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First DNA Barcoding-Based Record of *Tetranychus* and their Predators in Egypt

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ABSTRACT

Tetranychus sp. is a globally spread economic pest that affects multiple plant species affecting the quality and the quantity of the crop. Because of its economic importance, the ecological and molecular perspective was investigated deeply for this pest. From the ecological perspective, the numbers and the relative infestation of *Tetranychus* were investigated for different cultivars of maize, and we had found that there wer three cultivars that were relatively resistant in Egypt; namely, Pioneer 3057, 321 and 352 under the prevailing conditions of temperature and humidity. From the molecular perspective, the DNA barcoding technique was applied for the first time in Egypt for *Tetranychus* species and its predators, and we had found two species of *Tetranychus*; namely, *Tetranychus cinnabarinus* and *Tetranychus ludeni* Zacher which would have been mistakenly identified as *Tetranychus urticae* Koch., in most of the cases due to the high resemblance in morphology, and also we had found a predator ;namely, *Neoseiulus barkeri*, as one of the predators of *Tetranychus* species. This research proved that combining both ecological and molecular analysis of the pest is very important for understanding the best protection and prevention options. Moreover, the molecular DNA barcoding is a very reliable tool in *tetranychus* species identification and its predators.

INTRODUCTION

Tetranychus sp. is a globally spread economic pest that affects multiple plant species of herbaceous plants, fruit trees and crops of all kinds, affecting the quality and quantity of the crop. Therefore, it is of a great importance to combat it either by the standard chemical methods or by the selection of resistant varieties under the available environmental conditions (Abo-Karah, 1978; Hafez *et al*., 1989 and Gamiehm *et al*., 1993).

The use of molecular diagnostics for all types of organisms including mites has been used extensively in the recent years. DNA barcoding and various types of DNA sequence analyses have been used to determine species in various taxa. Restriction fragment length polymorphism (RFLP), has been used to discriminate closely related species in various taxa, by using universal primer sets and restriction enzymes including closely related species in the genus *Tetranychus* (Osakabe *et al*., 2008 and Arimoto *et al*., 2013). Microsatellite analysis has been used in population genetic analyses of tetranychid mites (Bailly *et al*., 2004). However, it is still very difficult to discriminate species of tetranychid mites because of both intraspecific variation and interspecific similarity. Species-specific primers containing unique nucleotides of the species are useful for species discrimination. However, it is difficult to design primers specific to each species. DNA regions for these primers must be common within species but discriminate from other species belonging to the same genus. Nevertheless, the use of species-specific primers would greatly increase the accuracy and speed of polymerase chain reaction (PCR)-based detection tools. Some representative sequences have been widely used for analysis. Barcode of life (BOLD) uses a 678-nucleotide sequence (the Folmer fragment) of the cytochrome-c oxidase subunit I (COI) sequence of mitochondrial DNA (mtDNA) in most species using the universal primer (Folmer *et al*., 1994). The COI sequence is also commonly used for species identification and phylogenetic relationships of spider mites (Navajas *et al*., 1996 and Ros and Breeuwer, 2007). Further, the complete mitochondrial genome was determined from six species of the genus *Tetranychus* (Chen *et al*., 2014). Misidentification of spider mites in exported products may lead to disputes in quarantine inspections. PCR-assisted diagnosis using species-specific primers can provide rapid and accurate species discrimination in the plant quarantine process.

The main objective of the study was to monitor the correlation relationship between the relative abundance and infestation of *Tetranychus* sp. and the resistance of the different varieties of maize also the correlation between the relative abundance and infestation *Tetranychus* sp. and variable environmental conditions of temperature and humidity throughout the growing season of maize. In addition to the ecological perspective of this work, we used the DNA barcoding technique, for *Tetranychus* species and their predators in Egypt. This pioneer use of DNA barcoding in Egypt will be the cornerstone for the molecular phylogenetic analysis of *Tetranychus* sp. and their predators in Egypt.

MATERIALS AND METHODS

Mite collection and morphological identification:

Fifteen leaves of each variety of maize were collected before noon, and leaves were placed in paper bags until they were transferred to the laboratory for inspection. The collection of mites and their predators was done according to the dates mentioned in Table 1.

Table 1. Dates of mites collection along with the tested meteorological characters during 2017 and 2018 seasons.

Dates	Max-Temp °C	Av-Temp °C	Min-Temp °C	RH-Max %	RH-Min%
		Season 2017			
5/5/2017	30.0	23.9	17.8	78	37
15/6/2017	28.9	25.6	22.8	73	42
25/6/2017	28.9	26.7	23.9	78	45
5/5/2017	30.0	27.8	25.6	69	42
15/7/2017	32.8	26.7	21.1	78	33
25/7/2017	31.7	28.9	25.6	74	40
5/4/2017	30.6	26.1	21.7	83	37
15/8/2017	31.7	28.9	26.1	74	34
		Season 2018			
5/5/2018	27.8	23.9	20.0	90	45
15/6/2018	40.6	30.6	20.6	83	19
25/6/2018	30.0	26.7	23.3	74	44
5/5/2018	30.6	27.8	25.0	89	52
15/7/2018	31.1	28.3	25.6	83	50
25/7/2018	31.1	28.9	26.1	84	40
5/4/2018	32.2	28.9	25.0	84	38
15/8/2018	31.1	28.3	25.6	65	37

Temp= Temperature Max= Maximum Min= Minimum
Av.= Average RH= Relative Humidity%

The numerical density of the lesion for the different maize varieties was then recorded.

Samples of mites and their predators were then separated and placed in vials containing 70% alcohol and 5% glycerin.

Correlation and multiple regression:

Five independent characters viz, maximum, minimum and mean temperature (x) as well as maximum and minimum relative humidity (RH) which assumed to have an effect on mite numbers per each maize variety were used. The correlation between mite numbers per each maize variety and the tested meteorological characters were applied. In regard to multiple regression, the mites number per each maize variety were applied as dependent characters (Y) and the tested meteorological characters as independent ones.

Statistical analysis:

Split plot was used for each trait in both seasons and was analyzed according to Steel and Torrie (1981).

The correlation values and multiple regression were analyzed by using Costat Program. Treatment means were compared by using L.S.D. at 0.05 level of probability.

DNA extraction and PCR amplification:

Three Samples of *Tetranychus* species and their predators (two for *Tetranchus sp.* and one sample for the predator) were randomly selected to be used for molecular analyses. Total DNA was extracted from the whole body of the samples *Tetranychus* species and their predator individuals by using G-Spin TM Total DNA Extraction Kit (iNtRoN). PCR amplification was performed with the following profile: 3 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 47 °C, and 1.5 min at 72 °C. An additional 10 min at 72 °C was allowed for last strand elongation.

The PCR primer used was as follows: primer name: COI – forward: 5' TTYGAYCCWAGAGGAGGAGG 3', reverse: 5' AAACCTARAAAATGTTGWGG 3' (Matsuda *et al*., 2012).

The PCR products were separated using 1% agarose gel electrophoresis, stained with ethidium bromide solution and visualized under UV light. and Sequenced in both directions using the amplifying primers with a BigDye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems) and on an ABI 3130xl automated sequencer.

DNA sequence analysis:

The data were analyzed using MEGA-X program. The GenBank database in the National Center for Biotechnology Information (NCBI) was searched using the BLASTn algorithm (Schäffer *et al*., 2001) and nucleotide sequences were aligned using CLUSTAL W (Thompson *et al*., 1994). Identified COI sequences from Egypt samples were submitted to the GenBank database.

RESULTS AND DISCUSSION

Seasonal population trend of *Tetranychus* species inhabiting maize varieties during two successive seasons

1) Season 2017

The seasonal fluctuation in population density of *Tetranychus* species were recorded in Table (2) and Fig. (1).

Table 2. Seasonal abundance of *Tetranychus* species on maize and relative infestation percentages per each date and each variety during 2017 season.

Maize variety	05-Jun	15-Jun	25-Jun	05-Jul	15-Jul	25-Jul	05-Aug	15-Aug	Mean*
324	3.3	9.3	11.3	16.7	3.0	50.0	60.0	4.0	19.7
%	2.1	5.9	7.2	10.6	1.9	31.7	38.1	2.5	18.4
Watania	2.0	4.3	9.0	5.0	5.0	50.0	53.7	3.0	16.5
%	1.5	3.3	6.8	3.8	3.8	37.9	40.7	2.3	15.4
321	2.0	5.3	8.0	5.0	3.0	44.0	55.0	3.3	15.7
%	1.6	4.2	6.4	4.0	2.4	35.0	43.8	2.7	14.7
Pioneer 3057	0.3	2.3	3.0	4.0	3.0	20.7	28.0	0.3	7.7
%	0.5	3.8	4.9	6.5	4.9	33.5	45.4	0.5	7.2
320	1.7	10.7	8.0	10.3	5.0	51.7	63.0	3.0	19.2
%	1.1	7.0	5.2	6.7	3.3	33.7	41.1	2.0	17.9
352	1.0	4.0	5.0	3.0	3.0	30.0	44.0	2.0	11.5
%	1.1	4.3	5.4	3.3	3.3	32.6	47.8	2.2	10.8
Nefertity	3.0	6.0	5.3	8.3	3.3	47.0	58.0	2.3	16.7
%	2.3	4.5	4.0	6.3	2.5	35.3	43.5	1.8	15.6
Mean	1.9	6.0	7.1	7.5	3.6	41.9	51.7	2.6	15.3
%	1.6	4.9	5.8	6.1	3.0	34.3	42.3	2.1	

*** Relative infestation/each variety**

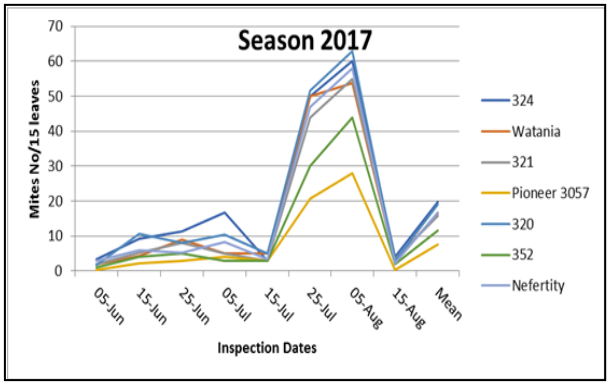

Fig. 1. Seasonal abundance of *Tetranychus* species on maize and relative infestation percentages per each date and each variety during 2017 season.

The mite numbers were relatively constant form 05-jun 2017 till 15-Jul 2017. During 25-Jul 2017 till 05-Aug 2017, the mite numbers suddenly increased then sharply decreased at 15-Aug 2017.

In regard to relative infestation/each variety, the highest susceptible variety was 324 (18.4%) followed by 320 (17.9%), Nefertity (15.6%), Watania (15.4%), 321 (14.7%), 352 (10.8%) and Pioneer 305 (7.2%). The highest Peak of infestation was recorded for 320 (63 mites/10 leaves) followed by 324 (60 mites/10 leaves) on 05-Aug 2017. The lowest infested date was on 05-Jun and 15-Aug 2017. The, relatively tolerant maize variety was Pioneer 3057 followed by 352.

2) Season 2018

Data presented in Table (3) and Fig. (2) showed that the mite numbers increased from 05-Jun 2018 up to

25-jun 2018 then decreased till 25-Jul 2018. The highest mite numbers were observed on 05 & 15-Aug 2018. The highest relative infestation/each variety was recorded on 324 then Nefertity, Watania,320, Pioneer 3057, 321 and 352, the respective values were 18.6, 17.1, 16, 15.5, 13.3, 11.9 and 7.7 relative infestation / each variety. Therefore, the susceptible and tolerant varieties were 324 and 352, respectively.

The highest infestation intervals were verified during August 2018 (05 & 15-Aug), their relative infestation/interval were 43.7 and 35.8%, respectively.

Generally, the highest susceptible variety was 324 during both tested seasons. However, the highest tolerant varieties were differed for season two another. on the other hand, the most the tolerant varieties were 321, 352 and Pioneer 3057.

Table 3. Seasonal abundance of *Tetranychus* species on maize and relative infestation percentages per each date and each variety during 2018 season.

Maize variety	05-Jun	15-Jun	25-Jun	05-Jul	15-Jul	25-Jul	05-Aug	15-Aug	Mean
324	6.0	12.0	12.0	14.0	6.0	6.0	56.7	70.3	22.9
%	3.3	6.6	6.6	7.7	3.3	3.3	31.0	38.4	18.6*
Watania	2.0	7.0	9.7	8.0	1.0	1.0	66.7	62.0	19.7
%	1.3	4.4	6.1	5.1	0.6	0.6	42.4	39.4	16.0
321	1.7	2.7	8.0	5.0	0.7	0.7	66.7	31.7	14.6
%	1.4	2.3	6.8	4.3	0.6	0.6	57.0	27.1	11.9
Pioneer 3057	0.0	2.0	4.0	0.3	0.3	0.3	53.3	70.3	16.3
%	0.0	1.5	3.1	0.3	0.3	0.3	40.8	53.8	13.3
320	2.7	12.3	10.0	13.0	5.0	5.0	66.7	38.3	19.1
%	1.7	8.1	6.5	8.5	3.3	3.3	43.6	25.1	15.5
352	0.3	3.0	5.0	0.0	0.3	0.3	53.3	13.0	9.4
%	0.4	4.0	6.6	0.0	0.4	0.4	70.8	17.3	7.7
Nefertity	3.0	10.0	9.7	10.0	1.0	1.0	66.7	67.0	21.0
%	1.8	5.9	5.7	5.9	0.6	0.6	39.6	39.8	17.1
Mean	2.2	7.0	8.3	7.2	2.0	2.0	61.4	50.4	17.6
%	1.6	5.0	5.9	5.1	1.5	1.5	43.7	35.8	

*** Relative infestation/each variety**

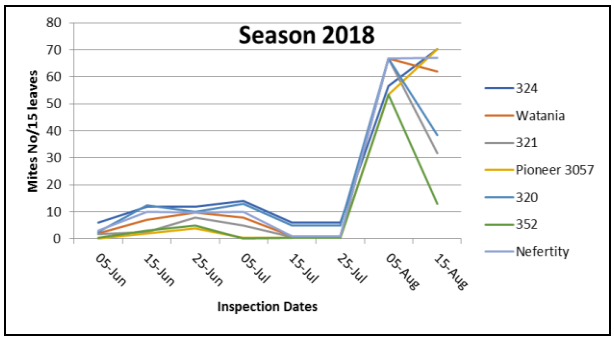

Fig. 2. Seasonal abundance of *Tetranychus* species on maize and relative infestation percentages per each date and each variety during 2018.

Simple Correlation and temperature:

Generally, in Table (4), the correlation between mite numbers for each maize variety and all tested meteorological characters were positive except minimum RH during the 2nd season and two tested seasons. However, these effects varied from variety to other in the 1st season.

It is interesting to mention that there were no significant different between mite numbers for each maize variety and all tested meteorological characters. These effects may be returned to long the intervals between inspections (10 days), the high changes in tested meteorological characters, etc.

There are five independent characters viz, maximum, minimum and mean temperature (x) as well as maximum and minimum relative humidity (RH) which presumed to have an influence on mites number per each maize variety (Table 5).

In general, R^2 (Regression of Determination) values for all tested varieties were not significant. In regard to independent characters, only minimum & maximum temperature and minimum RH had negative values which indicated that there were negative relations between mite numbers and these meteorological characters and vice versa for the remaining ones. In addition, minimum temperature had significant negative effects on mite number for 324, Watania, Pioner 3057 and Nefertity.

Table 4. Simple Correlation between mite numbers and temperature or relative humidity during each season and two tested ones.

	Max-Temp °C	Av-Temp °C	Min-Temp °C	RH-Max %	RH-Min %
Season 2017					
324	0.097	0.239	0.178	0.333	0.107
Watania	0.205	0.249	0.134	0.413	0.013
321	0.161	0.204	0.108	0.446	0.009
Pioneer 3057	0.182	0.187	0.087	0.444	-0.011
320	0.146	0.211	0.130	0.386	0.038
352	0.158	0.155	0.060	0.497	-0.029
Nefertity	0.170	0.200	0.102	0.406	0.003
Season 2018					
324	0.034	0.340	0.401	0.225	-0.520
Watania	0.006	0.322	0.415	0.222	-0.515
321	-0.062	0.267	0.440	0.192	-0.445
Pioneer 3057	0.003	0.308	0.398	0.193	-0.567
320	0.048	0.359	0.418	0.257	-0.385
352	-0.034	0.260	0.397	0.131	-0.402
Nefertity	0.034	0.335	0.392	0.239	-0.509
Two Seasons					
324	0.061	0.303	0.291	0.270	-0.181
Watania	0.062	0.297	0.280	0.290	-0.225
321	-0.011	0.219	0.257	0.249	-0.217
Pioneer 3057	0.071	0.307	0.289	0.306	-0.240
320	0.063	0.269	0.251	0.266	-0.165
352	-0.002	0.184	0.207	0.207	-0.234
Nefertity	0.080	0.293	0.257	0.304	-0.218

Temp= Temperature Max= Maximum Min= Minimum Av.= Average RH= Relative Humidity%

Table 5. Multiple regression two seasons.

Maize variety (Y)	a	T-Max (X1)	T-av (X2)	T-Min (X3)	Max-RH(X4)	Min-RH (X5)	R^2
324	29.2	-34.2	59.8	-25.9*	1.5	-2.3	0.518
Watania	42.1	-36.9	63.4	-27.3*	1.8	-2.7	0.566
321	71.3	-29.3	47.8	-19.9	1.5	-2.5	0.481
Pioneer 3057	23.3	-28.6	47.4	-19.2*	1.7	-2.6	0.566
320	24.6	-27.2	45.9	-18.9	1.6	-2.3	0.430
352	67.1	-19.4	30.0	-12.0	1.1	-2.1	0.400
Nefertity	34.4	-35.2	59.9	-25.4*	1.9	-2.8	0.543

T= Temperature Max= Maximum Min= Minimum A.= Average RH= Relative Humidity% R^2=Regression of determination

The experiment proved that (Pioneer 3057, 321, 352) were the most resistant although the variation in temperature in the last 15 years proved instability, the lesion was still active during the same period of time in the 6-7-8 months, where the highest density was recorded, followed by fluctuating elevations of the numerical density of the lesion during the spring period coming after a dormancy phase during the winter period.

DNA barcoding of *Tetranychus* species and their predators:

1- PCR and DNA electrophoresis

The amplified fragments of the isolated DNA of *Tetranychus* species and their predators show a successful amplification at the correct size of 300 bp as shown in figure 3.

PCR product amplified by COI primer in and their predator. COI primer gave a PCR product at 300bp.

2- Sequencing and species identification:

The sequences obtained from the PCR products previously amplified were analyzed with BLASTn software tool against the NCBI database and the results of the first and the second samples showed the presence of two species of *Tetranychus* namely *Tetranychus cinnabarinus* and *Tetranychus ludeni* which would have been mistakenly identified as *Tetranychus urticae* in most of the cases due to the high resemblance in morphology (Mansour and Bar Zur, 1992). Moreover, the third sample was identified to be *Neoseiulus barkeri*, as one of the predators of *Tetranychus* species. The results of BLASTn were shown in Table (6).

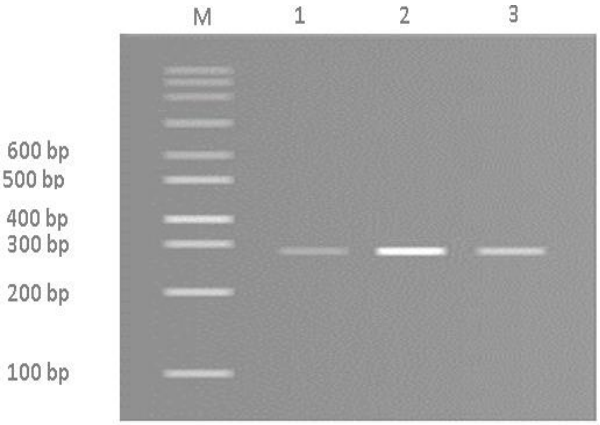

Fig. 3. DNA fragments generated by COI prime, M: DNA ladder (100-1000 bp), followed by two species of *Tetranychus* and their predators.

Table 6. The BLASTn results for the three isolated sequences for COI for Tetranychus and its predator

Sample No.	Description	Per Ident.	Accession
1	*Tetranychus ludeni* isolate ST cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	99.51%	KP828059.1
2	*Neoseiulus barkeri* isolate Syngenta cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	91.73%	KU342794.1
3	*Tetranychus cinnabarinus* genotype 1 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	100.00%	DQ656483.1

3- Phylogenetic analysis:

Sequences obtained from the three samples were aligned and the dendrogram showed the presence of two main clusters, the first cluster includes the two species of *Tetranychus*; namely, *Tetranychus cinnabarinus* and *Tetranychus ludeni* and the second cluster includes the identified predator namely *Neoseiulus barkeri*. The dendrogram showed the relative evolutionary distance between the *Tetranychus* species at 0.225 while the evolutionary distance of between the predator cluster and the *Tetranychus* species cluster (figure 4) .

The DNA barcoding techniques using the COI proved to be a reliable method in species discrimination and identification in *Tetranychus* species, and this can overcome the problem of difficult morphological discrimination in this genus. The success of DNA barcoding technique will be a cornerstone for many different pests in Egypt as to accurately identify the pest which will be followed by the proper management for pest control or eradication on the various crops.

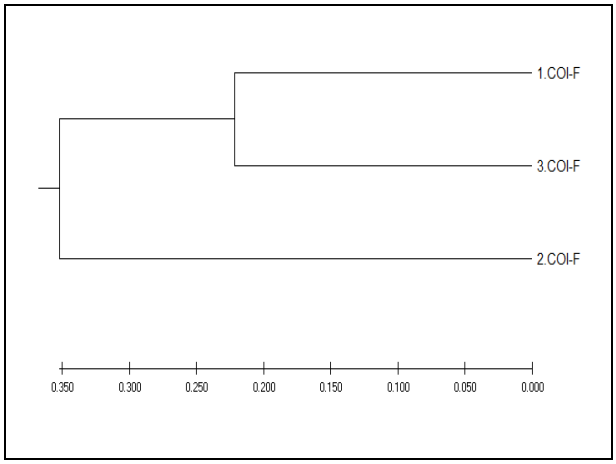

Fig. 4. Dendrogram showing the phylogenetic analysis of the three samples of *Tetranychus* species and their predator (Tamura *et al.*, 2004 and Kumar *et al.*, 2018).

The DNA barcoding technique will help us in the future make species specific primers that will make the process of identification easier and more reliable and will overcome any problems with the morphological identification.

From this piece of work combining the field and molecular analysis, we recommend the use of DNA barcoding in morphologically close species for accurate and reliable results.

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إستخدام تكنيك DNA Barcoding لاول مرة في مصر على انواع الـ Tetranychus ومفتراساتها

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اكاروسات المحاصيل الحقلية والقطن – معهد بحوث وقاية النباتات- القاهرة – دقى - جيزة

يعتبر *Tetranychus* افة اقتصادية منتشرة حول العالم على معظم المحاصيل. سواء كانت خضرية او شجرية حيث تؤثر على المحصول كما و نوعا . و لذلك تمت دراستها ايكولوجيا و جزيئيا. حيث تم رصد الكثافة العددية لتلك الافة على اصناف مختلفة من الذرة تحت الظروف البيئية الحقلية. حيث وجد ان اصناف الـ Pioneer 3057 و 321 و 352 هي الاكثر مقاومة لتلك الافة. و لأول مرة تم استخدام تكنيك الـ DNA barcoding في مصر تأكيدا لنوع الاصابة. حيث وجد نوعين من *Tetranychus* هما *Tetranychus ludeni* و *Tetranychus cinnabarinus* و اللذان كانا يعرفان على انهما *Tetranychus urticae* و ذلك لعدم مقدرتنا لتميزهم بالطرق التقليدية (و هو ما اثبته العلم) بالاضافة لذلك وجدنا مفترس اخر للـ *Tetranychus* يسمى *Neoseiulus barkeri* و هذا عكس المفترس الشائع المعروف باسم *Phytosenilus persimilus* حيث اثبتت الدراسة انه لا غنى من دراسة الافة جزيئيا لاثباتها و من ناحية اخرى اختيار طرق المكافحة المثلى لها و ان تكنيك الـ DNA barcoding هو من اصدق الطرق في التعرف على الافة و تحديد طرق مكافحتها.