمية نسبة منخفضة من التباين ما عدا إنزيم البيروكسيداز الذى سجل 67%. بينما أظهرت تقنية إنزيم البلمرة لقطع الدنا المكثرة عشوائياً تبايناً كبيراً بين الأنواع الثلاثة، حيث تم تسجيل عدد 28 من حزمة متفردة من بين 39 حزمة متباينة، منها 14 حزمة في النوع المصري وستة في النوع الايطالي وثمانية في النوع الكرينولي. وقد اعتبرت هذه الحزم المتفردة ككاشفات وراثية جزئية لهذه الأنواع الثلاثة. وفرقت شجرة القرابة بين الثلاثة على النحو التالي: حيث فصلت النوع المصري بعيداً عن النوعين الآخرين بمسافة وراثية كبيرة قدرها 0.25، بينما جاء النوعان الإيطالي والكرينولي فى مجموعة واحدة بمسافة وراثية تقدر ب 0.01. وأظهر البحث كفاءة هذه التقنيات فى توصيف وإظهار الفروق الوراثية وعلاقات القرابة فى شغالات نحل العسل أبيس ميليفيرا.