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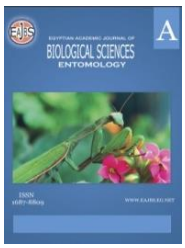

Usage of RAPD-PCR Analysis of DNA to Differentiate Lady Bird Beetles Among Family Coccinelidae

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ABSTRACT

Random amplified polymorphic DNA (RAPD) analysis, analysed by polymerase chain reaction (RAPD-PCR), as a tool to differentiate between six coccinellid beetles (*Coccinella undecimpunctata* L. 1758*; C. Novemnotata* Herbst 1793; *Hippodamia tredecimpunctata* L. 1758; *Cheilomenes propinqua vicina* (Mulsant, 1850); *Scymnus (Pullus) subvillosus* (Goeze, 1777) and *Cydonia vicina nilotic*a Mulsant 1850) evaluate the variation and the genetic structure of the different species with three decamer oligonucleotide primers (MWG1, MWG2, and MWG3). MWG1 primer was successfully used to evaluate the similarity and compare genetic variation among the six insects, the total number of amplicons ranged from 10 to 16 per primer with an average of 14 amplicons. Amplicons size (bp) were ranged from 100 bp to 1311 bp. Polymorphic amplicons were ranged from 9 to 16. The maximum of polymorphic bands belonged to MWG2 primer 16 bands with 100% polymorphism, and the minimum of polymorphic bands belonged to MWG3 primer (9bands) with 90% polymorphism. In addition, MWG1 primer was produced (14 bands) with 100% polymorphism. The RAPD analysis data grouped into two main clusters and an independent one, the first cluster was *C. vicina nilotica,* and *C. novemnotata* classified as the most related species with 72%, and they close to *H. tradecimpunctata* with 55%. The second cluster was *C. undecimpunctata,* and *S. subvillosus* were closed with 58% while, *C. vicina* is relatively distant from the other five species, and meet with them at common ancestor with 45% using RAPD-PCR analysis and the results were cleared genetic variations between the six coccinellid spp. The polymorphic percentage ranged between 90% and 100%, and the similarity percentage ranged between 0.32 and 0.71.

INTRODUCTION

Coccinellid species are frequently used as very important agents of a biological way for controlling harmful pests (aphids, white flies, thrips, eggs, and small larvae of Lepidoptera (Victor, 1997).

Also, aphid alfalfa weevil larvae are frequently alternative prey of Coccinellids (Evans *et al.*, 2004; Deligeorgidis *et al.*, 2005; Evans and Tolerance, 2007). *Coccinella undecimpunctata* L. is one of the common in Egypt; it is considered an important

predator of eggs and newly hatched larvae of cotton leaf worms and aphids. It is characterized by black colour, orange elytra spotted with 11 black spots (Dobzahansky, 1933; Ibrahim, 1955 and Brown, 1962).

DNA marker is used to bromide raw information, based on which an ecologist makes estimates of genetic diversity and gene flow between species (Behura *et al.*, 2001). Molecular data provide the means to differentiate sympatric species from allopatric (Ballingr-Crabtree *et al.*, 1992). Numerical taxonomy has been used in the classification of insects by the phenetic methods such as mosquitoes, honey-bees, sand flies and soldiers fly (De Sousa *et al*., 2001*;* Alattal *et al*., 2014; Adamson et al., 1993 and Badrawy *et al*., 2006). Molecular markers were applied to identify different insects such as aphids (Black *et al*., 1992), strains of Mediterranean fruit fly (Hamerton & Me Inn, 1994), termite casts (El-Gohary *et al.*, 2000), and *Coccinella septumpunctata* (Haubruge, 2002). Few molecular studies have been reported on the family Coccinellidae, compared to other insect groups in the world belonging to four subfamilies which could not be resolved by the phenotypic studies or behavioral studies. Kobayashi *et al.* (1998) investigated Cytochrome Oxidase 1 (CO1) gene region of Epilachninae studied the relationships among several species focusing on host shifts. Von der Schulenburg *et al.* (2001) found extreme lengths and length variation of Internal Transcribed Spacer Region (ITS1) gen region of twelve species belonging to five subfamilies of Coccinellidae and reported that the extreme length variations of some species were due to internal repeats. High variability of the ITS1 sequences was observed among tribes, genera and species. Fu and Zhand (2006) published the first study on a molecular systematic analysis of family Coccinellidae, and sequencing partial CO1 gene region to infer the higher taxonomic level relationships of sixteen species belonging to four subfamilies. These molecular studies of Coccinellidae have produced interesting outcomes about the hidden relationships among species which could not be resolved by the phenotypic studies or behavioral studies. Numerical phenotypic method and Random Amplified Polyphonic DNA (RAPD) molecular analysis were applied to compare between *Coccinella undecimpunctata* and its 13 aberrations which were collected from 10 localities in Egypt (Badrway *et al.*, 2009). Amplified fragment length polymorphism (AFLP) markers were used to evaluate polymorphism in elytra colour patterns in *Harmonia axyridis* (Coccinellidae) and the influence of environmental factors on colour differentiation (Lee *et al.*,2011). El-Bassiony & Abu El-Ghiet (2014) used RAPD-PCR to determine the genetic diversity in *C. septempunctata L.* associated with alfalfa crop in two Egyptian oases, the study confirmed to be very valuable for better understanding of population complexity of *C. septempunctata* and the genetic distance within *C. septempunctata* natural populations of diverse geographic origins.

The present study aims to differentiate between six coccinellid beetles based on morphological traits and (RAPD) analysis added powerful tools for studying and evaluating the variation and the genetic structure of the different species.

MATERIALS AND METHODS

A sampling of the Specimens:

The ladybird beetles were collected during the spring season throughout April – June 2018 by aspirator. The collected samples were put in an icebox filled with ice. The specimens were transferred to the Biological Control Res. Dep. laboratory, PPRI, ARC. They were isolated, divided and distributed into groups in tubes (2 cm in diameter * 7 cm in height). They were preserved in the freezer at (-24°C). The group *Coccinella. undecimpunctata* L., 1758; *C. Novemnotata* Herbst, 1793 *and Hippodamia*

tredecimpunctata L., 1758 were collected from *Triticum aestivum* L. Where, *Cheilomenes propinqua vicina* (Mulsant, 1850), *Scymnus (Pullus) subvillosus* (Goeze, 1777) and *Cydonia vicina nilotica* Mulsant, 1850 were collected from *Nerium oleander* L. The samples were sent to Bio-Technology Lab., Plant Pathology Res. Inst., ARC.

DNA Extraction:

A modification of the traditional sodium dodecyl sulfate (SDS) extraction procedure was adopted. Fresh fungal pellets were homogenized in 400 µL sterile salt homogenizing buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). Next, 6 µL 20 mg/mL RNase A was added and mixed well. The samples were incubated at 65°C for 10 min, after which 130 µL 3 M sodium acetate, pH 5.2, was added to each sample. Samples were whirled for 30 sec. at maximum speed and incubated at (-20°C) for 10 min. The lysate was centrifuged at 13,000 rpm at 4°C for 15 min., and the supernatant was transferred to fresh tubes. An equal volume of isopropanol was added to each sample, and after mixing well, and samples were incubated at (-20°C) for 10 min. Samples were then centrifuged for 20 min. at 4°C, at 6000 rpm. The DNA pellets were washed twice using 700 µL washing solution (100 and 70% ethanol, respectively). The DNA pellets were subsequently air-dried in an oven at 40°C for at least 10 min. The resultant DNA pellet was then resuspended in 100 µL 1X TE (10 mM Tris-HCl, 1 mM EDTA) buffer, pH 8.0 (Abd-Elsalam et al., 2007).

DNA Quantification and Gel Documentation:

Seven microliters of the isolated DNA and 3 µL of 10X loading dye were loaded in a lane of 1.5% (w/v) agarose gel containing 0.05 µg/mL ethidium bromide, to check the quality of the DNA. For quantitative measurements, a charge-coupled device camera imaging system and UVI-soft analysis (Gel Documentation and Analysis Systems, Uvitec, Cambridge, UK) were used to capture the image and to calculate the band intensities.

RAPD-PCR Analysis:

RAPD-PCR analysis was undertaken using 10 10-mer primers (MWG, Germany; Table 1). RAPD analysis was performed in 25-µl reaction volumes containing PCR buffer (Promega, Mannheim, Germany), 0.2 mmol/l dNTPs, 0.5 mmol/l primers, 4.0 mmol/l MgCl2, 1.25 units of Taq Polymerase (Promega, Mannheim, Germany) and 10–20 ng genomic DNA. PCR reactions were carried out in a T-Gradient thermal cycler (Biometra, Germany) using the following profile: 94 °C for 1 min, 36 °C for 1 min and 72 °C for 1 min for 30 cycles, and a final extension at 72 °C for 5 min. Following amplification, the samples were separated by electrophoresis in 1.4 % agarose gel, stained with 0.5 µg/ml of ethidium bromide and viewed under ultra-violet light. A 100- to 1500-bp ladder (Promega, Mannheim, Germany) was used as a molecular mass marker, (Abd-Elsalam *et al.*, 2007).

Data Analysis:

Each group of beetles was scored for the presence or absence of all bands identified. RAPD markers were scored (1) or (0) for each sample (1) was assigned for the presence of a band and (0) for its absence. Bands were considered as those migrating the same distance relative to a molecular weight marker, although could have included co-migration of different loci (Black 1993). A similarity matrix was calculated from the data on the presence or absence of bands for each sample. Based on the similarity matrix by using on-line (MAFFT version 7) un-weighted pair-group method analysis (UPGMA).

RESULTS AND DISCUSSION

Genomic DNA of six coccinellid species that were subjected to RAPD-PCR analysis with three decamer oligonucleotide primers (MWG1, MWG2, and MWG 3).

The total number of amplicons obtained ranged from 10 to 16 per primer with an average of 14 amplicons (Table 1). Amplicons size (bp) ranged from 100 bp to 1311 bp. Polymorphic amplicons ranged from 9 to 16. The maximum of polymorphic bands belonged to MWG 2 primer 16 bands with 100% polymorphism, and the minimum of polymorphic bands belonged to MWG3 primer (9bands) with 90% polymorphism. In addition, MWG1 primer was produced (14 bands) with 100% polymorphism.

Table 1: RAPD-PCR analysis with three primers to differentiate between six coccinellid species were collected from wheat (*Triticum aestivum)* field and oleander (*Nerium oleander)* plants in Giza, Egypt during spring, 2018.

Primer name	Nucleotide sequence (5'to 3')	Number of amplicons	Amplicons size (bp)	Monomorphic amplicons	Polymorphic amplicons	% polymorphism
MWG1	5'CAA CTC TCT CTC TCT3'	14	122–1311	0	14	100
MWG2	5'ACA CAC ACA CAC ACA TAT ATA3'	16	107–1209	0	16	100
MWG3	5'ACA CAC ACA CTC TCT CTC3'	10	100 – 727	1	9	90

The similarity of DNA bands ranged from 0.32 to 0.71 (Table 2). The lowest genetic distance of (100bp) was observed of *C. novemnotata, and C. vicina nilotica,* and the highest genetic distance of (1311bp) exhibited by *C.undecimpunctata* and *C. novemnotata.*

Table 2: Genetic similarity matrix for six coccinellid spp. were collected from wheat, *Triticum aestivum* field, and oleanders, *Nerium oleander* plant, Giza, using three RAPD primers.

Lane	1	2	3	4	5	6
1	1					
2	0.48	1				
3	0.37	0.46	1			
4	0.57	0.44	0.54	1		
5	0.47	0.5	0.49	0.52	1	
6	0.32	0.51	0.6	0.43	0.71	1

All primers produced a large number of discrete bands with different intensities (Fig. 1). Species-specific DNA bands varied with the different primers and the level of amplification of some bands was not uniform. MWG1 primer was successfully used to evaluate the similarity and compare genetic variation among the six insects (Fig. 1a), where it separated species-specific band clearly (122bp) only of *H. tredecimpunctata*, three bright and clear bands at (209 bp, 276 bp and 312 bp). *C. undecimpunctata* separated the largest number of the clear band (945 bp, 1029 bp, 1131 bp and 1311 bp)

also, four clear and bright bands (276 bp, 312 bp, 360 bp and 468 bp) for *C. novemnotata.* On the contrary, MWG2 primer confirmed the possibility of easy discrimination between species of Coccinellidae (Fig. 1b) where it gave very different profiles for the different species and the species-specific band could be distinguished only in *C. undecimpunctata* absent band (175bp) and bright clear bands (211 bp and 262 bp). Also, *C. vicina* could be distinguished only has a present band (969 bp), *S. subvillosus* (133bp) and *C. novemnotata* (1209 bp) , respectively. MWG3 primer gave one species-specific band at (158 bp) for all species and *C. vicina* (217bp) (Fig.1c) and *H. tredecimpunctata* has two species-specific bands at (138 bp, 396 bp).

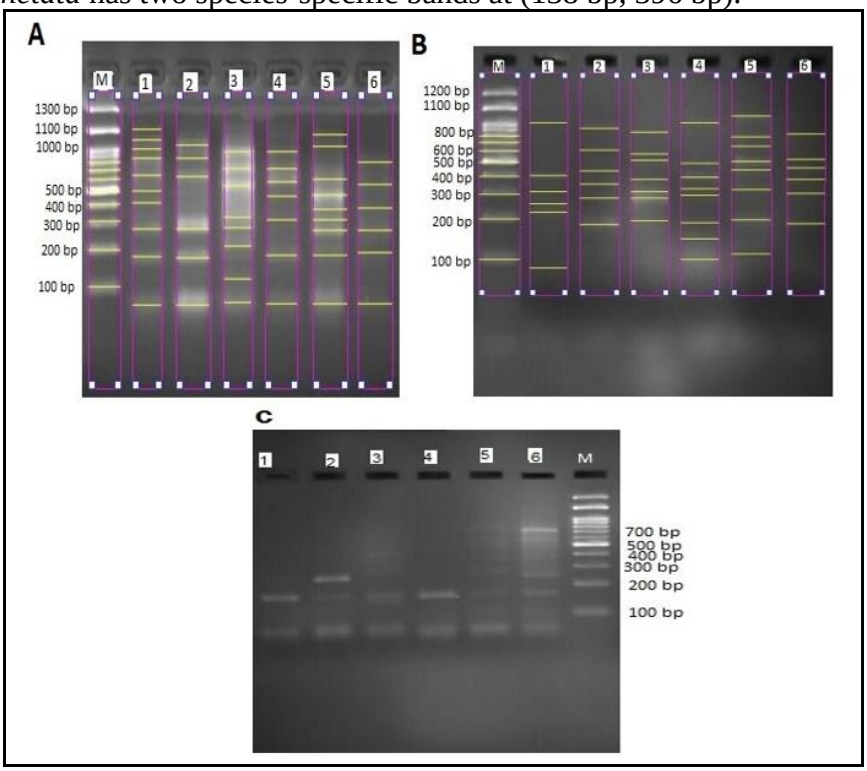

Fig 1: RAPD amplified fragment produced by three primers were used to evaluate and to compare genetic variation among the six species: (1) *C. undecimpunctata,* (2) *C. vicina,* (3) *H. tredecimpunctata,* (4) *S. subvillosus,* (5) *C. novemnotata* (6)*C. vicina nilotica.*

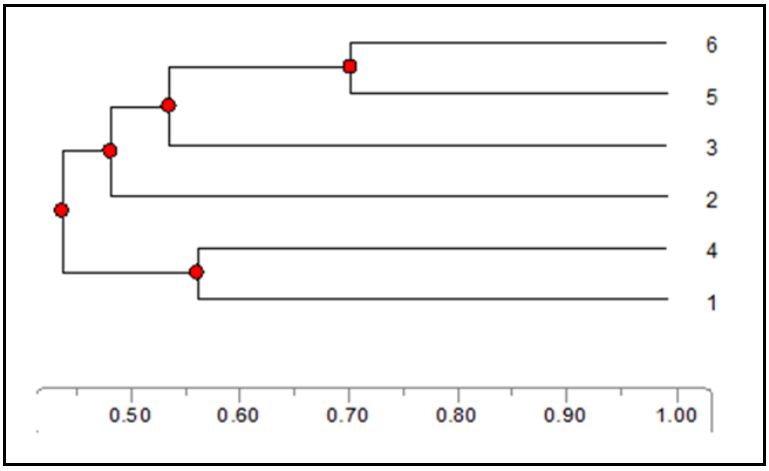

Fig 2: Phylogenetic tree was constructed based on the multiply aligned sequence data for six coccinellid species from Egypt: (1) *C. undecimpunctata* (2) *C. vicina,* (3) *H. tredecimpunctata,* (4) *S. subvillosus* (5) *C. novemnotata* (6) *C. vicina nilotica.*

Cluster analysis reveals several characters of coccinellid spp. (Fig.2). The RAPD analysis data grouped into two main clusters and an independent one, the first cluster was *C. vicina nilotica,* and *C. novemnotata* classified as the most related species with 72%, and they close to *H. tradecimpunctata* with 55%. The second cluster was *C. undecimpunctata,* and *S. subvillosus* were closed with 58% while, *C. vicina* is relatively distant from the other five species, and meet with them at common ancestor with 45%.

The molecular studies of coccinellid spp. have produced interesting outcomes about hidden relationships among species which could not be observed well by the phenotypic or behavioral studies (Aruggoda, *et al.,* 2010). Also, Harde (1981) mentions that the external morphology of adult Coccinellidae is fairly simple, their identification can – in some cases – be surprisingly difficult because of the variability within many species, especially in coloring. In the present study of usage of RAPD-PCR analysis, the results were shown that they revealed genetic variations between the six coccinellid spp. The polymorphic percentage ranged between 90% and 100%, and the similarity percentage ranged between 0.32 and 0.71.

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ARABIC SUMMARY

تحليل الحمض النووي DNA بإستخدام تقنية (RAPD-PCR)للتمييز بين أنواع خنافس أبو العيد عائلة Coccinelidae

إيمان فتحي سيد المهدي، حازم عبد الرءوف أبو الفضل، منى عبد الحميد شعيب
قسم المكافحة الحيوية – معهد بحوث وقاية النبات – مركز البحوث الزراعية-الجيزة -مصر

تحليل الحمض النووي DNA بإستخدام تقنية (RAPD-PCR) بواسطة ثلاث أنواع من البادئات Primers MWG3, MWG2, MWG1 للتمييز بين ستة أنواع من خنافس أبو العيد
, C. *Novemnotata* Herbst, Hippodamia *tredecimpunctata* . (*Coccinella undecimpunctata* L ,), *Scymnus (Pullus) subvillosus* (Goeze)L., *Cheilomenes propinqua vicina* (Mulsant and *Cydonia vicina nilotic*a Mulsant
لتقييم التباين وأيضا التركيب الوراثي لهذه الأنواع وإستخدم البادئ MWG1 بنجاح وكانت نسبة الأشكال المتعددة 100% وأنتجت (14 bands) لتقييم كل من التشابه والتباين الوراثي بين الأنواع الستة. وسجل MWG2 نسبة 100% أشكال متعددة (polymorphism) وأنتجت (16 bands)، بينما كان تعدد الأشكال (9 bands) بنسبة 90% في حالة MWG3.
وقد أوضح الشكل الشجري لهذه الأنواع الستة درجة عالية من التباين تراوحت بين 45% إلى 72% ونتج عنها مجموعتين أساسيتين ونوع واحد مستقل. كانت المجموعة الأولى *C. vicina nilotic*a بنسبة تقارب 72% مع *C. Novemnotata* وبنسبة 55% مع *H. tredecimpunctata*.
أما المجموعة الثانية كانت *C. undecimpunctata* بنسبة تقارب 58% مع *S. subvillosus* . بينما *C. propinqua vicina* بعيد نسبيا عن باقى الأنواع الخمسة ويلتقي معهم في الأسلاف المشتركون بنسبة 45%.
وأوضحت النتائج للتباين الجيني بين الستة أنواع أبو العيد وتراوحت نسبة الأشكال المتعددة (من 90 الى 100%) ونسبة التماثل من (0.32 – 0.71)