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In vivo and *in vitro* nuclear and mitochondrial DNA analyses of red palm weevil *Rhynchophorus ferrugineus* populations in Egypt

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ABSTRACT

One of the most damaging invasive insects is the red palm weevil *Rhynchophorus ferrugineus* (RPW). It considers the most damaging insect pest of date palm. In the present study, in vivo and in vitro genetic differences among RPW females from five different regions of Egypt were investigated through RAPD markers and the partial sequence of CO1 gene. Ten primers were used in RAPD analysis. 46 of the 60 produced fragments with a ratio of 76.67% were polymorphic and 14 fragments with a ratio of 23.33% were monomorphic between the samples of the different studied regions. There was no difference between in vivo and in vitro four samples collected from the five studied geographic regions by using RAPD analysis. The multi-alignment between in vivo and in vitro samples of CO1 partial sequence of studied populations revealed the absence of any nucleotide differences between the samples of the same region. The neighbor-joining tree between the studied RPW populations and the closed sequences from Gen Bank placed them in two different clusters with different haplotypes from Egypt and the Kingdom of Saudi Arabia. According to CO1 gene partial sequence and RAPD analysis results, it can be concluded that there are slight effects of cell culture media compositions on the DNA of cells. The study results suggest that cell culture methods can be used in biological control studies instead of in vivo ones. Also, there may be more than one haplotype of RPW in Egypt and it may be introduced from the same or different sources.

***Keywords*:** Red palm weevil, RAPD-PCR, Cytochrome oxidase subunit 1 (CO1).

INTRODUCTION

Invasive species of insects have many economic and negative impacts on biodiversity (Sakai *et al*., 2001). One of the most damaging invasive insect is *Rhynchophorus ferrugineus* (Faleiro, 2006; Gonzalez, 2019) which is the most destructive insect pest of date palm (Rugman-Jones, *et al*., 2013, Diagne *et al*., 2021; Montiel *et al*., 2022).

It was reported that Red palm weevil (RPW) has a high diversity worldwide (Ijaz *et al*., 2018; Torky *et al*., 2023). RPW has reached the United Arab Emirates, Sultanate of Oman, and the Kingdom of Saudi Arabia in 1985. In 1992, it reached Egypt (Cox, 1993). Ferry and Gomez (2002) reported that the United Arab Emirates is the source of RPW in Egypt.

In addition to nuclear genes sequences, data analyses of Cytochrome

oxidase subunit 1 (COI) provide definite indication for the presence of at minimum two species of RPW; *Rhynchophorus ferrugineus* and *R. vulneratus* (Rugman-Jones *et al*., 2013). *R. ferrugineus* is accountable for all invasive populations in the world, while *R. vulneratus*, has a more southern spreading across Indonesia. It is currently synonymized under *R. ferrugineus* and accountable for the invasive insects in California. Hashem (2016) studied the mitochondrial CO1 gene sequence of RPW in Egypt and he reported that it may be introduced from different origins. Movement and import of plants within Egypt are the main ways to the insect introduction and spread (FAO, 2019).

Genetic variations among RPW are essential for the insect managing approach comprehensive biosecurity. It gives rapid and accurate identification of invasive species and their populations (Sharma *et al*., 2009). Nuclear DNA analysis of RPW was carried out by random amplified polymorphic DNA marker (RAPD) to study the relationship among RPW individuals from Egypt, UAE, Indonesia and KSA (El-Mergawy *et al*., 2011a).

DNA of mitochondria was effectively used in genetic analysis studies and investigates the invasion history of different insects (Behura, 2006, El-Mergawy *et al*., 2011b; Rugman-Jones *et al*., 2013). The sequence of COI mitochondrial gene was assured as a bio-identification tool and it was used to identify the phylogeny, Barcode studies, genetic differences, and geographical distribution for different insect species (Hebert *et al*., 2003). Mitochondrial DNA markers in the insects have the advantages of their haploid status, maternal inheritance, and high evolution rate. Also, universal primers for the mitochondrial genes are obtainable and can be used in insects in which the sequences of their genomes are not known (Zhang and Hewitt, 2003).

On the other hand, for over 35 years, Insects cells have been successfully cultured *in vitro* as continuous cell lines. Most of insect cell lines are derived from dipteran and lepidopteran insects. The higher degree of species divergence within the order Coleoptera, needs more coleopteran cell lines to be established to allow study of this order insects (Hoshino *et al.*, 2009). Rizwan-ul-Haq and Aljabr (2014) established mid-gut epithelial cells culture from RPW (named as RPW-1) that can be cultured successfully on four different commercial media at optimum conditions.

Biological control is the best choice method to improve the management options against the RPW (Almasoudi *et al*., 2022; Hajjaji *et al.*, 2023). In this case, DNA analysis of the weevil is very important to determine any differences between *In vivo* and *in vitro* cells genomes. Using cell culture methods in studies of biological control of the insect without the need to bring large numbers of insects to the laboratory will reduce the threat of the insect spread to the environment.

For oases inhabitants, date palm is the main source of their income. It's a common food in many regions of the world (Abd El-Azeem *et al*., 2011, Ibrahim *et al*., 2014, Hashem, 2016; Manee *et al*., 2023). So, the present work aimed to study nuclear and mitochondrial DNA analyses of *in vivo* and *in vitro* of RPW genotypes collected from five regions of Egypt using RAPD-PCR and CO1 gene partial sequence.

MATERIALS AND METHODS

1. RPW samples

Randomly, RPW female samples were collected from five governments of Egypt; Al Sharqiya, Cairo, El-Dakahlia, Kaliobeya, and Qina governorates.

2. Establishment of primary cell culture from RPW

The primary culture was initiated from the red palm weevil pupa ovaries as described by Khamiss and Hashem (2012) and Khamiss and Abdel Badeea (2013). Pupae from each region were obtained from RPW rearing room, Animal Biotechnology Department, GEBRI, USC. All processes were achieved under disinfection. Grace's insect medium (pH 6.2 - 6.4) supplemented with FBS (Bio west Cat N: S0750) in different concentrations (30, 20%, 15 %, 10% and 5%) were used. For subcultures, cells were removed from the flask surfaces by frequently surging the medium with a pipette till individual cells suspension was obtained. Then, 2.5 ml of suspended cells were transferred to a 25 cm² flasks containing 2.5 ml of fresh medium and incubated at 28°C.

3. Genomic DNA extraction

RPW female's legs tissues and cultured cells were used for genomic DNA extraction for *in vivo* and *in vitro* studies, respectively. Extraction of DNA was conducted using the genomic DNA extraction kit (iNtRON Biotechnology, Inc. Korea).

4. RAPD analysis

Analysis of RAPD was carried out using ten oligonucleotides primers (Williams *et al*., 1990) for *in vivo* and *in vitro*. The primers that were chosen from Operon Kit (Operon Technologies Inc., Alaba meda, CA), are presented in Table (1).

Table 1: The primers nucleotides sequence that were used for RAPD analysis.

Primer	Sequences	Primer	Sequences
OPA-02	TGCCGAGCTG	**OPD-03**	GTCGCCGTCA
OPB-02	TGATCCCTGG	**OPD-04**	TCTGGTGAGG
OPB-03	CATCCCCCTG	**OPH-01**	GGTCGGAGAA
OPC-01	TTCGAGCCAG	**OPO-01**	GGCACGTAAG
OPC-02	GTGAGGCGTC	**OPO-02**	ACGTAGCGTC

A total volume of 25µl PCR reaction mixture was used. The reaction consisted of 12.5µl of master mix (GeneON, Germany), 0.4µM of primer, 100ng of DNA and the volume was completed to 25µl with deionized water. Thirty cycles of PCR amplification were performed in a Biometra*T1* gradient thermal cycler after the initial denaturation step for 3 min at 94°C. Each PCR cycle consists of denaturation at 94°C for 1 min; annealing at 35°C for 0.5 min and extension at 72°C for 2 min and at 72°C for 7 min as a final extension step (Soliman *et al*., 2003; Hashem, 2016). The end products of PCR were analyzed on 1.0% agarose gel. Marker with 100 bp was used to detect different DNA fragments lengths. The present and absent bands of RAPD were scored as 1 and 0, respectively. Data matrices were analyzed and dendrogram construction was done using NTSYS program, version 2.1, Applied Biostatistics Inc. (Rohlf, 2000).

a. PCR for CO1 gene

For the detection of mitochondrial CO1gene, the mixture of PCR with a total volume of 25µl consists of 12.5µl of master mix (GeneON, Germany), 0.5µM of each primer and 100ng of DNA. The primers sequences were (5'-GGATCACCTGATATAGCATTCCC-3') as a forward primer and the reverse primer sequence was (5'-TCCAATGCACTAATCTGCCATATTA-3') (O'meara, 2001). The program of PCR was achieved as follows: 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 0.5 min and extension at 72°C for 0.5 min, and a

final extension step at 72°C for 7 min. The obtained bands of PCR were detected by using 2% agarose gel electrophoresis.

b.CO1 sequence analysis

The partial sequence of *in vivo* and *in vitro* RPW CO1 gene from the five regions of Egypt was achieved through the BigDye® Terminator v3.1 Cycle Sequencing Kit. Applied Biosystems 373xl DNA Analyzer was used. The sequencing of DNA was performed using the above-mentioned forward primer. Sequence analysis was conducted by using Finch TV 1.4 Software.

Blast program from NCBI, USA (http://www.ncbi.nlm.nih.gov/Blast) was used to retrieval the GenBank related sequences. One hundred CO1 gene partial sequences were recovered and aligned with the current study sequences to construct a Neighbor-Joining tree (Jaccard, 1908). MEGA version 10.1.7 (Kumar *et al*., 2018) was used for the conduction of Phylogenetic analyses. In the comparisons of pairwise sequence (Pairwise deletion option) the all positions that have alignment gaps and missing data were eliminated. 500 replicas Bootstrapping (Felsenstein, 1985) and multiple alignments were carried out.

RESULTS

Primary Cell Culture

In the present study, the mechanically method was used with pupa ovaries tissues to obtain a mix of individual cells and explants. It was found that the ovary tissue is a good tissue that can produce a high number of stable cultures. The ovary cell culture was successfully established in Grace's medium supplemented with 10% FBS. The cells began to attach to the T-flask after incubation for 24 h at 28°C. Figure (1) shows the cell culture post-seeding and Figure (2) presents the cell division and migration. More cells migrated from the explants and several cells were in mitosis during subsequent days.

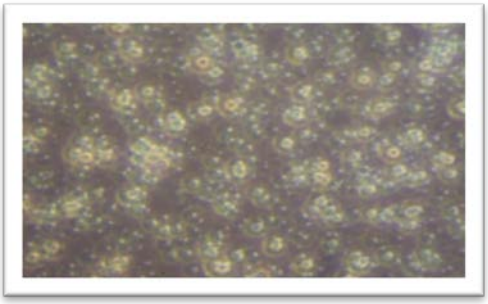

Fig. 1. Phase-contrast micrographs of the primary culture of RPW pupae ovaries tissues culture post seeding.

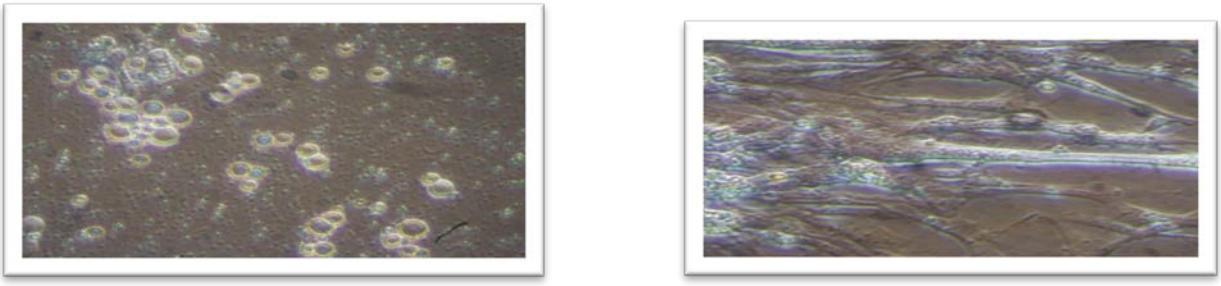

Fig. 2. Phase-contrast micrographs of the primary culture of RPW cell culture showed migration or cell division.

In subcultures of the first generation, cell growth was slow, and the cells were subcultured after 20 days and were maintained in the medium with 10% FBS, pH 6.2 at 28°C. Then cells after 7 days adhered tightly to the flask.

RAPD analysis

Ten primers of RAPD were used to estimate the *in vivo* and *in vitro* genetic variability among different samples of RPW from the five investigated Egyptan regions. The number of produced bands per primer was wide-ranging between 2 bands for primer OPO-01 to 9 for primers OPH-01with a total of 60 bands (Fig. 3 and Table 2).

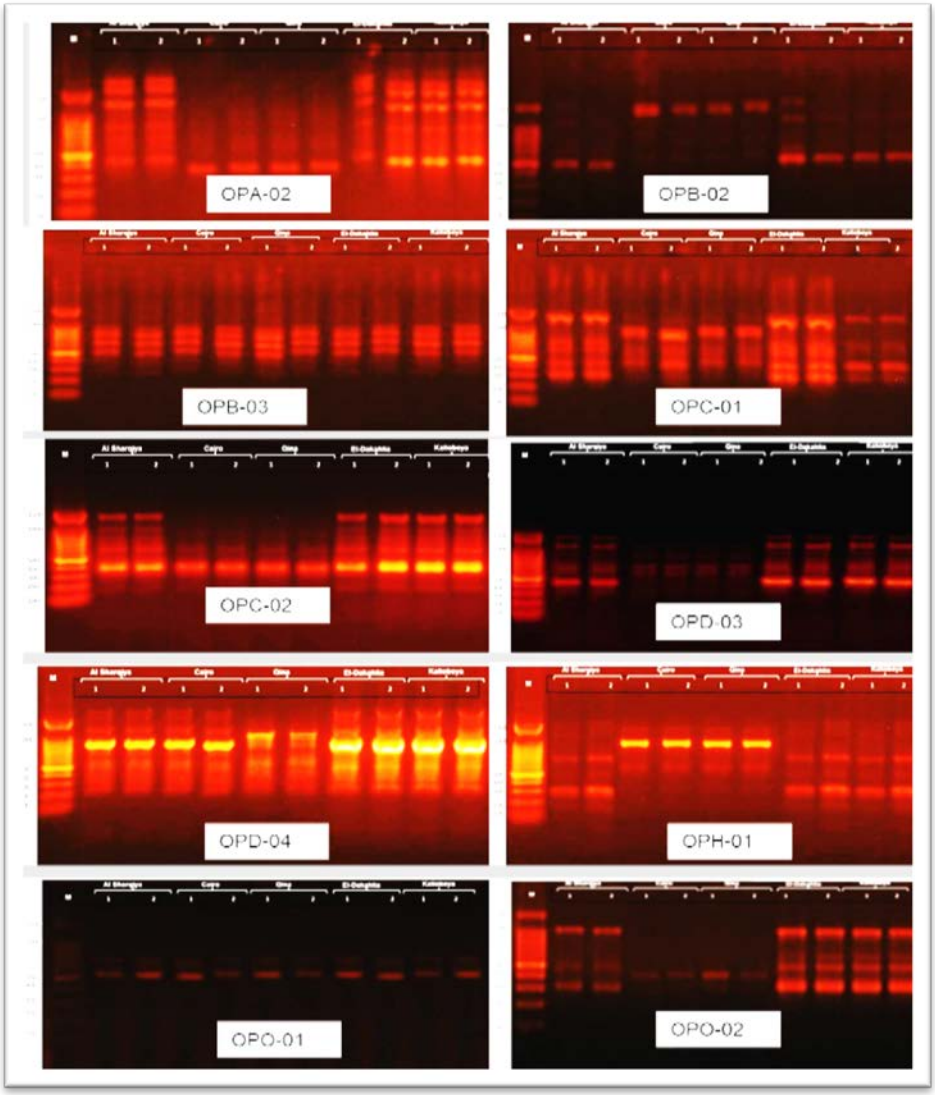

Fig. 3. The RAPD patterns of *in vivo* and *in vitro R. ferrugineus* populations collected from the five regions of Egypt, Al Sharqiya, Cairo, El-Dakahlia, Kaliobeya, and Qina governorates analyzed using different primers. M: DNA marker, 1: *in vivo* samples, 2: *in vitro* samples.

Table (2). RAPD analysis of RPW samples from five regions of Egypt.

PM	Name of primers										Total
	OPA-02	OPB-02	OPB-03	OPC-01	OPC-02	OPD-03	OPD-04	OPH-01	OPO-01	OPO-02	
AF	7	5	6	6	5	7	8	9	2	5	60
PF	5	5	3	5	2	6	6	9	0	5	46
MF	2	0	3	1	3	1	2	0	2	0	14
PF%	71.4	100	50.0	83.3	40.0	85,7	75.0	100	0.0	100	76.7

PM: polymorphism.
AF: amplified fragments, **PF**: polymorphic fragments, **MF**: monomorphic fragments.

There were no differences between *in vivo* and *in vitro* samples with nine from the ten primers. The primer OPB-02 is the only primer that gave 2 bands of 700 and 1500 bp with *in vivo* El-Dakahlia compare with *in vitro* samples. The obtained results in Table (2) indicated that 46 of the 60 produced amplified fragments with a ratio of 76.67% were polymorphic and 14 bands with a ratio of 23.33% were monomorphic fragments. The polymorphism ranged from 0.00% in primer OPO-01 to 100% in primers OPB-02, OPH-01 and OPO-02. Additionally, Figure (4) presents the dendrogram of the studied RPW populations based on RAPD results.

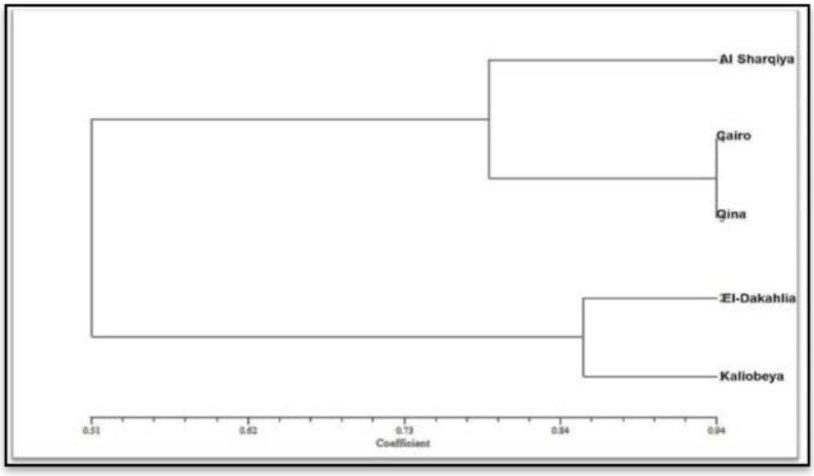

Fig. 4. Dendrogram relationship between *R. ferrugineus* populations from the five studied regions of Egypt generated from RAPD analysis.

The dendrogram revealed that the five populations fall into two main clusters. The first cluster contains the populations of Al Sharqiya, Cairo and Qina. The second one contains both El-Dakahlia and Kaliobeya populations.

Genetic variations among RPW populations using partial sequence of CO1 gene

Fragment of 1200 bp of CO1 gene was obtained by PCR amplification for all *in vivo* and *in vitro* RPW samples. After the elimination of uninformative nucleotides, 369 nucleotides were used in the comparison. The multi-alignment between *in vivo* and *in vitro* samples of CO1 partial sequence of the studied populations, revealed the absence of any nucleotides differences as shown in Figure (5).

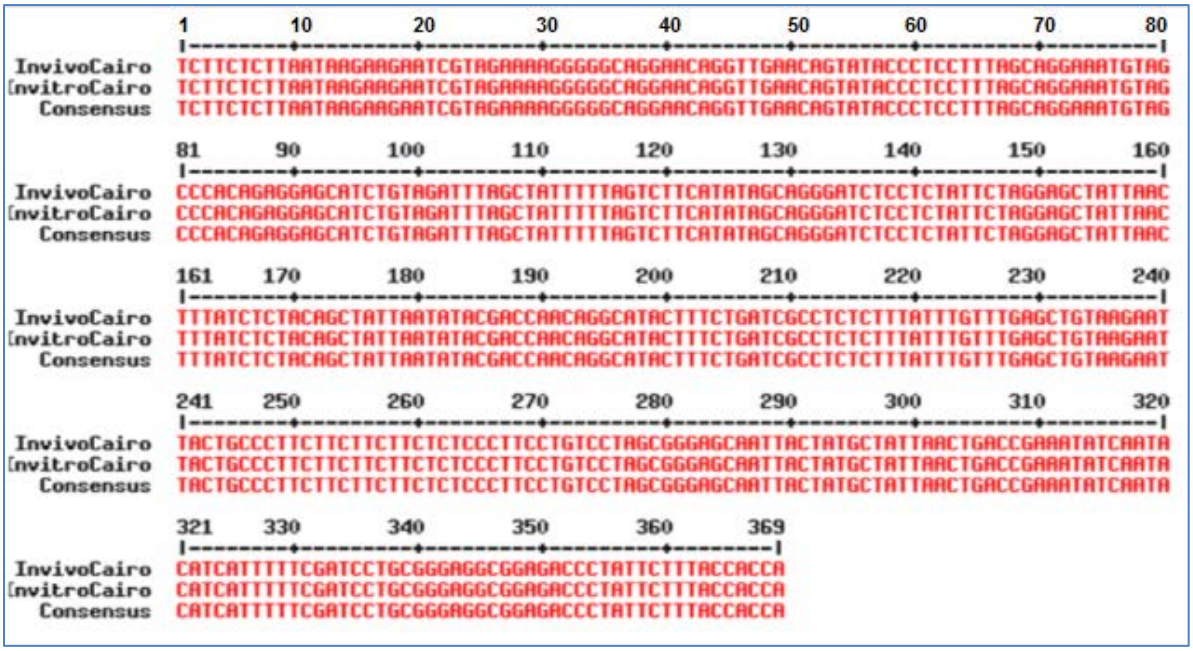

Fig. 5. Nucleotides sequence comparison of CO1 partial sequence between *in vivo* and *in vitro* Cairo samples of *R. ferrugineus*.

The composition of nucleotide was 58.8% of A-T and 41.2 % of G-C for the of 369 nts partial sequence. Sequence analysis indicated that there were no nucleotide deletions or insertions. From the multi-alignment result (Fig. 6), it was observed that there are one substitution (G instead of T) in the Qina sample and A instead of C in each of Kaliobeya and Qina samples.

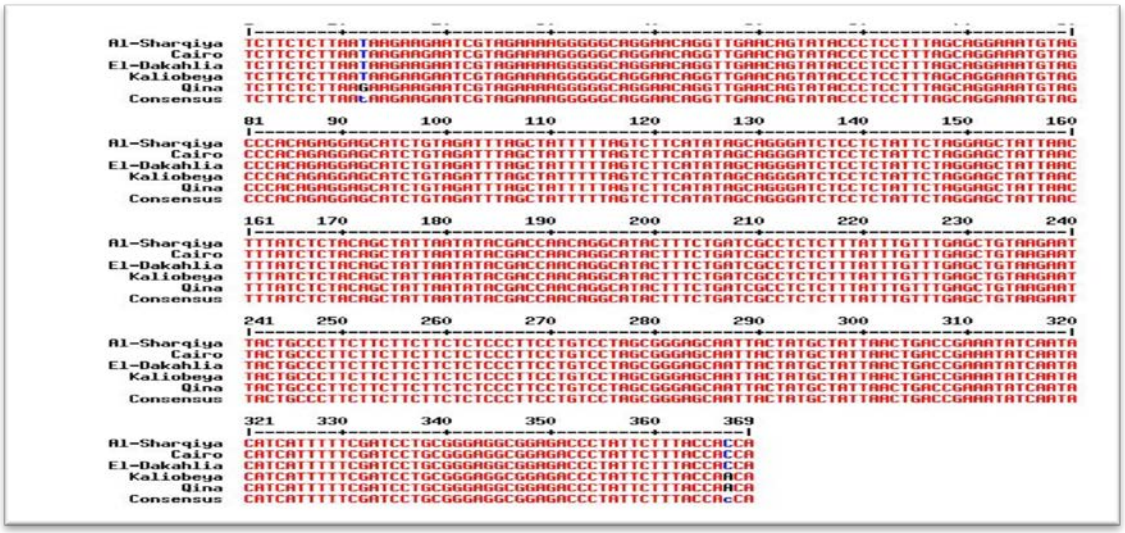

Fig. 6. Nucleotide sequences multi-alignment of CO1 partial sequence between *R. ferrugineus* populations from different regions.

The phylogenetic tree between the studied RPW populations and the closed sequences from Genbank placed them in two different clusters with different haplotypes from Egypt (*R. ferrugineus* Whahat, *R. ferrugineus* Behera) and Kingdom of Saudi Arabia (*R. ferrugineus* voucher) (Fig.7).

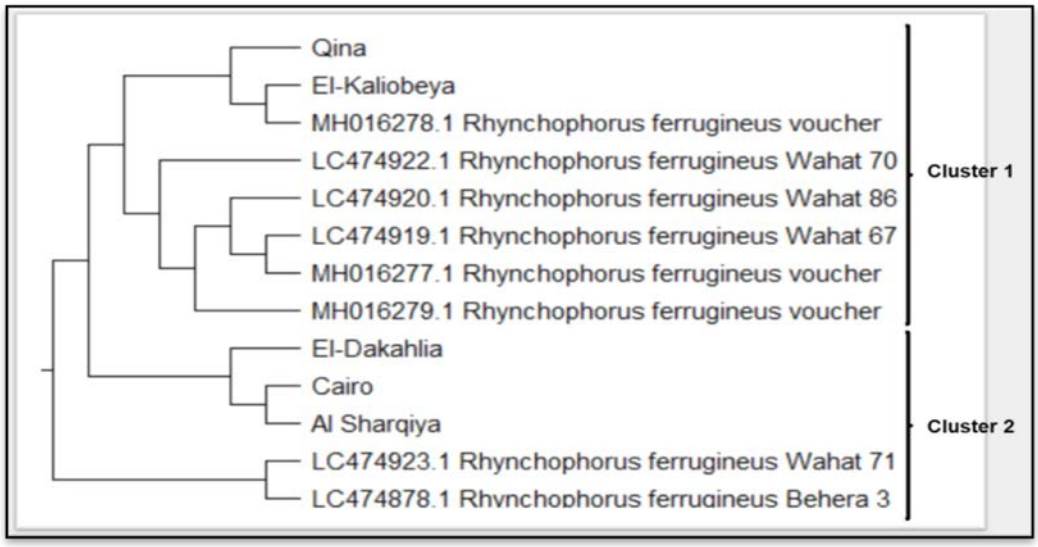

Fig. 7: Phylogenetic tree between *R. ferrugineus* populations from the five studied regions of Egypt and that of GeneBank based on partial sequence of CO1 gene.

Qina and El-Kaliobeya *R. ferrugineus* populations with one from KSA in cluster 1 and El-Dakahlia, Cairo and Al-Sharqiya with populations from Egypt (Wahat and Behera) in cluster 2. Partial sequence of CO1 gene of Qina and El-Kaliobeya populations participate in one SNP (position 367) as observed from multi-alignment. This observation placed the studied populations in two different haplotypes. One includes Qina and El-Kaliobeya *R. ferrugineus* populations and El-Dakahli, Cairo and Al-Sharqiya in the other.

DISCUSSION

For the mechanical method that used with pupa ovaries tissues to obtain a mix of individual cells and explants, it was found that the ovary tissue is a good tissue and produced a high number of stable cultures. Goodman *et al*. (2001) and Lynn (1999) indicated that the success of established culture is not dependent on the species but also on the selected tissues that were used. In the current investigation the ovary cell culture was successfully established in Grace's medium supplemented with 10% FBS. This result is in agreement with those given by Lynn (1996), Aljabr *et al*. (2014) and Khamiss and Abdel Badeea (2013). The cells began to attach to the T-flask after incubating at 28°C for 24 h. More cells migrated from the explants and several of cells were in mitosis during subsequent days. Xu *et al.* (2011) established and characterized a cell line derived from the embryos of *Sarcophaga peregrina* (Diptera: Sarcophagidae) at 28°C. In subcultures of the first generation, cell growth was slow, and the cells were subcultured after 20 days

as the results reported by Khamiss and Abdel Badeea (2013). Subcultures were maintained in the medium with 10% FBS, pH 6.2 and at 28°C. The cells after 7 days adhered tightly to the flask in agreement with the results of Lin-Hua *et al*. (2011).

In RAPD data analysis, the number of produced bands per primer was wide-ranging between 2 to 9 with a total of 60 bands. There were no differences between *in vivo* and *in vitro* samples with ten primers. The primer OPB-02 is the only primer that gave 2 bands of 700 and 1500 bp for samples from El-Dakahlia *in vivo* compare with *in vitro*. Also, the results indicated that the polymorphism ranged from 0.00% to 100% which reflect the genetic homogeneity absence among the studied populations. Hashem (2016) observed the same results when studied the genetic differences between three populations of RPW from Egypt. He found that the polymorphism ranged from 14.8% to 100% when he used ten RAPD primers. In contrast, Gadelhak and Enan (2005) noticed 51.4% polymorphism in the comparison between seven RPW populations from UAE and the used primers did not generate any unique bands as RAPD markers for any studied population. Hashem (2016) obtained 42 unique bands and most of them were in one from the three examined populations. In another study, El-Mergawy *et al*., (2011a) found 17 markers of RAPD for the Egyptian populations of *Rhynchophorus ferrugineus*. Haymer and McInnis (1994) and Bardakci (2000) reported unique markers that can be used to distinguish among the geographic RPW populations. The differences between the present results and those of Prole *et al*. (2020) and Li *et al*. (2023) can be related to the variances in the used primers and the geographic regions of RPW.

On the other hand, the dendrogram of the studied RPW populations based on RAPD results revealed that the five populations clustered into two main clusters. The first cluster contains the populations of Al Sharqiya, Cairo and Qina. The second one contains both El-Dakahlia and Kaliobeya populations. Although Al Sharqiya governorate is nearest El-Dakahlia and Kaliobeya than Cairo and Qina, Al Sharqiya samples clustered with both Cairo and Qina governorates samples. This indicates the absence of a relationship between the RPW samples, distribution, and the samples region. El-Mergawy *et al*. (2011a) and Hashem (2016) reported that not all the samples of Egyptian populations have direct relations with the geographic regions as some populations from distant regions were clustered together. As well as, the high polymorphism among the studied populations (76.67%) shows that, these populations could have diverse origins. Vieira *et al*. (2007) concluded that the invasive populations resulting from several introductions from various origins are predictable to be genetically more differentiated.

The study of genetic variations among insects' populations from the studied samples using partial sequence of CO1 gene indicated there was no nucleotides differences between *in vivo* and *in vitro* samples of each region. The composition of nucleotide was 58.8% of A-T and 41.2 % of G-C for the partial sequence of 369 nts. In the previous studies on CO1 gene sequence analysis, the A-T percentage was 61.7% to 62.4% and the G-C was 37.6% to 38.3% in the haplotypes that were studied from different countries (El- Mergawy *et al.*, 2011b; Khawaja *et al.*, 2023). In addition, Smith (2005) and Li *et al*. (2009) reported that the composition of the bases of the CO1 gene of the other insects was biased towards A and T bases.

Sequence analysis indicated that there were not any nucleotide deletions or insertions. There was one substitution (G

instead of T) in the Qina sample and A instead of C in each of Kaliobeya and Qina samples. Phylogenetic tree between the studied RPW populations and the closed sequences from Genbank placed them in two different. El- Mergawy *et al*. (2011b) indicated that in Egypt, the RPW local populations were fixed for the same haplotype (H8) while only one haplotype (H17) was established in KSA and Israel. Ferry and Gomez (2002) reported that UAE is the RPW source in Egypt through the introduction of the infested offshoot to Egypt. With agreement with the present study, El- Mergawy *et al.* (2011b) and Hashem (2016) showed that the RPW haplotype from Egypt was not related to any haplotype that discovered in UAE. Additionally, Abbas (2010) proposed that Egypt might have received RPW from an earlier KSA population, where RPW populations were first discovered as early as 1986. As revealed before by Hashem (2016), the differences between studies results may be due to the differences of sample regions, the studied fragment of CO1 gene or the period between the different studies. As well as, in the present investigation and based on RAPD markers, there was a high polymorphism between the studied populations of RPW comparing with sequence analysis results of CO1 gene. This may be related to the size of genome that can be screened by RAPD primers compared with the small size of analyzed fragment of the CO1 gene.

Conclusion

According to CO1 gene partial sequence and RAPD analysis, it can be concluded that there are a slight effects of cell culture media compositions on the DNA of cells. The study results suggest that cell culture methods can be used in biological control studies instead of *in vivo* ones. Also, this technique could be compared with the former works, there may be more than one haplotype of RPW in Egypt based on mitochondrial CO1 gene sequence and it may be introduced from the same or different sources.

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تحليل المادة الوراثية للنواة والميتوكوندريا في الحشرة والخلايا المنزرعة لعشائر سوسة النخيل الحمراء في مصر

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المستخلص

سوسة النخيل الحمراء (RPW) *Rhynchophorus frrugineu*s هي واحدة من الحشرات الغازية الأكثر ضررا وهي من أخطر الآفات الحشرية التي تصيب نخيل البلح. في هذه الدراسة، تم دراسة الاختلافات الوراثية في الحشرة والخلايا المنزرعة بين سوسة النخيل الحمراء في خمس مناطق مختلفة في مصر من خلال الواسمات الجزيئية العشوائية RAPD والتسلسل الجزئي لجين الستوكروم اوكسيديز ١ (CO1). تم استخدام عشرة من البادئات العشوائية في تحليل الـ RAPD. وقد اشارت النتائج الى ان ٤٦ من ٦٠ حزمة منتجة بنسبة ٧٦.٦٧% كانت متعددة الأشكال و ١٤ حزمة بنسبة ٢٣.٣٣% أحادية الشكل بين عينات المناطق المختلفة المدروسة. وقد لوحظ انه لا يوجد هناك فرق بين عينات أربع مناطق في الحشرة وفي الخلايا المنزرعة والتى تم جمعها من المناطق الجغرافية الخمس المدروسة باستخدام تحليل RAPD. ويضا قد لوحظ عدم وجود اختلافات بين العينات الخاصة بالحشرة والخلايا المنزرعة للتسلسل الجزئي لجين CO1 في المجموعات المدروسة بين عينات نفس المنطقة. وقد اشارت شجرة القرابة الوراثية بين مجموعات الحشرة المدروسة وتلك القريبة منها من GenBank الى وجود العينات المصرية فى مجموعتين مختلفتين مع اخرى من المملكة العربية السعودية. ووفقا للتسلسل الجزئي لجين CO1 ونتائج تحليل RAPD، يمكن استنتاج أن هناك تأثيرات طفيفة للبيئات المستخدمة فى زراعة الخلايا على الحمض النووي للخلايا ومن ثم يمكن استخدام طرق زراعة الخلايا في دراسات المكافحة البيولوجية للحشرة بدلاً من الحشرة نفسها. أيضًا، قد يكون هناك أكثر من نمط haplotype لسوسة النخيل الحمراء في مصر والتى قد تكون دخلت مصرمن نفس المصدر أو من مصادر مختلفة.