GENETIC VARIATIONS AMONG THE RED PALM WEEVIL *Rhynchophorus ferrugineus* POPULATIONS COLLECTED FROM EGYPT

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Date palm is the main source of income of oases inhabitants and it's a common food in the Middle East, North African and many other tropical and subtropical regions (Abd El-Azeem *et al*., 2011; Amy *et al.*, 2012; Ibrahim *et al*., 2014).

The Red palm weevil (RPW) *R. ferrugineus* is one of the most damaging invasive insects (Faleiro, 2006; EL-Mergawy *et al*., 2011a). It is the most damaging insect pest of palms (Rugman-Jones *et al.*, 2013). Invasive insect species have an economic impact and a negative effect on biodiversity (Sakai *et al*., 2001).

Cytochrome c oxidase subunit 1 (COI) haplotype data analyses provide conclusive evidence, corroborated by additional nuclear gene regions sequences, for the existence of at least two species. *R. ferrugineus* is native only to the western and northern parts of continental Southeast Asia, the Philippines and Sri Lanka and is responsible for all invasive populations worldwide. In contrast, the second species *R. vulneratus* (Panzer), which is currently synonymized under *R. ferrugineus*, has a more southern distribution across Indonesia. It is responsible for the invasive population in California, USA (Rugman-Jones *et al*., 2013).

The Red palm weevil was reached the Sultanate of Oman, the United Arab Emirates (UAE) and the Kingdom of Saudi Arabia (KSA) in 1985 and Sharquiya governate of Egypt in 1992 (Cox, 1993). Ferry and Gomez (2002) indicated that UAE is the source of RPW in Egypt through introduction an infected offshoot to Egypt.

On the other hand, the study of genetic variations among the invasive species is essential for their management strategy including biosecurity. It gives rapid and accurate identification of invasive species and their populations (Sharma *et al*., 2009; Armstrong and Ball, 2005; Grapputo *et al*., 2005).

The genetic variations among RPW was revealed by random amplified polymorphic DNA marker (RAPD) in a comparison among individuals of RPW from UAE, Egypt, Indonesia and KSA (Abulyazid *et al*., 2002; Salama and Saker, 2002; Gadelhak and Enan, 2005;

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Al-Ayied *et al*., 2006; El-Mergawy *et al*., 2011a).

Mitochondrial DNA was successfully used in genetic variations studies of different insect species (Behura, 2006). COI mitochondrial gene sequence was confirmed as bio-identification tool and used to detect the genetic variations, phylogeny, Barcode studies and geographical distribution in different insect species (Hebert *et al*., 2003). The advantages of using mitochondrial DNA markers in insects are due to their maternal inheritance, high rate of evolution and haploid status. As well as, universal primers are available and can be used for species which their sequences are not known (Roehrdanz, 1993; Zhang and Hewitt, 2003). Also, the mitochondrial CO1 marker was used to investigate the invasion history and origins of *R. ferrugineus* (El-Mergawy *et al*., 2011b; Rugman-Jones *et al*., 2013).

In the present study, genetic variations among some genotypes of the Red palm weevil *Rhychophorus ferrugineus* collected from three different regions of Egypt were studied using random amplified polymorphic DNA marker (RAPD-PCR) and partial sequence of mitochondrial Cytochrome c oxidase subunit 1 gene (CO1).

MATERIALS AND METHODS

A. Red palm weevil (RPW) samples

Random female's samples from *Rhynchophorus ferrugineus* (Olivier) were collected from three geographical regions in Egypt (ten samples from each region): North Egypt (Rashed), East Egypt (Ismalia) and Upper Egypt (Qina).

B. Genomic DNA extraction

Genomic DNA was extracted from legs tissues of the RPW females samples (50 mg of each ten samples from each region) using genomic DNA extraction kit (G-Spin)^{TM} for cell/tissue (iNtRON Biotechnology, Inc. Korea). DNA was extracted according to the manufacturer's protocol.

C. RAPD analysis

RAPD analysis was carried out according to Williams *et al*. (1990) using ten oligonucleotides primers (Table 1) that were selected from the Operon Kit (Operon Technologies Inc., Alaba meda, CA). PCR reaction mixture with total volume of 25 µl consisted of 12.5 µl of Maximo Taq DNA Polymerase 2X-preMix (GeneON, Germany), 0.5 µM of primer, 50 ng of genomic DNA and the volume completed up to 25 µl with ddH_2O. PCR amplification was performed in a Biometra*T1* gradient thermal cycler for 40 cycles after initial denaturation for 3 min at 94°C. Each cycle of PCR consisted of denaturation at 94°C for 1 min; annealing at 36°C for 1 min and extension at 72°C for 2 min and a final extension step at 72°C for 10 min (Soliman *et al*., 2003). PCR products were separated on 1.5% agarose gel and photographed. Ladder with 100 bp (V-gene Biotechnology Limited, shiqao, P. R. China) was used to determine the lengths

of different DNA fragments. Each sample was duplicated to confirm the stability of PCR products.

RAPD's banding patterns were scored as (1) for the present band and (0) for the absent one. Data matrices were analysed using Numerical Taxonomic and Multivariate Analysis System program (NTSYS), version 2.1, Applied Biostatistics Inc. (Rohlf, 2000). Similarity coefficients were applied for dendrogram construction by using the UPGMA (Unweighted Pair Group Method with Arithmetic Average) as well as the SAHN (Sequential Agglomerative Hierarchical Nested Clustering) routine in the NTSYS program.

D. Cytochrome c oxidase subunit 1 (CO1) gene

To detect the presence of the mitochondrial gene *CO1*, PCR mixture with total volume of 20 µl was consisted of 10.0 µl of Maximo Taq DNA Polymerase 2X-preMix (GeneON, Germany), 1.0 µM of each primer and 50ng of genomic DNA. The CO1 primers sequence was (5'-GGATCACCTGATATAGCATTCCC-3') as a forward primer, while the reverse primer sequence was (5'-TCCAATGCACTAATCTGCCATATTA-3') (O'meara, 2001). The PCR program was performed as follows: 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min, and a final extension step at 72°C for 5 min. The products of PCR were analysed by electrophoresis in 1.5% agarose gel. The amplified products molecular size was determined using 100 bp DNA ladder.

E. Cytochrome c oxidase subunit 1 sequence analysis

The PCR product sequencing of *RPW CO1* gene from different regions of Egypt was carried out with the BigDye® Terminator v3.1 Cycle Sequencing Kit and an Applied Biosystems 373xl DNA Analyzer. DNA sequencing was performed by using the aforementioned specific primers. Finch TV 1.4 Software was used for sequence analysis. The GenBank accession numbers of the three CO1 sequences identified in the present study were KU366272, KU366273 and KU366274 for samples Qina-Eg, Ismailia-Eg and Rashed-Eg, respectively.

Blast program from National Center for Biotechnology Information (NCBI), USA (http://www.ncbi.nlm.nih.gov/Blast) was used to obtain Egyptian samples related sequences from GenBank. One hundred sequences of CO1 gene were retrieved from GenBank and were aligned with our RPW sequences from different regions of Egypt to construct a Neighbor-Joining tree (Jaccard, 1908). Phylogenetic analyses were conducted using MEGA4 (Rohlf, 2000). All positions containing alignment gaps and missing data were eliminated only in the pairwise sequence comparisons (Pairwise deletion option). Bootstrapping of 1000 replicas (Felsenstein, 1985) and multiple alignments (http://multalin.toulouse.inra.fr/multalin) were carried out.

RESULTS AND DISCUSSIONS

RAPD data analysis

In this investigation, ten RAPD primers were used to evaluate the genetic variability between different genomic-DNA of *R. ferrugineus* collected from three different regions of Egypt. Table (2) and Fig. (1) showed that, the number of reproducible bands per primer, varied between 5 for primer OPA-3 to 16 for primers OPC-3, OPR-06 and OPR-07 with a total of 120 bands. The results in Table (2) clearly indicated that 82 of the 120 produced fragments with ratio of (68.33%) were polymorphic and 38 bands with ratio of (31.67%) were monomorphic. The polymorphism ranged from 14.8% in primer OPC-02 to 100% in primer OPR- 07. The results indicated that, this percentage reflects the absence of genetic homogeneity among the examined populations. In contrast, Gadelhak and Enan (2005) detected 51.4% polymorphism in RAPD markers for comparison among seven RPW samples from UAE. In the meantime, the used primers generated 42 unique bands (RAPD markers). The largest number of these markers was specific for females weevils collected from Rashed, North Egypt. El-Mergawy *et al*. (2011a) found that 17 RAPD markers were unique for the Egyptian populations. As reported by Haymer and McInnis (1994) and Bardakci (2000), the unique RAPD markers may be used to produce genetic markers that can distinguish among the geographic populations of RPW.

Genetic similarity and dendrogram

Genetic similarity values between *R. ferrugineus* populations generated from RAPD marker and dendrogram based on similarity values (Fig. 2) were performed to reveal the similarities between the different populations. The dendrogram demonstrated that the three genomic samples fall into two main clusters. The first one contained the population of North Egypt (Rashed). The second one contained both East Egypt (Ismalia) and Upper Egypt (Qina). The average genetic similarity among the three regions populations of RPW ranged from 32% to 35%. These results were in agreement with El-Mergawy *et al*. (2011a) who detected genetic similarity among different geographic populations of RPW ranged from 20% to 70%. While, Gadelhak and Enan (2005) observed genetic similarity ranged from 38 to 94% among RPW populations from UAE.

The observed genetic similarity recorded among the tested Egyptian populations based on RAPD marker, showed that there is no relation between the genetic similarity and the geographical region. Although, Ismalia is near to Rashed than Qina, the population from Ismalia clustered with that from Qina. Similarly, El-Mergawy *et al*. (2011a) found that, not all the Egyptian individuals have direct relationships with geographic region as some individuals from distant regions were clustered together. Also, the highly polymorphism among the Egyptian populations (67.5%) indicates that, these populations

could be derived from different origins. Invasive populations derived from multiple introductions from various origins are expected to be genetically more diversified (Vieira *et al.*, 2007).

Genetic relationship among RPW using the sequence of Cytochrome c oxidase subunit 1 (CO1)

PCR product of *CO1* gene gave a single band of about 1200 bp for all populations. The nucleotide composition was 58% of A-T and 42% of G-C for the partial sequence of CO1 gene (340 nt). El-Mergawy *et al*. (2011b), found that the A-T frequencies were 61.7% to 62.4% and the G-C frequencies were 37.6% to 38.3 in the haplotypes that they studied from different countries. Also, Smith (2005) and Li *et al*. (2009) found that the base composition of the CO1 gene sequence of other insects was biased towards adenine and thymine.

Sequence analysis of *CO1* gene indicated that there was not any deletions, insertions, or substitutions and there was no difference between the investigated populations from the three regions of Egypt as observed from the multi-alignment result (Fig. 3). All of them were clustered together (Fig. 4) and were very close to H17 haplotype. Egyptian populations were also close to a cluster that contains El- Mergawy-H8 haplotype which was found in Mediterranean Basin and KSA. El- Mergawy *et al*. (2011b), reported that the local populations of RPW in Egypt were fixed for haplotype (H8) while, haplotype H17 was only found in KSA and Israel. Ferry and Gomez (2002), indicated that UAE is the source of RPW in Egypt through introduction an infected offshoot to Egypt. In addition to the present study, El- Mergawy *et al*. (2011b) indicated that the Egyptian haplotype was not similar to any of the haplotypes that they detected in the UAE. Furthermore, Abbas (2010) suggested that Egypt may have received RPW from an earlier population in KSA, where RPW was first discovered as early as 1986. As mentioned previously, El- Mergawy *et al*. (2011b) indicated that haplotype H8 is the only haplotype in Egypt. The difference between the present study and El- Mergawy results may be due to the differences in the regions that the samples were collected from, the fragment size of *CO1* gene that was analyzed or the time between the two studies. Also, in the present study and based on RAPD marker, there was a highly polymorphism and low genetic similarity between the analyzed populations of *R. ferrugineus* and in contrast with that of *CO1* gene sequence analysis results. This may be related to genome size that RAPD can be screened compared with the small fragment size of *CO1* gene that was analyzed.

In conclusion and according to RAPD analysis, unique RAPD markers may be used to produce genetic markers that can distinguish among the geographic populations of RPW. Also, the results of the present study and compared with the previous studies, indicated that there may be more than one mitochondrial CO1 hap-

lotype in Egypt and the RPW may be introduced from the same or different origins.

SUMMERY

In the present study, genetic variations among the Red palm weevil (RPW) *Rhychophorus ferrugineus* collected from three different regions of Egypt were studied using random amplified polymorphic DNA marker (RAPD) and partial sequence of mitochondrial Cytochrome c subunit 1 gene (*CO1*). RAPD analysis was carried out using ten oligonucleotides primers. The number of reproducible bands per primer varied between 5 and 16 bands with a total of 120 bands. From the 120 bands, 82 (68.33%) were polymorphic and 38 bands (31.67%) were monomorphic. The used primers generated 42 unique bands (RAPD markers).

Genetic similarity recorded among the three populations under investigation on the base of their banding patterns in RAPD indicated that there is no relation between the genetic similarity and the geographical region.

PCR product for amplification of *CO1* gene gave a single band of about 1200 bp. The nucleotide composition was 58% of A-T and 42% of G-C for the partial sequence of *CO1* gene (340 nt). In Neighbor-Joining tree between Egyptian and GenBank *CO1* sequences, the three Egyptian populations of RPW *CO1* haplotypes were clustered together and were very close to H17 haplotype.

According to RAPD analysis, unique markers may be used to produce genetic markers that can distinguish between the geographic populations of RPW. Also, the results of the present study and compared with the previous studies, indicated that there may be more than one mitochondrial *CO1* haplotype in Egypt. These results suggested that RPW may be introduced from the same or different origins.

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Table (1): Nucleotides sequence of the primers used for RAPD analysis.

Primers	Sequences	Primers	Sequences
OPA-01	CAGGCCCTTC	OPH-03	AGACGTCCAC
OPA-03	AGTCAGCCAC	OPR-05	GACCTAGTGG
OPC-02	GTGAGGCGTC	OPR-06	GTCTACGGCA
OPC-03	GGGGGTCTTT	OPR-07	ACTGGCCTGA
OPC-04	CCGCATCTAC	OPR-08	CCCGTTGCCT

Table (2): RAPD analysis of different genomic-DNA of *R. ferrugineus* populations collected from different regions of Egypt.

PM	Name of primers										Total
	A1	A3	C2	C3	C4	H3	R5	R6	R7	R8	
AF	12	5	7	16	11	13	10	16	16	14	120
P+U	9	3	1	10	7	7	7	13	16	9	82
Unique	7	2	1	6	2	1	4	4	9	6	42
mono	3	2	6	6	4	6	3	3	0	5	38
PF%	75.00	60.00	14.28	62.50	63.63	53.84	70.00	81.25	100.0	64.28	68.33

PM: polymorphism. AF: amplified fragments. P: polymorphic fragments.
U: unique fragments. PF: polymorphism frequency.

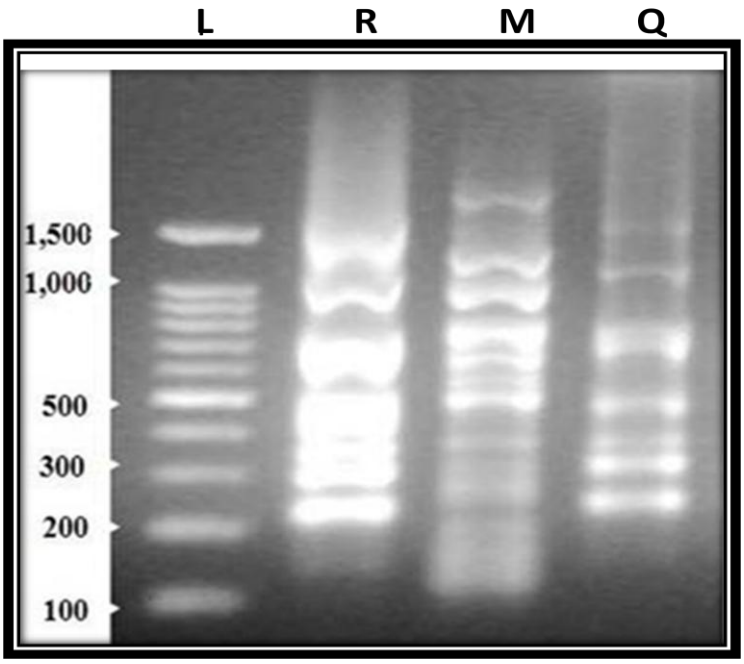
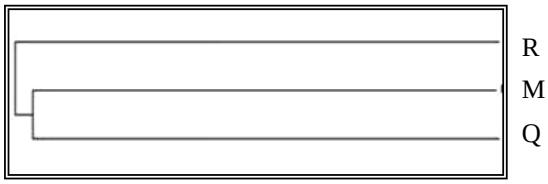


Fig. (1): Photograph showing RAPD patters from the *R. ferrugineus* populations collected from different regions of Egypt analyzed using OPC-03 primer. L = DNA ladder, R = North Egypt (Rashed), M = East Egypt (Ismalia) and Q = Upper Egypt (Qina).



	R	M
M	0.33	
O	0.32	0.35

Fig. (2): Genetic similarity values and dendrogram relationship between *R. ferrugineus* populations collected from different region of Egypt generated from RAPD analysis.
R = Rashed, M = Ismalia and Q = Qina.

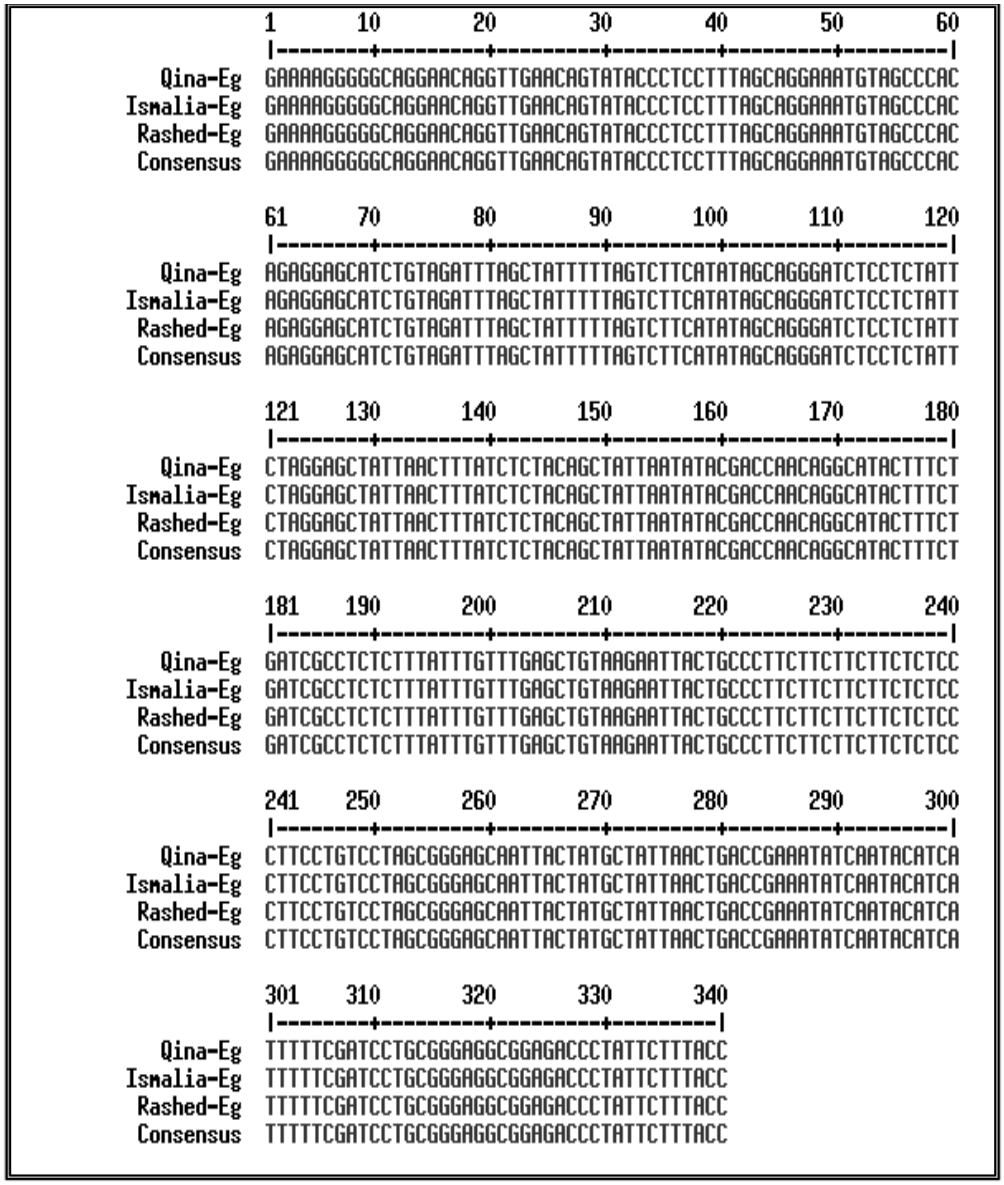


Fig. (3): Multiple sequence alignment of the investigated Egyptian populations (Qina-Eg, Ismailia-Eg and Rashed-Eg) of *R. ferrugineus CO1* gene partial sequences.

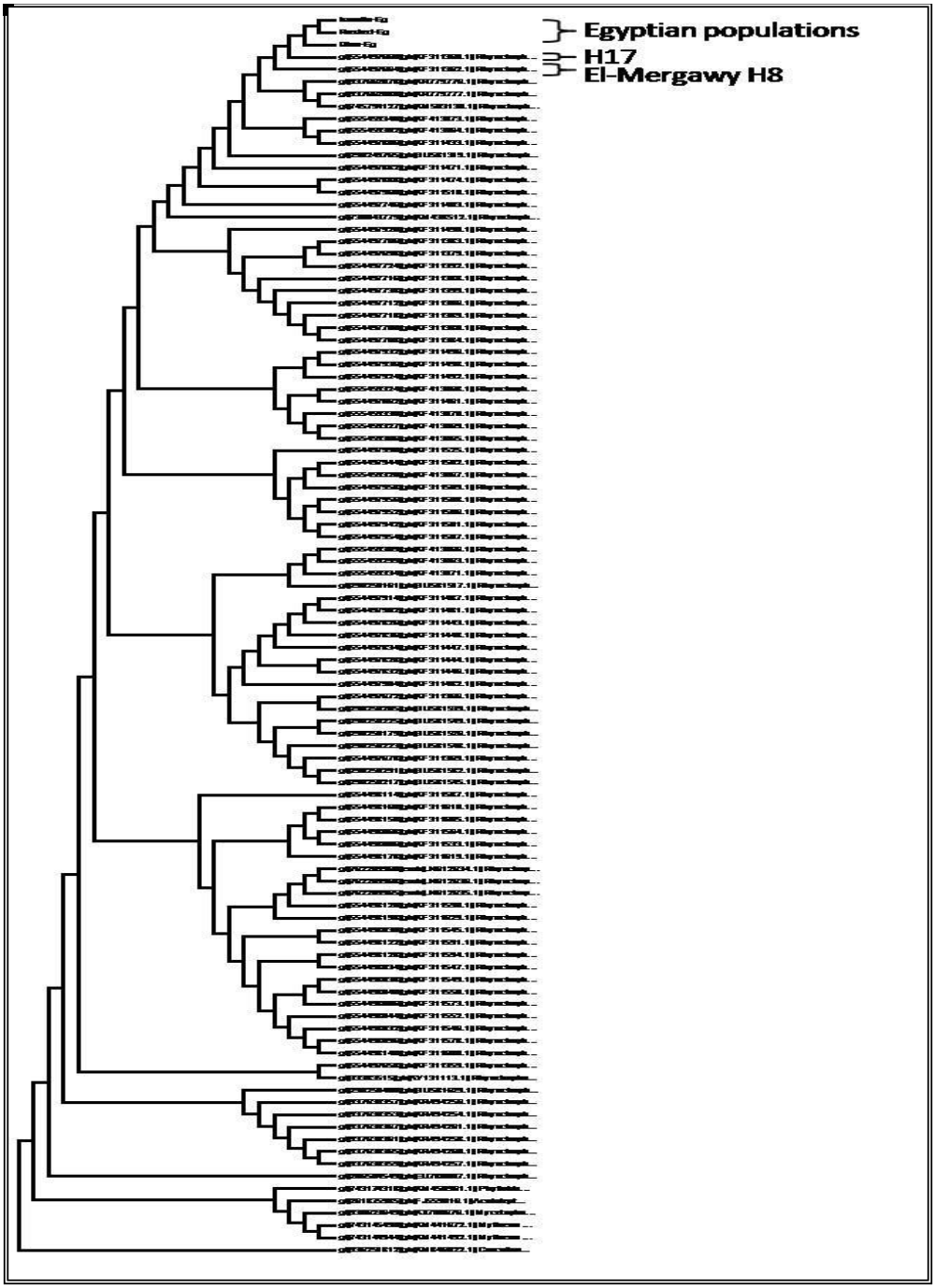


Fig. (4): Neighbor-Joining tree. Comparison between the investigated Egyptian populations (Qina-Eg, Ismailia-Eg and Rashed-Eg) and GeneBank populations of *R. ferrugineus* CO1 gene sequences.