

## Relatedness Among Geographical Populations of *Sitobion avenae*(fab.) (Hemiptera:aphididae) in Egypt, Based on Screening of Some Morphological Characters and Mitochondrial-DNA Coi Gene Analysis.

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### ABSTRACT

English Grain Aphid *Sitobion avenae*(Fab.) is important and migratory aphid species with wide distribution in wheat growing regions especially in Upper and Middle Egypt. Morphological criteria for aphid species identification may be affected by environmental factors. So, eleven geographical populations of *S. avenae* were collected from different localities of Egypt. Twenty five quantitative morphological characters (morphometric or numeric) were investigated and screened in the collected populations. On the other hand, genetic variations among those populations based on Mitochondrial-DNA COI gene sequencing and quantitative expression by using Real-time PCR (RT-qPCR) were studied. Finally, relatedness among those populations was also concerned on base of morphological or genetic variations among the populations. Ten morphological characters varied significantly among those populations; however populations of El-Gharbia and El-Qaloubia governorates were more related morphologically. Relative amounts of mRNA ranged between  $7.720 \pm 0.451$  to  $18.180 \pm 0.852$  as cycles thresholds (Ct) with high significant difference among those populations where the highest amounts of mRNA were detected in geographical population of Domiata followed by Beni-Suif Governorates. Moreover populations of El-Qaloubia and El-Sharqya Governorates were more related populations. Gene sequencing of mt-DNA COI in eight geographical populations showed 23 polymorphic sites that pairwise distance comparison among them ranged from 0.9% (difference in 6 nucleotides) to 2.7% (difference in 18 nucleotides). The largest sequence divergence was detected when population of Domiata was compared with populations of El-Sharqya or Kafer El-Shikh, while the lowest divergence was detected between populations of El-Behera and El-Monofia Governorates.

**Keywords:** *Sitobion avenae*; Morphology; Mitochondrial DNA; RT-PCR; Sequencing; Cytochrome oxidase I (COI); Geotypes; Egypt.

### INTRODUCTION

English Grain Aphid *Sitobion avenae* (Fab.) is one of the important aphid species that attack wheat and other Gramineae plants, and characterized with its wide spread because of great capability of winged adults to migrate to long distance. Europe is considered as the originated region of this species but widespread throughout the world, where prefer temperate climates so it was recorded in North Africa, the Middle East, Asia America, and Mediterranean area Emden and Harrington (2007). First record in Egypt was by Willcocks (1922) on blades and green ears of wheat and barley. It was also recorded on barely by El-Hariry (1979), Ibrahim and Afifi (1991) and El-Fatih (2000) in Giza, and Abd El-Salam (1999) in Sinai. Moreover, it was recorded on cereal weeds by Megahed *et al.* (1978). Identification of this species and its geographical populations is very important because it is a main vector of viruses to wheat plants such as barely yellow dwarf viruses (BYDV) Liu *et al.* (2004), which threat quantitative protection of grains.

DNA barcoding represents an important step towards improved pest identification molecular techniques, which may be more accurate than visual identification in cases where species or races are morphologically similar. Moreover, understanding the genetic variability of different geographical populations of insects provide us with important data about resistant -spread in populations especially in aphid populations. DNA fingerprinting is a good tool for studying *Sitobion avenae* populations and capable of distinguishing between different clones Barro *et al.* (1994). Many

studies concerned on genetic structures differentiation of aphid geographical populations such as *Rhopalosiphum padi* in Egypt Tabikha and Iddas (2016) and populations of *S. avenae* were studied in different regions around world as Britain, France, Chile, China, Romania and Denmark Li *et al.* (2001), Llewellyn *et al.* (2003) and (2004), Papura *et al.* (2003), Cai and Zhao (2004), Figueroa *et al.* (2005), Guo *et al.* (2005), Jensen (2008) and Zhao-huan *et al.* (2011).

Molecular markers are rapid identification technique for large numbers of individuals collected at immature stages Carew *et al.* (2003) and (2005). The nuclear rRNA, mitochondrial cytochrome b and cytochrome oxidase subunit I/II (COI/ COII) regions were proposed as the standard genes to be used when developing molecular markers to assist in identification of organisms as well as determining the phylogenetic relationships among taxa Hebert *et al.* (2003a,b) and Tautz *et al.* (2003). Mitochondrial-DNA is a good molecular marker to distinguish between *Sitobion avenae* and related species, while RAPD is suitable to estimate genetic diversity in addition to microsatellite can give information about genetic structure of population Figueroa *et al.* (1999). So mt-DNA cytochrome oxidase I (COI) gene was found to be a very promising tool for species identification, analysing aphid population structure, phylogenetic and phylogeography relationship intra or inter species of Aphididae Fottit *et al.* (2008), XU *et al.* (2009) and Lee *et al.* (2011) because its traits are maternal inherited, genetic recombination absence, availability of PCR primers Hebert *et al.* (2004) and abundant of comparative data Barrette *et al.* (1994). SO spatial and seasonal

patterns of mitochondrial DNA diversity for *R. padi* populations were examined in France Martinez-Torres *et al.*(1997) and has been also used to demonstrate the presence of two predominant lineages of *R. padi* in New Zealand Bulman *et al.* (2005) or populations that collected from different localities in Spain Martinez *et al.*(1992) and with geographical populations of *S. avenae* that collected from different localities of China Zhao-huan *et al.* (2011).

Therefore, DNA barcoding of the 5' end of the mitochondrial cytochrome c oxidase I (COI) gene has been used to differentiate species, moreover, COI-based RFLP profiles provide a useful tool for identifying aphid species Sunnucks *et al.* (1997), Shufran (2003) and Valenzuela *et al.*(2007). Over 700 bp of the mitochondrial COI gene are used to develop RFLP profiles for 25 aphid species from southern Australia Valenzuela *et al.*(2007). Fragment of 588 bp for mt-DNA COI was also sequenced and analysed among different geographic population of *S. avenae* in China Zhao-huan *et al.*(2011). Sequencing of mt-DNA COI was one of specific analyses that showed high level of polymorphism in insects such as *Bactocera cucurbitae* Hu *et al.*(2008).

Real-time PCR has also been used for pest insect identification Walsh *et al.*(2005), Yu *et al.*(2005) and Huang *et al.*(2010), including aphids Naaum *et al.* (2012). This technique is quicker and yields results that are easier to interpret than DNA barcoding. An additional benefit is that portable platforms exist, allowing these assays to be run in the field. A real-time PCR assay and combined with the ANDE DNA extraction are described for the portable, rapid and accurate identification of the soybean aphid Naaum *et al.*(2014).

As a result of being *S. avenae* migratory pest with wide distribution in wheat growing regions of Egypt, the current study aimed to throw some light on morphological, genetic variation and relatedness among geographical populations of *S. avenae* collected from different regions of Egypt by using more specific genetic techniques such RT-PCR and DNA sequencing that depend on mt-DNA COI gene.

## MATERIALS AND METHODS

### 1. Samples collection and preservation:

Apterous forms of English Grain Aphid *Sitobion avenae* (Fab.) from leaves and spinks of wheat, *Triticum aestivum* were monitored and collected from different administrative regions in Egypt (between latitudes of 22° and 32°N and longitudes 25°E and 35°E) during March, 2015. Data about latitudes and longitudes of regions and date of collection and amount of each specimen were recorded and presented in Table (1). Samples were preserved in Eppendorf tubes with ethyl alcohol 70% till further specimen mounting and morphological investigation. Ten adult females were caged separately on wheat leaves by using leaf cages under field conditions of each region. After three days, offsprings of the next generation for each stem mother were collected by hair brush and preserved in Eppendorf tubes with ethyl alcohol 70% and transferred to

laboratory under cooling and then preserved under -20°C till further use in molecular studies.

### 2. Morphological studies:

The preserved adults apterae were macerated, dehydrated then cleared according the described procedure of Blackman and Eastop (2000). The prepared specimens were mounted on glass slide with swan's gum chloral media for further microscopic investigations. The species was identified by using taxonomic keys of Blackman and Eastop (1984) and (2000) and confirmed by using taxonomic keys of Fathi and El-Fatih (2009). Twenty five quantitative morphological characters (morphometric or numeric) were investigated and screened in all specimens (20 apterae adults) from each tested locality as follow:-

1. Lengths of antennae and antennal segments (I, II, III, IV, V, Basal part of VI and Unguis of VI).
2. Number of sensorial seta on antennae and each antennal segment (I, II, III, IV, V and Basal part of VI).
3. Lengths of the fourth and fifth rostral segments.
4. Number of seta on tibia of fore legs.
5. Lengths of dark apex and clear part of tibia and total length of tibia for fore legs.
6. Lengths of spheniculi and reticulated apex of spheniculi.
7. Lengths of cauda base (triangular region) and total length of cauda.

**Table 1: Samples series of *Sitobion avenae* geographical populations and associated information about samples sizes and localities.**

| Sample No. | Locality (Governorate) | GPS           |                  | Sample Size |
|------------|------------------------|---------------|------------------|-------------|
|            |                        | Latitudes     | Longitudes       |             |
| 1          | *Beni-Suif             | 29°05' 15.55" | N31°06' 37.54" E | 15          |
| 2          | El-Fayoum              | 29°26' 19.88" | N30°46' 19.06" E | 17          |
| 3          | *El-Giza               | 30°01' 03.64" | N31°12' 17.60" E | 14          |
| 4          | *El-Qaloubia           | 30°17' 11.36" | N31°11' 59.49" E | 20          |
| 5          | *El-Sharqya            | 30°35' 43.89" | N31°27' 46.04" E | 18          |
| 6          | *El-Monofia            | 30°36' 23.18" | N31°00' 00.55" E | 13          |
| 7          | El-Gharbia             | 30°49' 15.06" | N30°59' 33.52" E | 16          |
| 8          | El-Dakahlia            | 31°03' 55.40" | N31°22' 15.66" E | 19          |
| 9          | *KaferEl-Shikh         | 31°07' 27.38" | N30°57' 12.12" E | 22          |
| 10         | *El-Behera             | 31°19' 42.13" | N30°24' 16.66" E | 18          |
| 11         | *Domiat                | 31°24' 01.19" | N31°41' 59.04" E | 32          |

\*: This geographical population subjected to Mitochondrial-DNA COI gene sequencing.

Obtained numeric and morphometric data were processed by using COSTAT (2008) statistical software computer program. Relations between tested parameters with each others were studied through applying simple correlation analysis. In addition, all generated data of each tested parameter were subjected to ANOVA test analysis. Data of tested morphological characters that differ significantly from geographical population to another were subjected to hierarchical clusters analysis that based average linkage method and Euclidean distance, which was performed by SYSTAT 13 Computer program.

### 3. Mitochondrial-DNA COI analysis:

Eleven specimens of geographical populations for *Sitobion avenae*, collected from wheat plants in eleven Governorates of Middle and Lower Egypt, were subjected to Real Time – Polymerase Chain Reaction

RT-PCR for quantification of cytochrome oxidase subunit I (COI) genes expression. Moreover, Cytochrome Oxidase subunit I (COI) gene in eight geographical populations of grain aphid was sequenced by using specific primers of the gene (LepF) and (LepR) with primers sequence as in Table (2) Hebert *et al.* (2004) and Zhao-huan *et al.* (2011).

**Table 2: Sequence of specific two primers for cytochrome oxidase subunit I (COI) gene that used in real-time PCR and gene sequencing.**

| Primers | Primer sequence 5'→3'      |
|---------|----------------------------|
| Lep (F) | ATTCAACCAATCATAAAGATATTGG  |
| Lep (R) | TAAACTTCTGGATGTCCAAAAAATCA |

**Quantification of the cytochrome oxidase subunit I (COI) genes expression using real-time PCR:**

**a) RNA isolation protocol:**

Total RNA was extracted from tissues of single individual of aphid by using GStract™ RNA Isolation kit II (Guanidium Thiocyanate methods) according to the manufacture procedures.

**b) Reverse transcription-polymerase chain reaction (RT-PCR) of mRNA:**

Reverse transcription (RT) or first strand reaction was performed for converting the mRNA to complementary DNA (cDNA) in the presence of deoxynucleotide triphosphates (dNTPS) and reverse transcriptase enzyme. The components are combined with DNA primer in a reverse transcriptase buffer for an hour at 42°C. The exponential amplification via reverse transcription polymerase chain reaction provides a highly sensitive technique, where a very low copy number of RNA molecules can be detected.

Reverse transcription reaction was performed using oligo(dT) primer (5'-TTTTTTTTTTTTTTTT-3'). Each 25 µl reaction mixture contained 2.5 µl (5x) buffer with MgCl<sub>2</sub>, 2.5 µl (2.5 mM) dNTPs, 1 µl (10 pmol) primer, 2.5 µl RNA (2mg/ml) and 0.5 unit reverse transcriptase enzyme. PCR amplification was performed in a thermal cycler programmed at 42 °C for 1 hr, 72 °C for 10 min (enzyme inactivation) and the product was stored at 4 °C until use.

**c) Quantitative estimation for cytochrome oxidase subunit I (COI) genes expression using RT-qPCR:**

Samples were analysed using the Fermentase kit: Each reaction contained 12.5 µl of 2x Quantitech SYBR® Green RT Mix, 1 µl of 25 pm/µl forward primer (Lep F), 1 µl of 25 pm/µl reverse primer (Lep R), 1 µl of the cDNA (50ng), 9.25 µl of RNase free water for a total of 25 µl. Samples were spun before loading in the Rotor's wells.

The real time PCR program was set up as follows : initial denaturation at 95 °C for 10 min.; 40 cycles of denaturation at 95°C for 15 sec.; annealing at 60°C for 30 sec. and extension at 72 °C for 30 sec. Data acquisition was performed during the extension step. This reaction was performed using Rotor-Gene- 6000-system (Qiagen, USA).

**d) Data analysis:**

After real-time PCR reactions were completed, the means of threshold cycle (Ct) values were calculated from three replicates. Obtained means were subjected to ANOVA test analysis by using SAS Software (2008) then hierarchical clusters analysis based on single linkage method and Euclidean distance for them singularly or combined with the tested morphological characters, were performed by SYSTAT 13 Computer program.

**Cytochrome oxidase subunit I (COI) gene sequencing:**

**a) DNA extraction and amplification**

DNA from single individual of apterus aphid adult was extracted using a Cetyl Trimethyl Ammonium Bromide (CTAB) protocol Weeks *et al.* (2000) with some modifications as described by Tabikha and adds (2016). To detect the extracted DNA, 1.2% agarose gel in TBE buffer was used and then 5 µl of DNA with double amount of loading buffer were mixed and electrophoresed under a constant voltage of 80 volts for 1.5 hours. Quantity and quality of extracted DNA were determined by spectrophotometer and agarose gel electrophoresis.

The cytochrome oxidase subunit I region with approximately molecular size of 700bp was amplified by using the previous mentioned primers in Table (2) according to Zahao-huan *et al.* (2011). The PCR reaction was performed using total volume of 25 µl, containing 12.5 µl 2x Taq PCR Master mix (Biomed biotech, Beijing, China), 2 µl 10 µmol L<sup>-1</sup> of each primer, 2 µl DNA template and 6.5 µl H<sub>2</sub>O. PCR conditions were as follow, initial cycle with 94 °C for 5 min. and 40 cycles of 94 °C for 30 sec., 50 °C for 30 sec. and 72°C for 40sec., followed by extension cycle at 72°C for 10 min. The PCR product was visualized using 1% ethidium bromide on agarose gel and photographed using gel documentation system.

**b) DNA sequencing of COI gene:**

**PCR product purification:** PCR clean up column kit (Maxim Biotech INC, USA) was used to purify the amplified PCR products of the COI gene as follow:

1. Three volumes of Membrane Binding Buffer were added to one volume of PCR product and mixed well. One volume of isopropanol was added to the mixture and mixed by inverting tube 5 times.
2. The mixture was transferred into a spin column in a 2.0 ml micro-centrifuge tube and centrifuged for one min. at 15.000 rpm.
3. The spin column was transferred into a fresh 2.0 ml micro-centrifuge tube carefully and 500 µl of washing buffer were added into the spin column and centrifugation was done for one min. at 15.000 rpm.
4. The spin column was transferred into a new 1.5 ml centrifuge tube and centrifuged for one min. at 15.000 rpm.
5. The spin column was transferred into a new 1.5 ml centrifuge tube and 50-120 µl of elution buffer were added to the spin column, the column was stranded for one min. and centrifuged for one min. at 15.000 rpm

Sequence alignment: Pairwise and multiple DNA sequence alignment were carried out using CLUSTALW multiple sequence alignment programme version 1.82 (<http://www.ebi.ac.uk/clustalw>), Thompson *et al.* (1994).

**c) Phylogenetic analysis**

Bootstrap neighbour joining tree was generated using MEGA 4 Kamura *et al.* (2004) from CLUSTALW alignment. Comparison with sequences in the GenBank Database was achieved in BLASTN searches at the National Centre for Biotechnology Information site (<http://ncbi.nlm.nih.gov>).

**RESULTS**

Searching missions for monitoring, collecting, screening morphometric, numeric morphological characters and studying genetic variations of *Sitobion avenae* (Fab.) in Egypt Governorates, declared that this species was observed with abundant numbers in Middle and Lower Governorates of Egypt, while its presence was reared in Upper Egypt Governorates during March, 2015. So current study based on 11 geographical populations collected from Governorates located in

Middle and Lower Egypt. Twenty five morphometric or numeric morphological characters were investigated and screened in the collected populations; in addition to forty ratios among those characters were also concerned. On the other hand, genetic variations among those populations were based on mt-DNA COI analysis for extracted DNA and RNA from each population. Finally, relatedness among those populations was also concerned on base of morphological or genetic variations among the populations.

**1. Relatedness among populations based on screening morphological characters.**

Although most of the tested morphometric and numeric morphological characters don't differ significantly from geographical populations to another, there are ten characters varied significantly among the tested geographical populations. So Table (3) presented summary of screening of morphometric and numeric morphological character data for *S. avenae* and validated ranges of each characters that characterized Egyptian populations and that is nearly constant in local populations.

**Table 3: Summary for some morphometric and numeric morphological traits characterize *Sitobion avenae* populations in Egypt.**

| Morphometric characters "Lengths" range (mm) |             |                               |             |
|--|-------------|-------------------------------|-------------|
| Ant. Seg. I                                  | 0.09 - 0.12 | Dark region of tibia          | 0.16 - 0.33 |
| Ant. Seg. II                                 | 0.07 - 0.10 | Clear region of tibia         | 0.79 - 1.22 |
| Ant. Seg. III                                | 0.59 - 0.77 | Reticulated Apex of Sphinculi | 0.11 - 0.18 |
| Ant. Seg. IV                                 | 0.39 - 0.70 | Sphinculi                     | 0.43 - 0.64 |
| Ant. Seg. V                                  | 0.34 - 0.59 | Base of Cauda                 | 0.10 - 0.18 |
| Ant. Seg. VI (Base)                          | 0.11 - 0.18 | Cauda                         | 0.28 - 0.51 |
| Ant. Seg. VI (Unguis )                       | 0.60 - 0.82 | Rostral Seg. IV               | 0.11 - 0.12 |
| Antenna                                      | 2.30 - 3.11 | Rostral Seg. V                | 0.10 - 0.12 |
| Tibia of fore Leg                            | 0.95 - 1.44 |                               |             |
| Numeric characters (range of setae numbers)  |             |                               |             |
| Ant. Seg. I                                  | 4 - 7       | Ant. Seg. V                   | 3 - 10      |
| Ant. Seg. II                                 | 3 - 5       | Ant. Seg. VI (Base)           | 2 - 5       |
| Ant. Seg. III                                | 9 - 19      | Antenna                       | 33 - 54     |
| Ant. Seg. IV                                 | 6 - 13      | Tibia of fore Leg             | 50 - 81     |

The varied morphological characters and results of ANOVA test analysis among the geographical populations were presented in Table (4), which reflected that each of the following characters showed high significant difference among 11 populations:-

- 1- Ratio between length of tibia to length of fourth rostral segment.
- 2- Ratio between length of unguis to length of fourth rostral segment.
- 3- Ratio between length of tibia to length of unguis.
- 4- Ratio between length of tibia to length basal part of the sixth antennal segment.
- 5- Ratio between length of the fourth antennal segment to length of unguis.
- 6- Ratio between length of basal part of the sixth antennal segment to length of clear part of tibia.
- 7- Ratio between length basal part of the sixth antennal segment to length the fourth rostral segment.
- 8- Ratio of sensorial setae numbers on the third antennal segment to antenna.

9- Numbers of sensorial setae on the third antennal segments.

10- Number of setae on tibia.

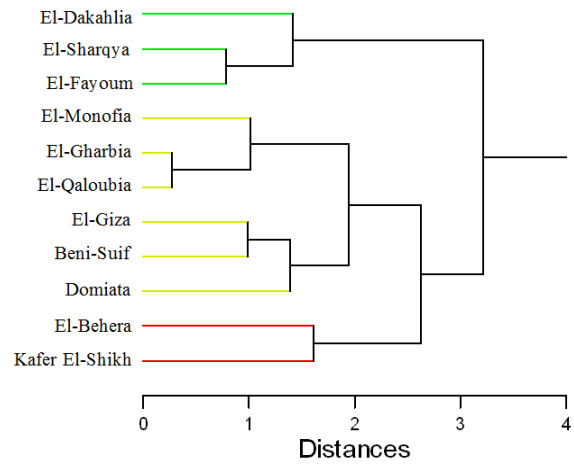
So it will be recommended avoiding using those characters in discriminate *Sitobion avenae* species. In contrary the rest constant tested characters can be used as taxonomic characters for *Sitobion avenae* species as possible or at least characterized *Sitobion avenae* populations of Egypt, but it is unsuitable to discriminate geographical populations of *Sitobion avenae* inside Egypt.

Hierarchical clusters, which showed relatedness among the eleven geographical populations of *Sitobion avenae* and based on the ten variable morphometric and numeric morphological characters among the geographical populations were graphically illustrated in Figure (1). Results reflected that the eleven geographical populations can be classified into two main clusters. The first cluster includes two sub-clusters, which separate El-Fayoum and El-Sharqya populations from El-Dakahlia population. The second cluster includes the

most tested populations. It was separated into two sub-clusters; the first sub-cluster includes populations of El-Behera and Kafer El-Shikh. While the second sub-cluster divided into two groups, the first group includes two sub-groups that the first sub-group includes Domiata population, while the second one includes populations of Beni-Suif and El-Giza; the second group separates population of El-Monofia from the most related populations of El-Gharbia and El-Qaloubia.

**2. Relatedness among populations based on mt-DNA COI analysis:**

To study genetic variations and relationships among the eleven geographical populations of *S.avenae* that collected from different localities in Middle and Lower Egypt, quantification of the cytochrome oxidase subunit I (COI) genes expression of mt-DNA was assayed by using real-time PCR (RT-qPCR). For more investigation of genetic variations, one fragment of 700 bp of this gene was amplified and sequenced by using DNA sequencer, in eight out of the eleven tested geographical populations because of abundance of specimens in those populations. All obtained data were subjected to hierarchical cluster analysing to show the degree of homology or analogy among those populations



**Figure 1:** Hierarchical clusters show relatedness among eleven geographical populations of *Sitobionavenae*, based on investigation of ten morphometric or numeric morphological characters, varied significantly among the tested populations.

**Table (4):** Variations among geographical populations of *Sitobion avenae* in eleven Egyptian Governorates based on ten morphometric and numeric characters of adult apterus viviparous.

| Locality (Governorate) | Characters (Means ±S.D.)   |                          |                          |                              |                           |  |                                     |                            |                             |                            |
|------------------------|----------------------------|--------------------------|--------------------------|------------------------------|---------------------------|--|-------------------------------------|----------------------------|-----------------------------|----------------------------|
|                        | Ratios of Lengths          |                          |                          |                              |                           | Numbers of setae on                        |                                     |                            |                             |                            |
|                        | Tibia / Ros. Seg. V        | Unguis / Ros. Seg. V     | Tibia / Unguis           | Tibia / Base of Ant. Seg. VI | Ant. Seg. IV / Unguis     | Base of Ant. Seg. VI / Clear part of Tibia | Base of Ant. Seg. VI / Ros. Seg. IV | Ant. Seg. III / Antenna    | Ant. Seg. III               | Tibiae                     |
| Beni-Suif              | 10.62±0.92 <sup>abc</sup>  | 6.16±0.29 <sup>bc</sup>  | 1.77±0.04 <sup>ab</sup>  | 7.82±0.27 <sup>b</sup>       | 0.822±0.05 <sup>ab</sup>  | 6.38±0.51 <sup>c</sup>                     | 1.39±0.09 <sup>ab</sup>             | 30.68±2.67 <sup>abcd</sup> | 12.25±1.89 <sup>abcde</sup> | 67.50±7.90 <sup>abcd</sup> |
| El-Fayoum              | 11.50±0.53 <sup>a</sup>    | 6.41±0.16 <sup>abc</sup> | 1.79±0.04 <sup>a</sup>   | 7.76±0.19 <sup>b</sup>       | 0.835±0.07 <sup>ab</sup>  | 6.50±0.29 <sup>bc</sup>                    | 1.48±0.03 <sup>a</sup>              | 30.16±2.75 <sup>abcd</sup> | 11.67±0.58 <sup>bcde</sup>  | 74.33±8.08 <sup>ab</sup>   |
| El-Giza                | 10.99±1.14 <sup>ab</sup>   | 6.26±0.34 <sup>abc</sup> | 1.79±0.06 <sup>a</sup>   | 7.75±0.28 <sup>b</sup>       | 0.835±0.05 <sup>a</sup>   | 6.24±0.55 <sup>c</sup>                     | 1.45±0.15 <sup>ab</sup>             | 29.28±3.89 <sup>abcd</sup> | 11.6±2.19 <sup>cde</sup>    | 70.20±9.12 <sup>abc</sup>  |
| El-Qaloubia            | 10.14±0.67 <sup>bc</sup>   | 6.29±0.46 <sup>abc</sup> | 1.68±0.10 <sup>abc</sup> | 7.97±0.46 <sup>b</sup>       | 0.795±0.07 <sup>abc</sup> | 6.50±0.64 <sup>bc</sup>                    | 1.33±0.01 <sup>abc</sup>            | 28.11±4.90 <sup>cd</sup>   | 11.40±2.30 <sup>de</sup>    | 65.60±5.37 <sup>bcd</sup>  |
| El-Sharqya             | 9.85±1.15 <sup>cd</sup>    | 6.53±0.56 <sup>ab</sup>  | 1.61±0.08 <sup>c</sup>   | 7.63±0.86 <sup>b</sup>       | 0.739±0.08 <sup>cd</sup>  | 6.28±0.67 <sup>c</sup>                     | 1.39±0.12 <sup>ab</sup>             | 30.42±2.83 <sup>abcd</sup> | 12.40±1.14 <sup>abcde</sup> | 76.00±4.64 <sup>a</sup>    |
| El-Monofia             | 10.28±0.61 <sup>bcd</sup>  | 6.00±0.29 <sup>c</sup>   | 1.75±0.12 <sup>ab</sup>  | 7.52±0.57 <sup>b</sup>       | 0.799±0.04 <sup>abc</sup> | 6.17±0.54 <sup>c</sup>                     | 1.40±0.10 <sup>ab</sup>             | 25.52±4.24 <sup>abcd</sup> | 10.20±1.30 <sup>e</sup>     | 63.80±5.97 <sup>cd</sup>   |
| El-Gharbia             | 10.06±0.37 <sup>bcd</sup>  | 5.94±0.32 <sup>c</sup>   | 1.77±0.12 <sup>a</sup>   | 7.58±0.62 <sup>b</sup>       | 0.781±0.02 <sup>abc</sup> | 6.22±0.52 <sup>c</sup>                     | 1.39±0.12 <sup>ab</sup>             | 27.96±4.06 <sup>abcd</sup> | 11.40±1.82 <sup>de</sup>    | 65.00±8.03 <sup>bcd</sup>  |
| El-Dakahlia            | 10.72±0.51 <sup>abc</sup>  | 6.53±0.17 <sup>ab</sup>  | 1.64±0.10 <sup>bc</sup>  | 8.74±0.71 <sup>a</sup>       | 0.752±0.05 <sup>bcd</sup> | 7.38±0.53 <sup>a</sup>                     | 1.23±0.12 <sup>cd</sup>             | 33.46±2.86 <sup>ab</sup>   | 14.25±1.71 <sup>abc</sup>   | 74.00±4.55 <sup>ab</sup>   |
| Kafer El-Shikh         | 10.11±0.33 <sup>bcd</sup>  | 5.98±0.21 <sup>c</sup>   | 1.73±0.09 <sup>ab</sup>  | 8.84±0.61 <sup>a</sup>       | 0.740±0.04 <sup>cd</sup>  | 7.25±0.53 <sup>ab</sup>                    | 1.18±0.13 <sup>d</sup>              | 34.46±1.31 <sup>a</sup>    | 15.00±2.74 <sup>a</sup>     | 59.40±5.41 <sup>d</sup>    |
| El-Behera              | 9.60±0.71 <sup>d</sup>     | 5.96±0.17 <sup>c</sup>   | 1.64±0.08 <sup>bc</sup>  | 7.42±0.52 <sup>b</sup>       | 0.756±0.06 <sup>bcd</sup> | 5.95±0.36 <sup>c</sup>                     | 1.32±0.10 <sup>bc</sup>             | 32.72±5.76 <sup>abc</sup>  | 13.80±3.11 <sup>abcd</sup>  | 63.60±7.50 <sup>cd</sup>   |
| Domiata                | 10.44±0.58 <sup>abcd</sup> | 6.66±0.24 <sup>a</sup>   | 1.58±0.09 <sup>c</sup>   | 9.05±0.17 <sup>a</sup>       | 0.706±0.02 <sup>d</sup>   | 7.54±0.24 <sup>a</sup>                     | 1.17±0.08 <sup>d</sup>              | 32.56±3.96 <sup>abc</sup>  | 14.60±2.07 <sup>ab</sup>    | 68.80±6.02 <sup>abc</sup>  |
| "F" Value              | 2.156*                     | 3.077**                  | 3.327**                  | 5.687***                     | 2.587*                    | 5.348***                                   | 4.506***                            | 2.386*                     | 2.757*                      | 2.743*                     |
| L.S.D. 0.05            | 0.55                       | 0.106                    | 0.008                    | 0.286                        | 0.093                     | 0.85                                       | 0.175                               | 14.743                     | 4.294                       | 45.042                     |

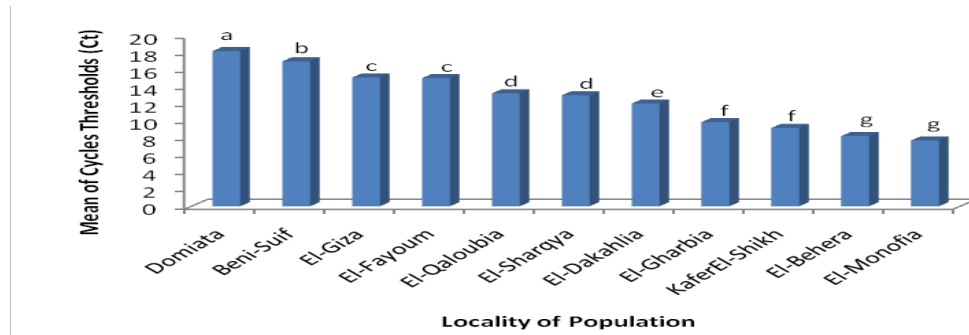
**Quantification of the cytochrome oxidase subunit I (COI) gene expression:**

To detect the relative amounts of mRNA for COI gene using RT-PCR in the eleven geographical populations of *S.avenae*, results illustrated in Figure (2), showed that cycles thresholds (Ct) of collected populations ranged between 7.720±0.451 to 18.180±0.852. In addition, results of ANOVA test reflected presence of high significant differences among those populations (F=126.688\*\*\*, LSD0.05=0.922) where the highest amounts of mRNA were detected from COI gene in geographical population of Domiata followed significantly by Beni-Suif while the lowest amount was observed in populations of El-Monofia and El-Behera. The level of gene expression was moderated in other populations especially in populations of El-Sharqya, El-Qaloubia and El-Dakahlia.

Relatedness among the eleven geographical populations of *S.avenae*, based on quantification of the COI gene expression by using RT-PCR was graphically illustrated in Figure (3A). It reflected that the eleven geographical populations can be classified into two main clusters. The first cluster was divided into two sub-cluster, the first sub-cluster contained populations of El-Monofia and El-Behera, while the second sub-cluster contained populations of Kafer El-Shikh and El-Gharbia. On the other hand, the second cluster was divided into two sub-clusters, the first one included populations of Domiata and Beni-Suif, while the second sub-cluster branched into two groups, the first group contained populations of Giza and El-Fayoum with moderated level of similarity, while the second group contained El-Dakahlia, El-Sharqya and El-Qaloubia populations, with high level of similarity between the last two populations.

In case of combining quantitative data of morphological analysis for some morphometric characters with quantitative data of COI gene expression as shown in Figure (3B), the eleven population could be classified into two clusters, the first cluster contained populations of Kafer El-Shikh and El-Behera; while the second cluster was divided into two sub-clusters, the first sub-clusters contained populations

of El-Monofia, El- Gharbia and El-Qaloubia, while the second sub-clusters contained two groups, the first one included two sub-groups one for populations of Domiata, Beni-Suif and Giza and the other contained populations of El- Sharqya and El-Fayoum populations with high level of similarity; the second group included only population of El-Dakahlia.



- Columns with the same letter indicate no significant difference among Ct values of their populations

Figure 2: Histogram for quantitative estimation of COI gene expression for geographical populations of *S. avenae*, expressed as cycles thresholds (Ct) values.

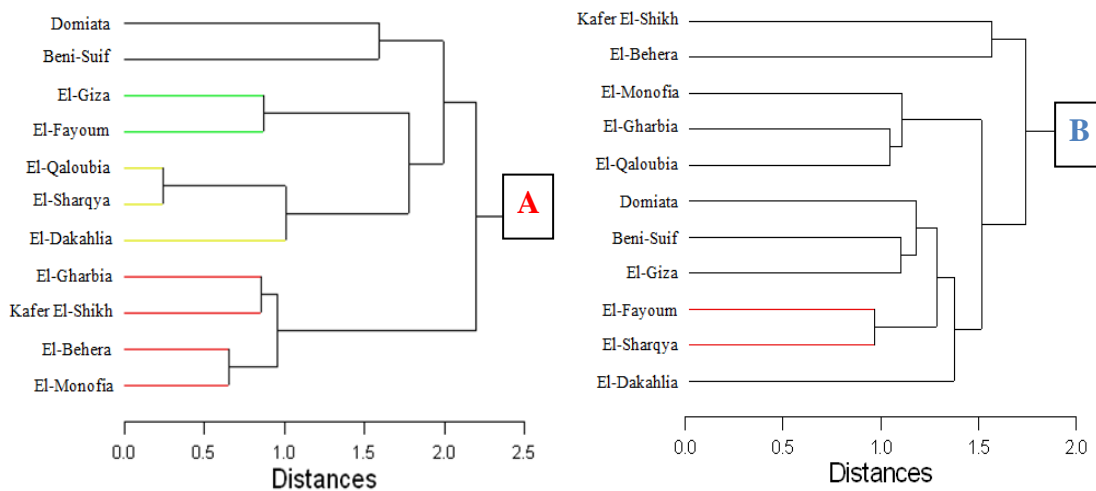


Figure 3. Hierarchical clusters show relatedness among eleven geographical populations of *S. avenae*, based on (A) quantification of the COI gene expression and (B) combined each of quantitative characters of morphological investigation and Ct values of COI gene expression.

**DNA sequencing of cytochrome oxidase subunit I (COI) gene:**

The amplified 700 bp DNA fragment of the COI gene in eight geographical populations of *S. avenae* was subjected to sequencing by using DNA sequencer at MacroGen Company (Korea). The results of sequence analysis as shown in Table (5), revealed that 23 polymorphic sites of this fragment were detected in examined specimens, which eight of them were transitions and transversion, six of them were in T/C transitions, four of them were A/C transversions, four of them were A/T transversions and one of them was G/T transversions. The percentages of the four nucleotides in sequence were 35.33% for Thymine (T), 31.52% for Cytosine (C), 27.72% for Adenine and 5.43% for

Guanine (G), which indicates that interchanged basis biased toward Thymine.

Pairwise distance comparison among the eight geographical populations of *S. avenae* shown in Table (6) ranged from 0.9% (difference in 6 nucleotides) to 2.7% (difference in 18 nucleotides). The largest sequence divergence was detected when population of Domiata compared with populations of El-Sharqya or Kafer El-Shikh, while the lowest one was detected between populations of El-Behera and El-Monofia.

Alignment and Phylogenetic analysis of COI gene sequences in the eight geographical populations of *S. avenae* were listed in GeneBank and were carried out using CLUSTAL W (1.82) (<http://www.ebi.ac.uk/clustalw>) Thompson *et al.* (1994). MEGA4 was used to

generate the Bootstrap neighbour-joining tree Kamura *et al.*(2004)as graphically illustrated in Figure (4). Phylogenetic tree for the eight geographical populations of *S. avenae* showed that the eight populations could be classified into two clusters. The first cluster included two sub-clusters; the first sub-cluster included population of El-Giza, while the second sub-cluster was divided into two groups, the first group included

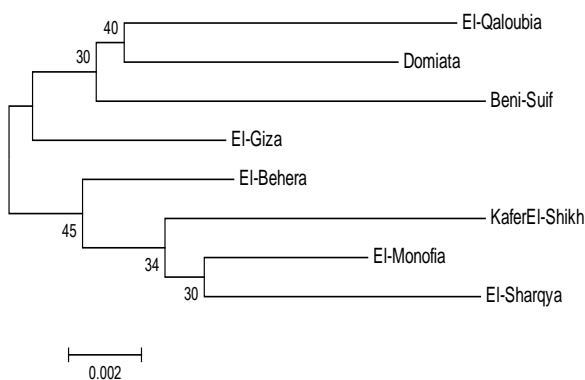
population of Beni-Suif while the other one included populations of El-Qaloubia and Domiata. On the other hand, the second cluster branched into two sub-clusters, the first sub-cluster included only population of El-Behera, while the second sub-cluster was divided into two groups the first group included only population of Kafer El-Shikh while the other included populations of El-Monofia and El-Sharqya

**Table 5: Variable position of COI gene sequence in eight geographical populations of *S. avenae*.**

| Population Locality | Nucleotides position beginning from 5→3 |    |    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |   |
|---------------------|---|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|---|
|                     | 45                                      | 80 | 97 | 139 | 142 | 190 | 201 | 211 | 227 | 252 | 271 | 303 | 350 | 360 | 410 | 415 | 465 | 484 | 540 | 575 | 599 | 621 | 645 |   |
| Beni-Suif           | A                                       | T  | T  | A   | A   | A   | G   | T   | T   | C   | T   | A   | A   | A   | A   | C   | T   | A   | T   | A   | C   | T   | C   |   |
| El-Giza             | A                                       | A  | T  | C   | A   | A   | T   | C   | G   | T   | C   | A   | A   | A   | C   | T   | T   | A   | C   | T   | C   | C   | C   |   |
| El-Qaloubia         | A                                       | T  | C  | A   | T   | T   | C   | C   | C   | T   | C   | A   | G   | A   | C   | T   | G   | A   | T   | T   | C   | A   | C   |   |
| El-Sharqya          | C                                       | A  | C  | A   | A   | T   | G   | A   | T   | C   | T   | C   | T   | T   | C   | C   | T   | A   | C   | A   | A   | A   | T   | C |
| El-Monofia          | A                                       | A  | C  | C   | T   | T   | T   | C   | T   | C   | C   | C   | T   | T   | C   | T   | G   | A   | C   | A   | A   | T   | C   |   |
| afer El-Shikh       | C                                       | A  | T  | C   | C   | T   | T   | C   | T   | T   | T   | C   | T   | A   | C   | C   | G   | A   | C   | T   | G   | T   | T   |   |
| El-Behera           | A                                       | A  | T  | C   | A   | T   | T   | C   | C   | T   | C   | A   | T   | T   | C   | T   | T   | A   | C   | A   | A   | T   | C   |   |
| Domiatia            | A                                       | T  | T  | T   | C   | A   | T   | T   | G   | T   | C   | A   | G   | T   | C   | T   | T   | C   | T   | A   | C   | A   | C   |   |

**Table 6:Pairwise comparisons of nucleotide sequence of mtDNA-COI gene of *S. avenae*, where mean and absolute distance are given below and above diagonal, respectively.**

|               | Beni-Suif | El-Qaloubia | Domiatia | El-Giza | El-Behera | El-Monofia | El-Sharqya | KaferEl-Shikh |
|---------------|-----------|-------------|----------|---------|-----------|------------|------------|---------------|
| Beni-Suif     |           | 14          | 12       | 12      | 14        | 16         | 11         | 16            |
| El-Qaloubia   | 0.021     |             | 11       | 11      | 12        | 12         | 17         | 15            |
| Domiatia      | 0.018     | 0.017       |          | 10      | 11        | 15         | 18         | 18            |
| El-Giza       | 0.018     | 0.017       | 0.015    |         | 7         | 12         | 16         | 12            |
| El-Behera     | 0.021     | 0.018       | 0.017    | 0.011   |           | 6          | 10         | 11            |
| El-Monofia    | 0.024     | 0.018       | 0.023    | 0.018   | 0.009     |            | 8          | 10            |
| El-Sharqya    | 0.017     | 0.026       | 0.027    | 0.024   | 0.015     | 0.012      |            | 11            |
| KaferEl-Shikh | 0.024     | 0.023       | 0.027    | 0.018   | 0.017     | 0.015      | 0.017      |               |



**Figure 4.Hierarchical clusters show relatedness among eight geographical populations of *S. avenae*, based on nucleotide sequences in mtDNA-COI gene using the Mega4 program.**

**DISCUSSION**

Colour in the English grain aphid is determined both genetically and in response to environmental factors Jenkins *et al.*(1999). Classical morphological criteria for aphid species identification may be affected by environmental factors such as climatic conditions

and physiological status of the host plantHelmi *et al.*(2011). Most of the tested morphometric and numeric morphological characters were nearly constant among 16 investigated geographical populations of *R.padi* in Egypt except five characters, which were varied significantly from geographical population to another. Those varied characters were ratios between second antennal segment length to total antenna length, first to second antennal segment width, length of first to second tarsal segment, and width to length of spheniculi, in addition numbers of sensorial hairs on fourth antennal segments. Population of El-Fayoum Governorate was nearly different morphologically than others Tabikha and Adss(2016), while current study reported that ten morphometric and numeric characters varied significantly among the eleven geographical populations of *S. avenae*.

Molecular techniques, such as DNA barcoding, may be more accurate than visual identification in cases where species or races are morphologically similar. DNA barcoding of the 5' end of the mitochondrial cytochrome oxidase I (COI) gene for species identification Hebert *et al.*(2003)has been shown to differentiate species of Aphididae Foottit *et al.*(2008) and Lee *et al.*(2011).Sequences from the cytochrome oxidase subunit II (COII) gene and elongation factor-I

alpha (EF-1) were used to examine variation within the *Myzus persicae* (Sulzer) complex. The low variation within this complex led to conclude that *M. nicotianae* and *M. persicae* were synonymous (all COII sequences were identical for all *M. persicae*) clones Clements *et al.* (2000). Although each of geographical and seasonal distribution of *S. avenae* populations had low effect on genetic variability of *S. avenae* in Chile Figueroa *et al.* (2005), in current study pairwise distance comparison among the eight geographical populations of *S. avenae* ranged from 0.9% to 2.7%, while it ranged from 0.2 (one nucleotide) to 1.7% (ten nucleotides) in sixteen haplotypes of *S. avenae* in China Zhao-huan *et al.* (2011). Divergence of only 0.4% was found in host races of *Acyrtosiphon pisum* by using the mt-DNA COI gene Boulding (1998) while in different species such as *S. avenae* and *S. miscanthi* are differ by 1.5% sequence divergence in mt-DNA COI gene Sunnucks and Hales (1996). In the current study Thymine nucleotide was the highest in sequence which agree with Simon *et al.* (1994), Dohlen *et al.* (2002) and Zhao-huan *et al.* (2011), who found that sequence biased toward Thymine and Adenine.

A region of 1.0 kb in the mitochondrial cytochrome oxidase subunit I gene from the greenbug aphid, *Schizaphis graminum* (Rondani), was sequenced in 24 field collected clones from non-cultivated and cultivated hosts Anstead *et al.* (2002). DNA sequences of the mt-DNA cytochrome oxidase I (COI) gene in greenbug, *Schizaphis graminum* revealed presence of 3 sub-specific clades. Each clade has been found to roughly correspond to a host ecotype Shufran (2003). Forty four multilocus genotypes were found from 1052 samples of *S. avenae* collected from wild and cultivated Poaceae in Chile Figueroa *et al.* (2004).

Real-time PCR has been used for pest insect identification Walsh *et al.* (2005) Yu *et al.* (2005) and Huang *et al.* (2010), including aphids Naaum *et al.* (2012) that signals were observed in the FAM fluorescence channel for *A. glycines* between Ct = 19.81–25.72 and for *Aphis oestlundii* between Ct = 30.75–32.51 Naaum *et al.* (2014), while in the current study Ct ranged between 7.720 to 18.180 in geographical populations of *S. avenae* in Egypt.

## CONCLUSION

Geographical populations of El-Gharbia and El-Qaloubia were more related morphologically. Relative amounts of mRNA from COI gene ranged between 7.720 to 18.180 as cycles thresholds (Ct) that the highest amount of mRNA was detected in geographical populations of Domiata, in addition to populations of El-Qaloubia and El-Sharqya were more related populations. Mitochondrial-DNA COI gene sequencing in eight geographical populations gave 23 polymorphic sites. The highest sequence divergence was between populations of Domiata and El-Sharqya or Kafer El-Shikh, while populations of El-Behera and El-Monofia Governorates were related.

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دراسة مدى تقارب العشائر الجغرافية لحشرة من الغلال الانجليزي *Sitobion avenae* المنتشرة بمصر وذلك بعمل مسح لبعض الصفات المورفولوجية وتحليل المواضع الجينية لحامض دانا الميتوكونديري والمسئولة عن تكوين انزيم **Cytochrome oxidase I**.

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تعتبر حشرة من الغلال الانجليزي من انواع الافات الحشرية المهمة والمنتشرة على مدى واسع في حقول القمح خاصنا في محافظات مصر الوسطى ومصر الدنيا. ولكن من المعروف ان الصفات المورفولوجية المستخدمة في تميز انواع المن ربما ان تتأثر بالظروف البيئة المحيطة. لذلك اهتمت الدراسة الحالية على جمع احدى عشر عشيرة جغرافية من حشرة من الغلال الانجليزي من مواقع مختلفة بجمهورية مصر العربية ودراسة خمسة وعشرون صفة مورفولوجية كمية بهم ( صفات مورفولوجية عددية او قياسية). بالاضافة الى دراسة الاختلافات الوراثية ما بين هذه العشائر على اساس دراسة تتابعات القواعد النروجينية المكونة للجين المسئول عن تكوين Cytochrome oxidase I بحامض الدواكسي ريبو نيوكليك اسيد المتواجد بالميتوكونديريا وكذلك مدى التعبير الجيني له باستخدام Real-time PCR (RT-qPCR). واخيرا قد تم دراسة مدى التقارب ما بين هذه العشائر على اساس الاختلافات المورفولوجية و الوراثية بينهم. حيث اظهرت النتائج ان هناك عشرة صفات مورفولوجية اختلفت معنويا من عشيرة الى اخرى، ولكن كانت العشائر الجغرافية المنتشرة بمحافظتي الغربية والشرقية اكثر تقاربا من الناحية المورفولوجية. اما بالنسبة لمدى التعبير الجيني الممثل في كمية حامض الريبو نيوكليك اسيد (الرسول) الناتج من الجين تراوحت ما بين 0.451 ± 0.720 الى 0.852 ± 18.180 (Ct) حيث اكبر كم منه لوحظ في العشيرة الجغرافية المنتشرة بمحافظة دمياط تبعتها عشيرة محافظة بنى سويف، كما ان عشيرتي محافظتي القليوبية والشرقية كانوا اكثر تقاربا. ودراسة تتابع القواعد النروجينية بهذا الجين وجد ثلاثة وعشرون موضع للاختلاف ما بين ثمانية عشائر مدروسة حيث مدى الاختلاف تراوح ما بين 0.9 % (6 قواعد نروجينية مختلفة) الى 2.7 % (18 قاعدة نروجينية مختلفة)، وعليه كانت عشيرة محافظة دمياط اكثر اختلافا عن عشائر محافظتي الغربية وكفر الشيخ، وعلى عكس ذلك كانت عشيرتي محافظة البحيرة ومحافظة المنوفية اكثر تقاربا.

**Keywords:** من الغلال - الشكل الظاهري - حامض دانا بالميتوكونديري - RT-PCR - تتابعات القواعد النروجينية - انزيم Cytochrome oxidase I (COI) - عشائر جغرافية - مصر.