

Genetic Diversity and Phylogenetic Relationship among Three Apocrita Species (Hymenoptera) From Qena Governorate, Egypt Based On RAPD Markers

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The genomic DNA of three Apocrita species (Hymenoptera) namely, *Vespa orientalis* [Vespoidea; Vespidae], *Xylocopa aestuans* [Apoidea; Apidae] and *Apis mellifera* [Apoidea; Apidae] has been subjected to RAPD-PCR analysis with 10 deca-oligonucleotides [A-01, A-02, A-03, A-04, A-05, A-07, A-08, A-09, A-11, and A-12]. All primers produced discrete and instructive RAPD bands of various lengths ranging from 200 to 3000 base pair. Ninety seven amplified fragments/bands were produced, from them 4 bands were common (monomorphic) and 93 were polymorphic; those correspond to a level of polymorphism of 95.88 % with an average number of polymorphic fragments per primer of 9.3 band. The *Xylocopa aestuans* recorded the highest band frequencies among the studied species.

Based on the Nei-72 distance matrix of genetic and the unweighted pair group method average (UPGMA), the three species under study are related to each other and have common ancestor. However, both *Vespa orientalis* (Vespidae) and *Apis mellifera* (Apidae) are genetically closer. The splitting up *Xylocopa austeanus* into sister clade to *Vespa orientalis* and *Apis mellifera* may be a sign to reconsider placement of genus *Xylocopa* within Apidae.

Key words: Genetic diversity, Phylogeny, Apocrita, *Vespa orientalis*, *Xylocopa aestuanus*, *Apis mellifera*, RAPD-PCR, Qena, Clade

Introduction

Hymenoptera is the most extensive insect order, with over 100,000 species described worldwide. They comprise of sawflies, wasps, bees and ants harbouring all terrestrial and some aquatic habitats. Hymenoptera classified into two suborders, the Symphyta (9 superfamilies) and the Apocrita (18 superfamilies) [1]. The Apocrita represent more than 90% of all described hymenopteran species. They are the most biologically diverse suborder of insects, exhibiting wide range and different lifestyles [2-3]. From the Apocrita, Apidae and Vespidae families undoubtedly have considerable economic and environmental benefits. Many species are pollinator biotic agents which contribute to the pollination of several plants, while others consider important predators (biological control agents) of pest insect species [4-5].

Several studies were carried out to figure out the phylogenetic relationships among the hymenopterans insects including apocritans [6-10]. However the pioneer to present the first cladogram of hymenoptera based on the cladistic approach was the German dipterist Willi Hennig [11].

RAPD assay is a DNA fingerprinting technique to amplify polymorphic DNA segments of the genome by PCR using single arbitrary primers homologues to random sites on the genome [12-13]. These polymorphic segments (RAPD markers) can be used to estimate genetic distances and similarities from data measured directly on DNA. RAPD markers have been widely used in entomological investigations including genetic diversity and identification of species and sub species [14-17].

Because of the higher organization level of relationships within the Apocrita remains controversial [18]. Therefore, figuring out kind of phylogenetic relationships among apocrita species could help to understand the evolution of the most biologically diverse suborder of hymenopteran insects. The aim of this work is to study the genetic diversity and phylogenetic relationships among three species genotypes from Apocrita (*Vespa orientalis* [Vespoidea; Vespidae], *Xylocopa aestuans* [Apoidea; Apidae] and *Apis mellifera* [Apoidea; Apidae]) using RAPD PCR assay.

Materials and Methods

Taxon sampling

Three Apocrita species (Hymenoptera): *Vespa orientalis* [Vespoidea; Vespidae], *Xylocopa aestuans* [Apoidea; Apidae] and *Apis mellifera* [Apoidea; Apidae] were collected by the author at the University Campus area near an apiary (*Vespa orientalis* and *Apis mellifera*) and from a nearby village El-Tramsa (*Xylocopa aestuans*) Qena, Egypt. The thoracic regions including attached legs were removed and washed with deionised water, then by absolute ethylalcohol to remove field contaminants thereafter, insect samples were subjected to the DNA extraction.

Insect Genomic DNA Extraction

Genomic DNA was extracted according to the published method [19] following the instructions given. DNA samples were stored at -20°C until used. The concentration and purity of DNA were estimated spectrophotometrically by UV absorption at A260 and A280 and visualized on 0.8 % agarose gel electrophoresis.

RAPD-PCR Assay

The RAPD assay was performed using total genomic DNA as described by Williams *et al.* [12]. Amplification reactions were carried out in a final volume of 25 µl containing final concentration $1.0 \times$ from the pre-mixed OnePCR™ 2X (GeneDireX Inc, USA) using ~50 ng genomic DNA of each sample, 10 pM of each 10-mer primer separately. Ten deca-nucleotide primers were used for the DNA amplification [A-01, A-02, A-03, A-04, A-05, A-07, A-08, A-09, A-11, and A-12] (Bio Basic Inc, Canada).

The PCR reaction was performed in a thermocycler (Primus 25 advanced, PEQLAB Biotechnologie GmbH) with the following cycling conditions, including initial denaturation at 95°C for 2 min, followed by 45 cycles of the following: 94°C for 1 min, 36°C for 1 min and 72°C for 2 min, then one cycle of final extension at 72°C for 10 min.

The amplification products of 15 µl were separated by electrophoresis on 1.5% (w/v) agarose gels with TAE buffer (0.40 mM Tris, 0.20 mM acetate, 2 mM EDTA pH 8), stained with ethidium bromide (0.5 µg/ml) and photographed under UV light using the Elttror M20 SaS Photo-Gel System (Italy) provided with Nikon Coolpix LB40 digital camera. The size of amplification products was determined with 100 bp DNA ladder (0.1 µg/µl, Solis BioDyne, Estonia).

Data Analysis

For RAPD analysis, the polymorphic DNA bands were screened and identified from RAPD images using PyElph gel image analysis software [20]. DNA fingerprints were scored for the presence (1) or absence (0) of bands of different molecular weight sizes in the form of binary matrix and analysed using POPGENE version 1.32 [21]. The program was used to calculate the Nei's original measures of genetic identity and genetic distance [22]. A dendrogram was constructed based on the results of Nei's genetic distance using the unweighted pair group method average 'UPGMA' clustering method [23], and displayed using the Molecular evolutionary genetics analysis Version 6.0 (MEGA6) software [24]. For each primer used in RAPD assay, the total number of scored bands, number of polymorphic bands, and percentage of polymorphism were computed.

Result

RAPD and genetic Polymorphism analysis

A total of 10 primers were screened for the ability to generate consistently amplified band patterns and to assess polymorphism among three insect genotypes (Table 1). Ninety seven bands were produced which is an average of 9.7 bands per primer and 4 bands were common in the studied samples. Out of whole amplified fragments, 93 were polymorphic. This corresponds to a level of polymorphism of 95.88 % and an average number of polymorphic fragments/primer of 9.3 calculated as NPBands/10 primers (Table 1).

All primers generated multiple banding patterns with 6 to 14 polymorphic amplified DNA bands ranging in size from 200 to 3000 bp as compared to a 100 bp DNA Ladder (Solis Bio Dyne). Interestingly, data showed high molecular weight band of ~ 3000 bp only recorded for *Xylocopa aestuans* with primer A-12. This band was not observed for both *Vespa orientalis* and *Apis mellifera* with any of the tested primers.

A maximum number of 14 amplicons were amplified with primer A-12, while the minimum number of 6 fragments was amplified with primer A-11. All primers exhibited fully percentage of polymorphism (%100) except primers A-04, A-05, A-08 and A-09 with percentage of polymorphism ranged from 85.71 % to 91.67%.

The highest number of polymorphic bands (14) was obtained with primer A-12 while the lowest number of polymorphic bands (6) was obtained with primers A-08 and A-11. The band frequency per species were 0.4227, 0.5052, and 0.40206 for *Vespa orientalis*, *Xylocopa aesteanus* and *Apis mellifera*, respectively while band frequency per primer ranged from 0.0617 to 0.1443 as shown in figure 1. The RAPD genotyping banding profile generated by all primers is presented in figure 2.

Table 1. Characteristic of RAPD primers used. Number of amplified bands per species (NABands/Species), Band frequency per primer (Band Freq/Primer), Band frequency per species (Band Freq/ Species), Total number of amplified bands (TNABands), Number of polymorphic bands (NPBands), Number of monomorphic bands (NMBands), Polymorphism Percentage (POL%), and Range of amplified fragment in base pair (RAF [bp]).

Primer Code	Sequence 5'-----3'	NABands/Species			TNA Bands	Band Freq/ Primer	NP Bands	NM Bands	% POL	RAF [bp]
		<i>Vespa orientalis</i>	<i>Xylocopa aestuans</i>	<i>Apis mellifera</i>						
A-01	CAGGCCCTTC	3	6	4	10	0.1031	10	----	% 100.00	300--2000
A-02	TGCCGAGCTG	4	3	3	9	0.0928	9	----	% 100.00	350--2200
A-03	AGTCAGCCAC	5	2	3	8	0.0825	8	----	% 100.00	400--1900
A-04	AATCGGCTG	5	6	5	10	0.1031	9	1	% 90.00	300--2700
A-05	AGGGGTCTTG	6	8	4	12	0.1237	11	1	% 91.67	350--2800
A-07	GAAACGGGTG	3	4	4	9	0.0928	9	----	% 100.00	400--2300
A-08	GTGACGTAGG	5	2	3	7	0.0722	6	1	% 85.71	300--1850
A-09	GGGTAACGCC	4	7	5	12	0.1237	11	1	% 91.67	200--2900
A-11	CAATCGCCGT	2	5	1	6	0.0619	6	---	% 100.00	300--1450
A-12	TCGGCGATAG	4	6	7	14	0.1444	14	---	% 100.00	300--3000
Total		41	49	39	97		93	4	% 95.88	
Band Freq/Species		0.4227	0.5052	0.4021						

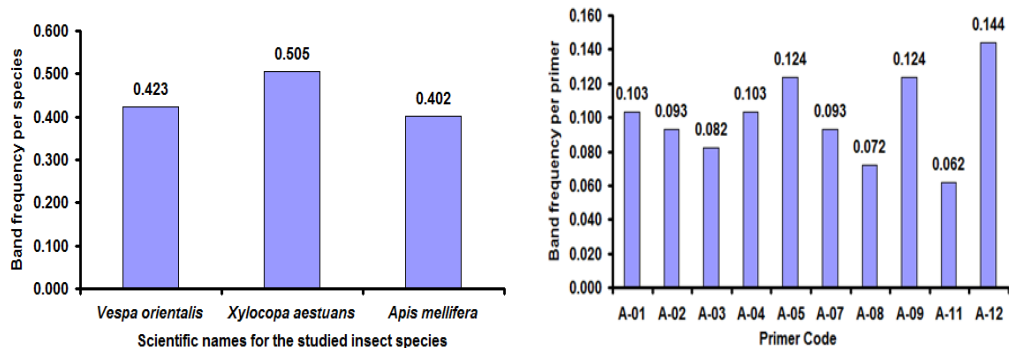


Figure 1. Band frequencies recorded for the Apocrita species, *Vespa orientalis*, *Xylocopa aestuans* and *Apis mellifera* (left) and per the 10 deca-nucleotide primers (right).

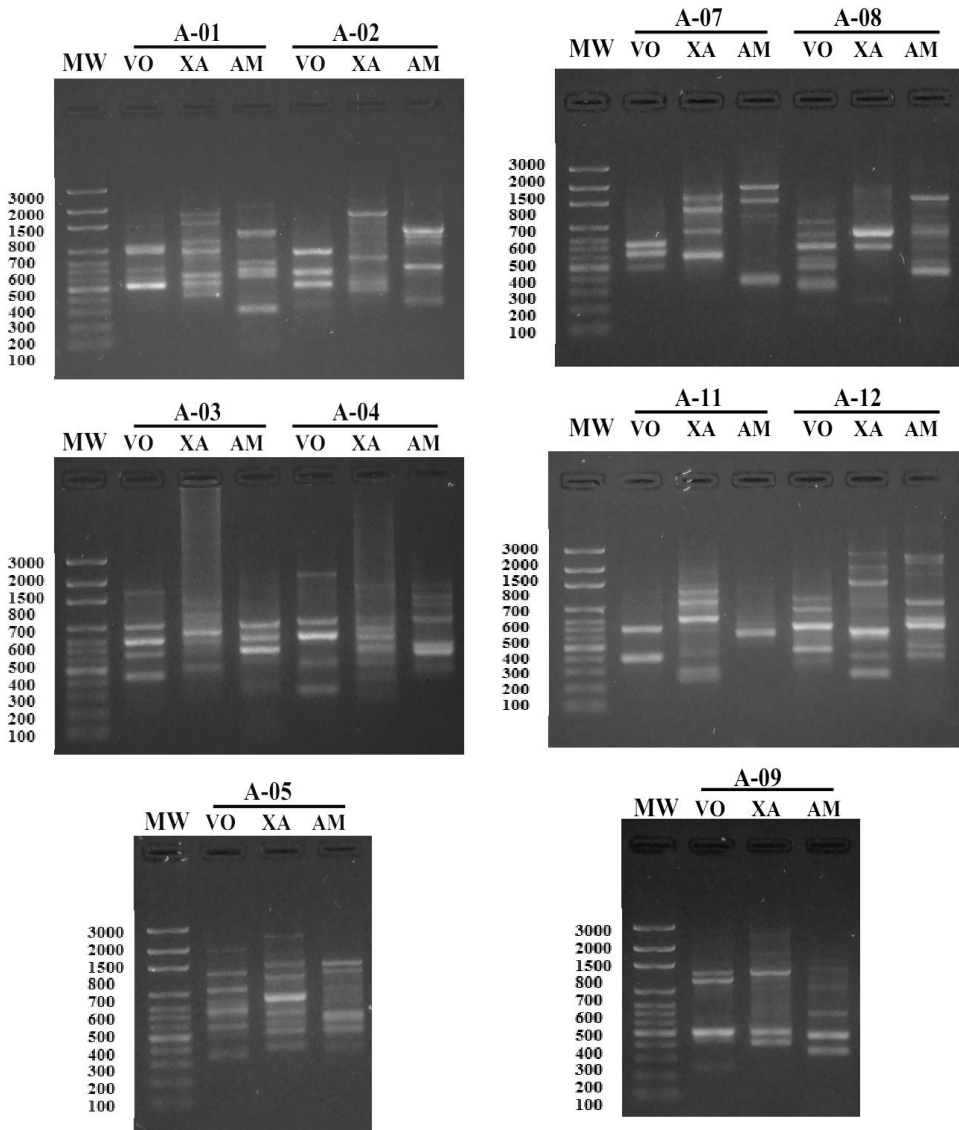


Figure 2. RAPD-PCR fingerprints obtained from three apocrita species generated by the 10 deca-nucleotides primers. MW: Molecular weight (100-3000 base pair), VO: *Vespa orientalis*, XA: *Xylocopa aestuans*, and AM: *Apis mellifera*

Phylogeny Analysis and Genetic relationships

Applying the unweighted pair group method average 'UPGMA' clustering method produced a tree based on the corresponding Nei-72 distance matrix of genetic using POPGENE version 1.32. The produced UPMGA tree (Figure 3) clearly distinguished both *Vespa orientalis*, and *Apis mellifera* grouped in single cluster as sister group having a closer relationship and share a common node (N1) in the phylogenetic tree (Figure 3). While *Xylocopa austeanus* split up as outgroup forming sister clade to them. The phylogenetic tree shows that *Vespa orientalis* is more closely related to *Apis mellifera* than to *Xylocopa austeanus* and all the three species having common ancestor (see N2).

According to the NEI-72 original measures of genetic identity and genetic distances (Table 2). The lowest genetic distances values of 0.9111 between *Vespa orientalis*, and *Apis mellifera*, while the highest genetic distance values of 1.1407 between *Xylocopa aestuans* and *Apis mellifera*. Based on their distance values, insect samples those closest to each other are *Vespa orientalis* and *Apis mellifera* with highest identity value of 0.4021, while insect species those are most distant to each other are *Xylocopa aestuans* and *Apis mellifera* with lowest identity value of 0.3196.

Table 2. Genetic identity and distance calculated for RAPD data binary character matrix based on Nei M, (1972). Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

	<i>Vespa orientalis</i>	<i>Xylocopa aestuans</i>	<i>Apis mellifera</i>
<i>Vespa orientalis</i>	****	0.3608	0.4021
<i>Xylocopa aestuans</i>	1.0194	****	0.3196
<i>Apis mellifera</i>	0.9111	1.1407	****

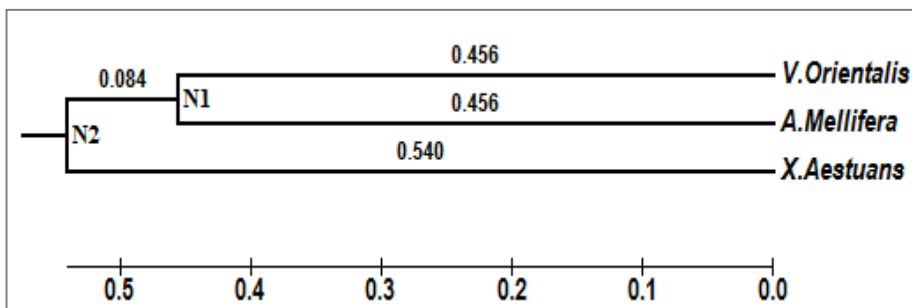


Figure 3. Dendrogram illustrating the genetic relationships among the three studied insect species, based on estimates of Nei's genetic distances for RAPD data (Table 2) and constructed using the UPGMA method of clustering. Branch lengths (1:100) and tree nodes (N1 and N2) are shown.

Discussion

RAPD markers were used in present study to detect genetic differences between three Apocrita species (hymenoptera) from Qena, Egypt. The RAPD and other molecular DNA-based assays showed to be useful for taxonomic relationships and genetic diversity studies at levels ranging from populations to species and subspecies [14-15].

Results obtained here demonstrated that the RAPD-PCR assay is powerful technique to detect polymorphism in the studied insect genotypes. All 10 deca-nucleotide random primers used produced at least 6 polymorphic fragments indicating the successful experimental PCR conditions used here for revealing instructive RAPD banding patterns. Some studies reported the effectiveness of PCR reaction conditions on fingerprint profiles causing false bands and non-reproducibility of assay [25-26].

Genetic variation in the three insect genotypes was examined using RAPD assay. The presence or absence of RAPD band (locus/marker) of identical molecular weight imply an interspecies genetic variation. Data presented here indicated that there is genetic relatedness among the three hymenopterans species studied. The selected primers displayed moderate to high frequencies of polymorphic bands. This may reflect that these species are to some level of the polymorphism are closely related and reflecting their internal genetic variations because the RAPD bands consider dominant genetic markers and are inherited in a Mendelian fashion [27].

From all primers used, primer A-12 exhibited highest primer band frequency of 0.14433 while lowest value of 0.06186 recorded for primer A-11. Interestingly, the high molecular fragment of about 3000 bp recorded only for *Xylocopa austeanus* with primer A-12. This band was not observed for both *Vespa orientalis* and *Apis mellifera*. It may consider primer-specific band for *Xylocopa austeanus* which indicate the presence of internal genotype-specific variation.

An average band frequency value of 0.4227, 0.5052, 0.40206 recorded for *Vespa orientalis*, *Xylocopa austeanus* and *Apis mellifera*, respectively. It could show the homogeneity and heterogeneity rate within the population of insect species under investigation [28]. Among them the *Xylocopa austeanus* showed high value of band frequency of 0.50515 that may indicate its high rate of homogeneity and heterogeneity due to encompass internal unique species trait differences.

The Nei's genetic distance [22] assumes that genetic differences are due to changes in genetic drift and mutation, this is biologically logic to be intended for comparing different species. Therefore, Nei-72 original measures of genetic identity and genetic distance was used. A Nei's closely related distance between *Vespa orientalis* and *Apis mellifera* obtained from RAPD data subjected to UPMGA positioned the two genotypes in single cluster as sister group with a common node in the phylogenetic tree (Figure 3) while *Xylocopa austeanus* split up as outgroup forming sister clade to them. These results may indicate more genetic makeup resemblance between *Vespa orientalis* and *Apis mellifera* than *Xylocopa austeanus*. For that *Vespa orientalis* is closely related to *Apis mellifera* than to *Xylocopa austeanus* and all the three species having common ancestor. Previous study [29] positioned the honeybees inside the Vespidae based on sequencing of the nuclear 28S ribosomal DNA and mitochondrial 16S ribosomal DNA. However, other study based on the phylogenomic analysis of aculeate hymenoptera argued about the position of the honeybees inside the Vespidae [30]. A great efforts have been made the attention to phylogeny and evolution of wasps, ants and bees [31], paraphyly of the vespoidea superfamily suggesting that Apoidea arises from within Vespoidea [32], monophyly of Xylocopinae suggesting its coming out from Apinae [33].

In conclusion, results showed the sensitivity and effectiveness of RAPD PCR assay in insect phylogenetic studies to the species level and generated distinctive, species-specific DNA profiles for the three insect species. The three studied apocrita species are closer to each other, however both *Vespa orientalis* (Vespidae) and *Apis mellifera* (Apidae) are genetically more

close. The splitting up of *Xylocopa austeanus* into sister clade to *Vespa orientalis* and *Apis mellifera* may be a sign to reconsider placement of genus *Xylocopa* within Apidae. Therefore, and beside to our results obtained by means of RAPD markers, it would be valuable using additional markers such as the mitochondrial cytochrome oxidase genes (COI and COII) for further phylogenetic analyses of Apocrita super families including Apoidea and Vespoidea. This will aid to address in deepest manner the phylogentic relations and taxonomic structures of that most biologically diverse suborder of hymenopteran insects.

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التنوع الوراثي و العلاقة التطورية الوراثية بين ثلاثة أنواع من حشرات ذوات الخصر (غشائية الأجنحة) من محافظة قنا، مصر باستخدام مؤشرات التفاعل العشوائي متعددة الأشكال لسلسلة الدنا (RAPD)

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تم اخضاع المادة الوراثية (DNA) لثلاثة أنواع من حشرات ذوات الخصر (غشائية الأجنحة) وهي دبور البلح (*Vespa orientalis*) من الدبابير الحقيقية (*Vespidae*)، وكل من دبور الخشب (*Xylocopa aestuans*) و نحل العسل (*Apis mellifera*) من النحلويات (*Apidae*) للتنميط الوراثي بواسطة مؤشرات التفاعل العشوائي متعددة الأشكال لسلسلة الدنا (RAPD) و ذلك باستخدام عشرة بادئات عشوائيه وهي

A-01،A-02،A-03،A-04،A-05،A-07،A-08،A-09،A-11 و A-12

أثبتت البادئات العشرة المستخدمة فعاليتها بإعطاء تعددية شكلية بين الأنواع المدروسة حيث أن كل البادئات اظهرت حزم مؤشرة فريدة مختلفة تتراوح من ٢٠٠-٣٠٠٠ زوج قاعدة.

انتج استعمال البادئات كليا عدد من الحزم بلغ سبع و تسعون حزمة منها اربع حزم أحادي الشكل (*Monomorphic*) اما البقية وهي الثلاثة و تسعون كانت متعددة الشكل (*polymorphic*) مع معدل تبايني بنسبة 95.88 و بلغ المتوسط 9.3 حزمة للبادئ الواحد. ولقد كان أعلى معدل للتردد الحزمي لحشرة دبور الخشب مقارنة بدبور البلح و نحل العسل.

بناء على معامل البعد الوراثي "معامل Nei72" و التحليل العنقودي باستخدام طريقة المجموعة الزوجية غير الموزونة مع المتوسط الحسابي (*UPGMA*)، وضح ارتباط الأنواع المدروسة وراثيا لبعضها البعض ولها سلف مشترك، لكن اظهرت الدراسة ان كل من دبور البلح و نحل العسل اقرب وراثيا لبعضهما البعض. وتجدر الإشارة الى ان إنعزال دبور الخشب في مجموعة مستقلة شقيقة لمجموعة دبور البلح و نحل العسل ربما يكون علامة لاعادة النظر لوضع دبابير الخشب ضمن النحلويات.