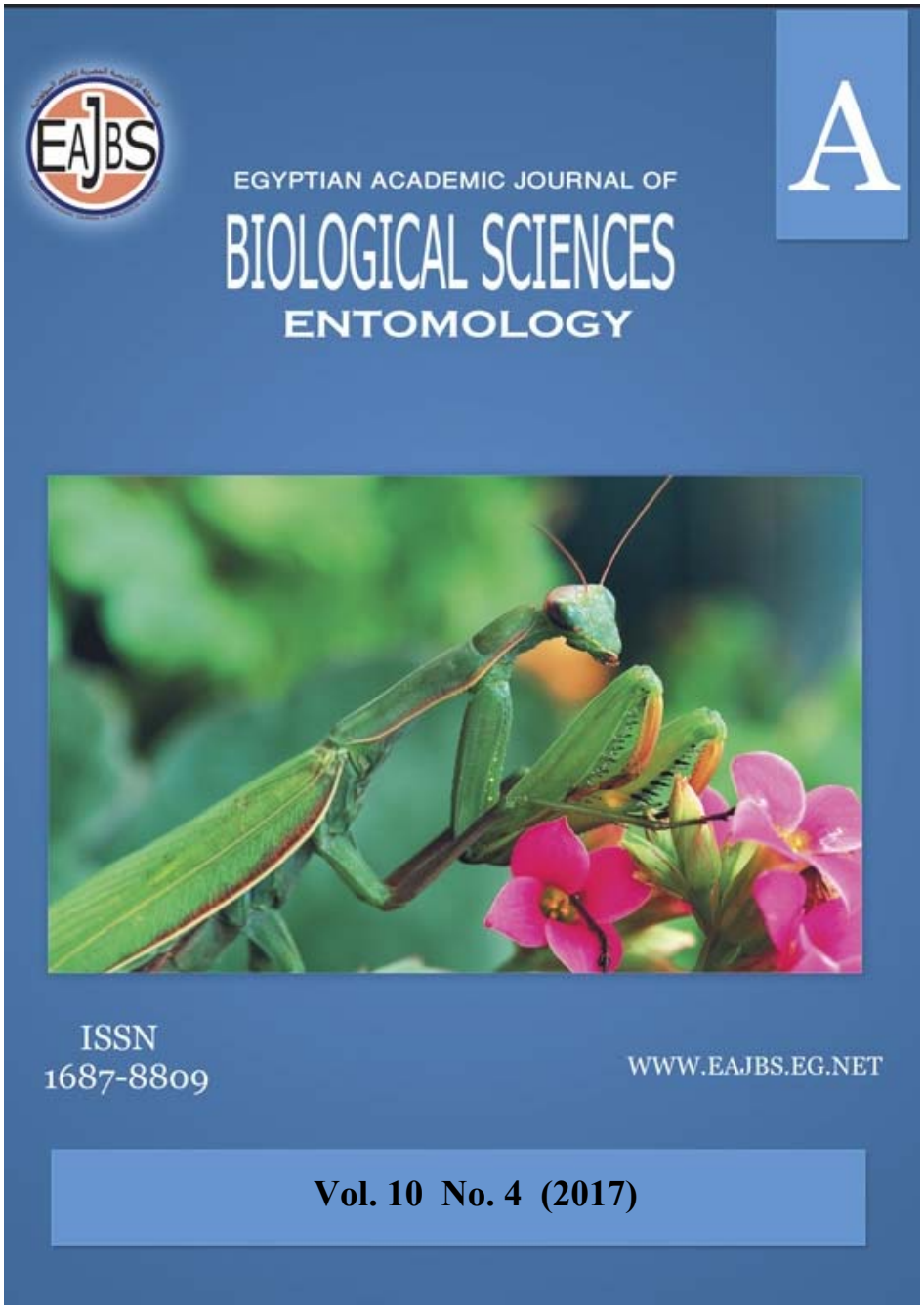

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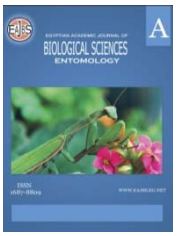

Molecular Identification of Thripids Attacking Olive Groves at Ismailia, Egypt

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ABSTRACT

A little attention has been given on molecular identification of thrips (Insecta: Thysanoptera) in Egypt. Thus, the primers ITS4A, 28S rRNA, 18S rRNA and the primer pairs COI-1 + COI-2 + COI-3 were used to identify the three dominant thrips species (*Haplothrips cahirensis* karny; *Dendrothrips eremicola* Priesener and *Hydatothrips kassimanus* Priesener). The samples were collected from a private olive grove during 2013 and 2014 at Ismailia governorate, Egypt. DNA sequence data for 28S rRNA gene showed a positive response to *H. cahirensis* and *D. eremicola* only, while 18S rRNA reacted with all species. ITS4A and COI-1 + COI-2 + COI-3 showed a negative response to the studied species. DNA sequences of the species based on 18S rRNA were recorded in the Gene Bank. The phylogenetic tree of the three thrips species was given also.

INTRODUCTION

Thrips (Thysanoptera) are very small insects, widespread throughout the world with a preponderance of tropical region, many temperate ones, and even a few are living in arctic regions. More than 6000 species were identified, only a few hundred are crop pests derived from two suborders: the Terebrantia, whose females have an external saw-like ovipositor, the last abdominal segment conical shaped and fore wing with microtrichia and veins. The Tubulifera, in which females without ovipositor, last abdominal segment tube shaped and fore wing without microtrichia or veins. The Tubulifera includes a single family, the Phlaeothripidae, whereas Terebrantia are classified into eight families: Uzelothripidae, Aeolothripidae, Adiheterothripidae, Fauriellidae, Heterothripidae, Merothripidae, Melanthripidae and Thripidae. In Egypt, there are about 140 described species derived from five families given in Figure (4) (Priesner, 1965; Parker *et al*., 1993; Lewis, 1997; El-Wakkad, 2007; Mound and Morris, 2007 and Thripswiki, 2014).

Thrips can induce a range of symptoms in plant tissue by their feeding as scaring on fruits or leaves and flower deformation or reducing pollens to critical levels. In addition, thrips are the only vectors of a series of plant damaging viruses (Tospoviruses). Moreover, thrips induce leaf gall in some ornamental plants. On olive tree, thrips feed on tender parts of the tree such as buds, developing leaves, developing inflorescences, flowers, fruits and tender bark causing leaves

deformation, yellowish white blotches on lower surfaces, leaves curl, leaves defoliation and fruit deformation with deep hollows appear in ripe olive, fruit defoliation when attacked in early season, infested fruit become smaller (Tzanakakis, 2003 and Tombesi *et al*., 2007).

Morphological identification of thrips, in both the adult and larval stages, is difficult because of their small size, high degree of similarity and polymorphism. In contrast, molecular identification is not hindered by phenotypic polymorphism, sex or developmental stage of a targeted species. Also, the interrelationships within Thysanoptera remain based entirely on morphological characters and cytotaxonomic studies are scarce in thrips (Brito *et al*., 2010 and Zhang *et al*., 2011). In addition, identification of species using molecular methods has become ubiquitous in diagnostics and ecological studies, especially with regard to insects for which morphological identification is difficult or time-consuming. Identification of thrips species based on morphology is usually tedious and laborious, requires a high level of taxonomic expertise and usually only adults can be identified. So, lots of research efforts using different molecular methods have hitherto been dedicated to this group of insects, especially to those with economic importance, in order to solve their morphological identification problems (Przybylska *et al.,* 2016).

From molecular data, the nuclear small subunit rRNA (18S) has played a dominant role in the estimation of relationships among insect orders (Kjer, 2004). In addition, the 18S rRNA gene has played an important role in resolving the deep phylogeny of insects (Yoshizawa and Johnson, 2005). Mound and Morris (2007) pointed out that gene regions such as 18S and 28S are used frequently because they exhibit levels of variation appropriate to higher-level systematics questions and because they are present in multiple copies in the genome and tend to amplify readily using standard primers.

Some studies addressed the relationships among order Thysanoptera based on the 18S rRNA gene. Crespi *et al.* (1996) were the first authors used molecular tools in studying the phylogenetic relationships of thrips, they involved eight in group taxa and were inferred from partial sequences of 18S ribosomal DNA (640 bp). Then, Morris & Mound (2003) involved 52 species (18 Tubulifera, 34 Terebrantia and seven of 9 families) with a 600 bp sequence of 18S rDNA. Mound and Morris (2007) added three thysanopteran in the Genebank with 1800bp of 18S rDNA. Brito *et al*. (2010) reported the cytogenetic data of 18S rDNA for seven species of Thysanoptera (4 Terebrantia, 3 Tubulifera of 2 families. Ninety-nine thrips species of seven families were sequenced based on 18S ribosomal DNA (640 bp) Buckman *et al*. (2013).

In 2009, the thrips species, *Dendrothrips eremicola* Priesner occurred as an outbreak on olive trees in some locations in Egypt (El-Kholy *et al*., 2009). So, this study aimed to identify thrips species on olive trees using molecular features supported a previous morphological study by Agamy *et al*. (2017) on the same species. Furthermore, the present study is considered as the first one on the molecular identification of olive thrips in Egypt.

MATERIALS AND METHODS

Sample collecting:

Thrips sampling in the present study were collected from private olive grove on different cultivars (El-Egazy; Manzanillo and Pequal) and associated weeds (*Cynanchum acutum* L.) at Ismailia governorate during 2013 and 2014.

Genomic DNA Isolation:

Total Genomic DNA of samples was extracted from 10-15 females' adults according to Dean *et al*. (2013) with some modification. The specimens were put into a 0.5 mL centrifuge tube with 40μL lysis buffer (50 mmol・L-1Tris-HCl (pH8.0), 20 mmol・L-1NaCl, 1 mmol・L-1EDTA, 1% SDS) and 40μL protease enzyme, then they incubated at 65 °C for one hour. One volume of chloroform: iso-amyl alcohol (24:1) was added, and the sample was emulsified by gentle inversion and centrifuged for 15 min at 13,000 rpm. The top aqueous phase was transferred to a clean tube. Two volumes of cold (-20°C) isopropanol were added to the sample, mixed well and incubated at -20°C. DNA strands were washed with 70% ethanol, centrifuged for 5 min at 13000 rpm, dried and finally eluted in milli Q H2O. It was stored at 20°C.

Quantification of DNA:

The concentration DNA samples were determined by Nano drop analysis (qualitative and quantitative) and electrophoresing DNA samples on 0.8% agarose gel. The concentration of the fragment of interest was estimated by comparing the intensity of ethidium bromide fluorescence to that of the known DNA concentration standards. Two µl of DNA sample was loaded on 0.8% agarose gel. The gel was run at 90 volts for 30 min. Thereafter, the DNA bands were visualized under ultraviolet light and photographed.

PCR amplification:

The amplification reactions were conducted using a thermal cycler (Eppendorf, CA). PCR was performed with 50µl volume master mix, containing 5 µl genomic DNA, 5µl primer F&R, 20µl water nuclease free and 25 µlof the master mix Kit (Cat No. Bio-52066) (Bio line) UK, with the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of (95°C, 1 min ; 50–52 °C , 60s; 72°C, 60s) and a final extension of 72°C for 10 min. The PCR negative control contained the identical amount of PCR mixture with 5 µl distilled water instead of DNA template. The primers ITS4A, 28S rRNA, 18S rRNA and the primer pairsCOI-1 + COI-2 + COI-3 were used to identify the three dominate thrips species (*Haplothrips cahirensis* karny; *Dendrothrips eremicola* Priesener and *Hydatothrips kassimanus* Priesener).The amplified products were run on 1.5% agarose gel (stained with ethidium bromide) with DNA ladder (100bp). Gels were visualized in a gel doc system.

Sequencing and Phylogenetic tree:

The PCR amplified fragments were eluted using Agarose Gel Extraction Kit (Jena Bioscience) according to the manufactures protocol. Direct sequencing of the above fragments was carried out using the forward and reversed of the gene-specific primers used in this study in an automated sequencer (Macrogen Korea). Sequences were checked in a sequence alignment editor, (Bio edit.) to look for any introduced errors during sequencing. Then, homology search was carried out using BLAST X version 2.2.6 (http://www.ncbi.nlm.nih.gov) and were deposited with NCBI. The sequence was obtained and the alignment analysis was performed using MAFFT (Katoh, 2002). The phylogenic tree was constructed using the method by forester software (Han and Zmasek, 2009).

Sequence analysis and accession number:

All sequences generated in this study were submitted to Gene Bank as shown in (Table 1).

Table (1): The sequencing of the used primers and their response to thrips species

Primers name	Sequence	Response to thrips species		
		H. cahirensis	*D. eremicola*	*Hy. kassimanus*
ITS4A	**ITS4A(F):**CGCCGTTACTGGGGCAATCCCTG	-	-	-
	ITSIF(R):CTTGGTCATTTAGAGGAAGTAA			
28S r RNA	**F:GACCCGTCTTGAAACACGGA**	+	+	-
	R:**TCGGAAGGAACCAGCTACTA**			
18S r RNA	**F:CCTGAGAAACGGCTACCACATC**	+	+	+
	R:**GAGTCTCGTTCGTTATCGGA**			
COI-1	**F:CACCTCAGGGTGTCCGAARAAYCARAA**	-	-	-
	R:TCTCCACCAACCACAARGAYATYGG			
COI-2	**F:TGTAAAACGACGGCCAGTATTCAACCAATCATAAAGATATTGG**	-	-	-
	R:CAGGAAACAGCTATGACTAAACTTCTGGATGTCCAAAAAATCA			
COI-3	**F:TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC**	-	-	-
	R:CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA			

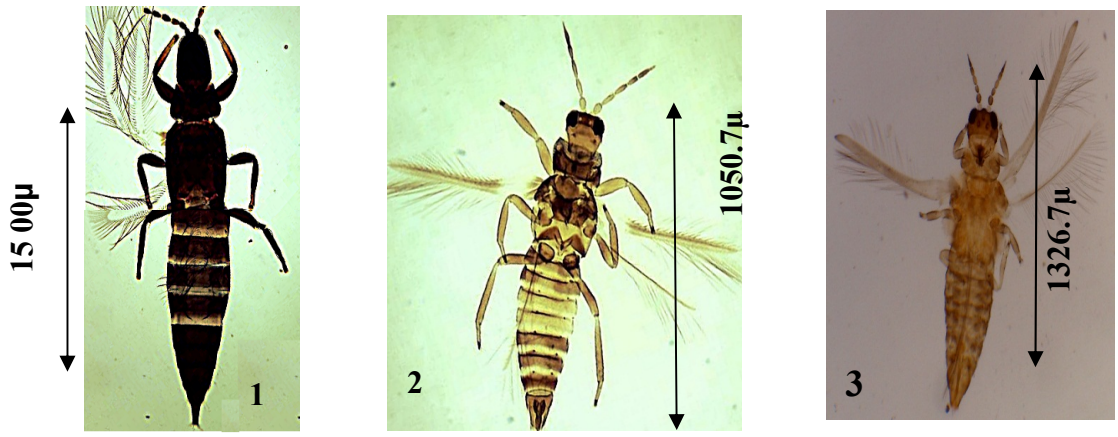
RESULTS AND DISCUSSION

Identification of studied species:

The examined specimens were identified and compared with the preserved specimens in Collection of the Ministry of Agriculture, Plant Protection Institute, Taxonomy department (MAC). The images and classification of species are given in Figures (1-4). The studied species were previously identified by morphological characters (Agamy *et al*., 2017). The data analysis showed that nucleotide sequence of 18s rDNA of the three tested samples were similar in size 700 bases (Figure 5).

The sequencing and response to four primers used in molecular identification of the thrips species was presented in Table (1). Not all of the primers tested produced PCR band pattern in the examined thrips. The primers ITS4A and the primer pairs COI-1 + COI-2 + COI-3 resulted a negative response to the studied species. DNA sequence data for 28S rRNA gene showed a positive response to *Haplothrips cahirensis* and *Dendrothrips eremicola* only, while 18S rRNA reacted with all examined species.

The DNA sequencing based on 18S r RNA gene of the examined species was compared with other sequences of the species published on Gene Bank database in Table (2) and Figures (6-12) as follow:



Figs. (1-3) thrips species: 1-*Haplothrips cahirensis; 2- Hydatothrips kassimanus*
3- Dendrothrips eremicola.

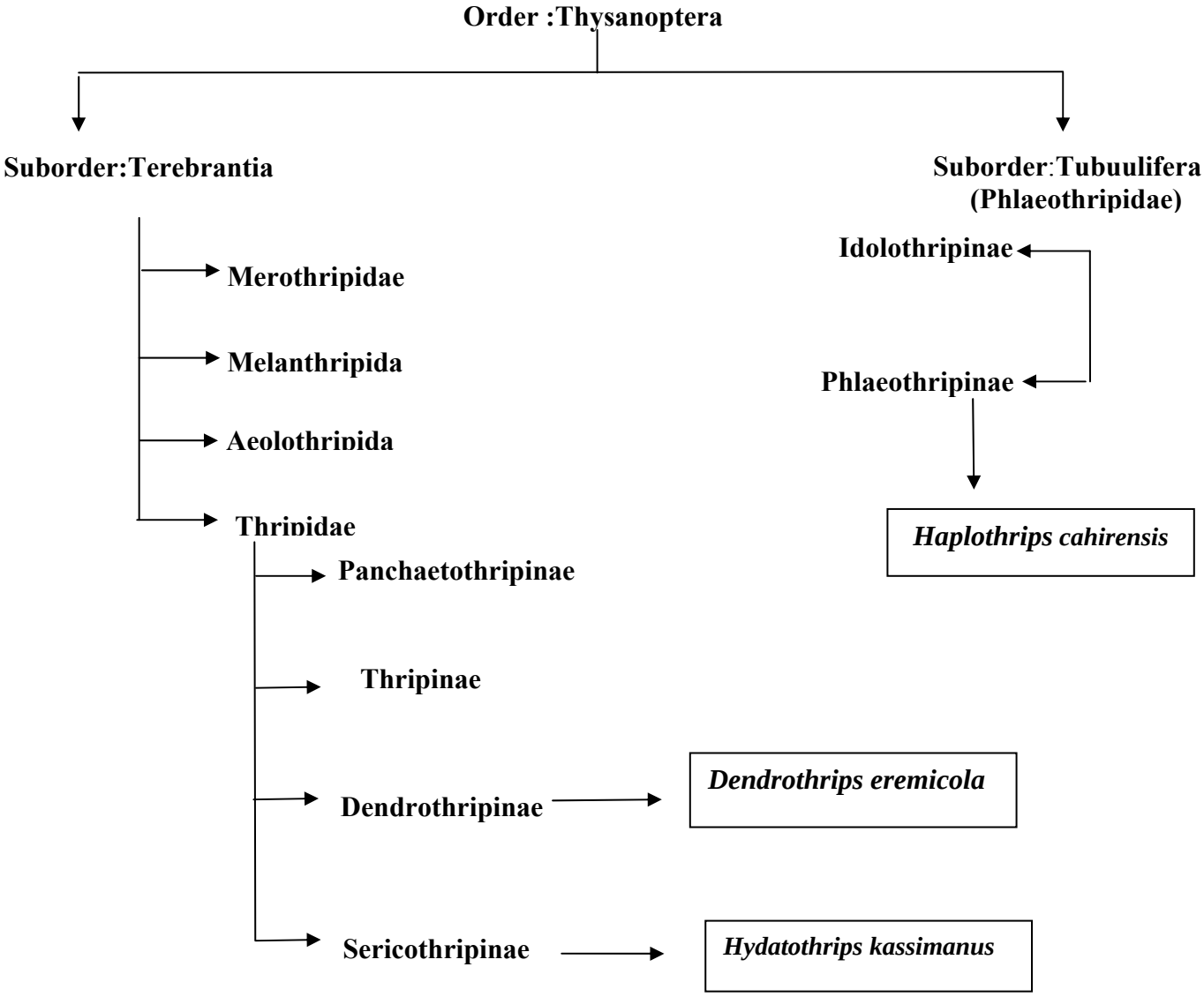


Fig.4. Thysanoptera family relationships

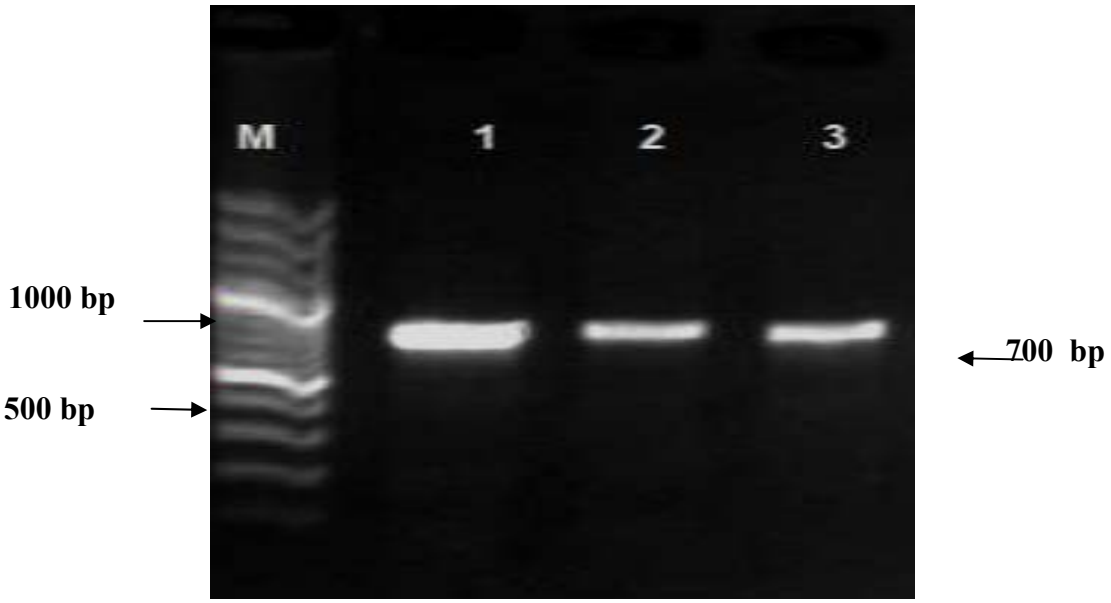


Fig 5. Agarose gel DNA electrophoresis for PCR of thrips species: 1- *H. cahirensis*, 2- *D. eremicola*, 3- *Hy. kassimanus.*

Table (2): 18S r RNA gene of the thrips species compared with othersequences from National Center for Biotechnology Information (NCBI).

Thrips species	Accession numbers	Agreement in GenBank	% Identical	Query cover
H. cahirensis	LC198516.1	*Dyothrips pallescens*	80	99
		Haplothrips froggatti	79	99
		Leptothripsmali	78	99
D. eremicola	LC198515.1	*Dendrothrips* sp.	78	81
		Pseudodendrothrips mori	78	81
Hy. kassimanus	LC198514.1	*Sericothrips staphylinus*	98	86
		Neohydatothrips annulipes	98	86
		Hydatothrips argenticinctus	97	86

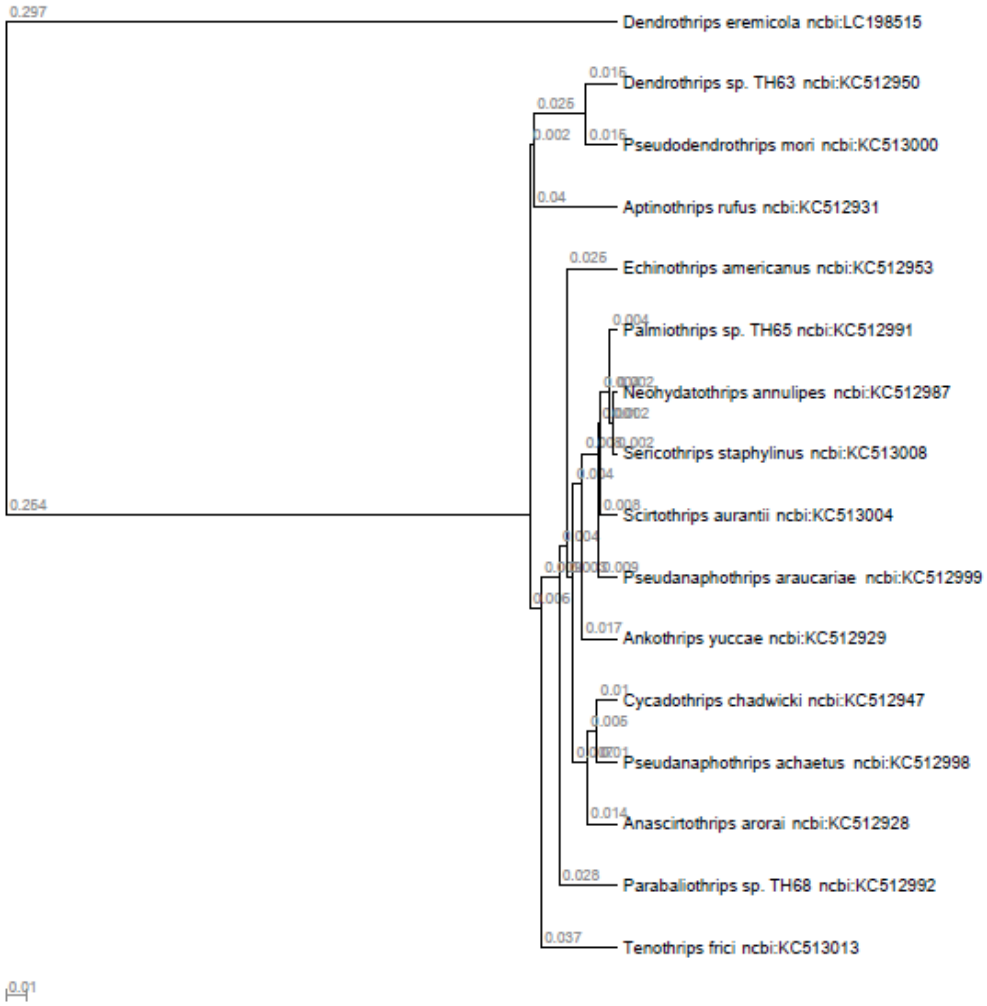


Fig. (6) Phylogenetic tree analysis for the relationships between *D. eremicola* and other related species recorded in NCBI based on18S r RNA gene

***Dendrothrips eremicola* Priesener:**

This species was recorded in Gene Bank database for the first time with Accession number (LC198515.1). The sequencing obtained in Table (2) showed the relation between the species and the nearest species (*Dendrothrips* sp. and *Pseudodendrothrips mori*), there are 87% similarity to each other. The phylogenetic tree in figure (6) emphasized that the three species were placed in the same clade in the tree. The results confirm the previous study of Mound and Morris (2007) and Buckma *et al*. (2013), whereas; *Dendrothrips* sp. and *Pseudodendrothrips mori* were placed in the same clade. This result agree with the morphological studies carried out by other authors (Mound, 1999**;** Alavi, 2014; Balou and Dosty, 2015 ; Mound and Tree, 2016 and Agamy *et al*., 2017) which reported that both genus *Dendrothrips* and *Pseudodendrothrips* were classified under the same

subfamily Dendrothripinae. While, *Aptinothrips rufus* was placed under the other subfamily (Thripinae).

***Hydatothrips kassimianus* (Priesener):**

In addition, *Hydatothrips kassimianus* was recorded in Gene Bank database for the first time with Accession number (LC198514.1). The phylogenetic tree (Fig.7) and Table (2) showed a strong relation between the studied species and the nearest ones (*Hydatothrips argenticinctus*.; *Sericothrips staphylinus* and *Neohydatothrips annulipes*) with similarity of 97,98% in the clade of the tree. These results are compatible with the study of Buckman *et al*. (2013), whereas the phylogenic tree showed a strong relation between the above species (88%). The above species were derived from the same subfamily Sericothripinae according to morphological studies done by (Mound and Tree, 2009; Minaei, 2013; Poorkashkooli *et al*., 2015 and Agamy *et al*., 2017).

***Haplothrips cahirensis* karny**:

This species was recorded in Gene Bank database as *Haplothrips* sp. with Accession number (LC198516.1) whereas it was registrant in Gene Bank before full morphological identification. The Phylogenetic tree in Figure (8) showed the relation between *Haplothrips cahirensis* and the related species. Whereas, a strong supported clade (059) includes *Haplothrips* sp, *Dyothrips pallescens*, *Haplothrips froggatti* and *Leptothrips mali* compared with other species derived from the subfamily *Phlaeothrip*inae. This tree agree with the tree obtained by Buckman *et al*. (2013) whose report showed a strong relation (98%) between *Haplothrips* spp. (*H. graminis*, *H. leucanthemi* and *H. victoriensis*), *Dyothrips pallescens* and *Leptothrips mali*. Based on morphological characters published by Mound and Minaei (2007), the all above species are belonging to the subfamily *Phlaeothrip*inae and *Haplothrips pallescens* were known as synonym of *Dyothrips pallescens*, so they considered to being as derived from the same genus.

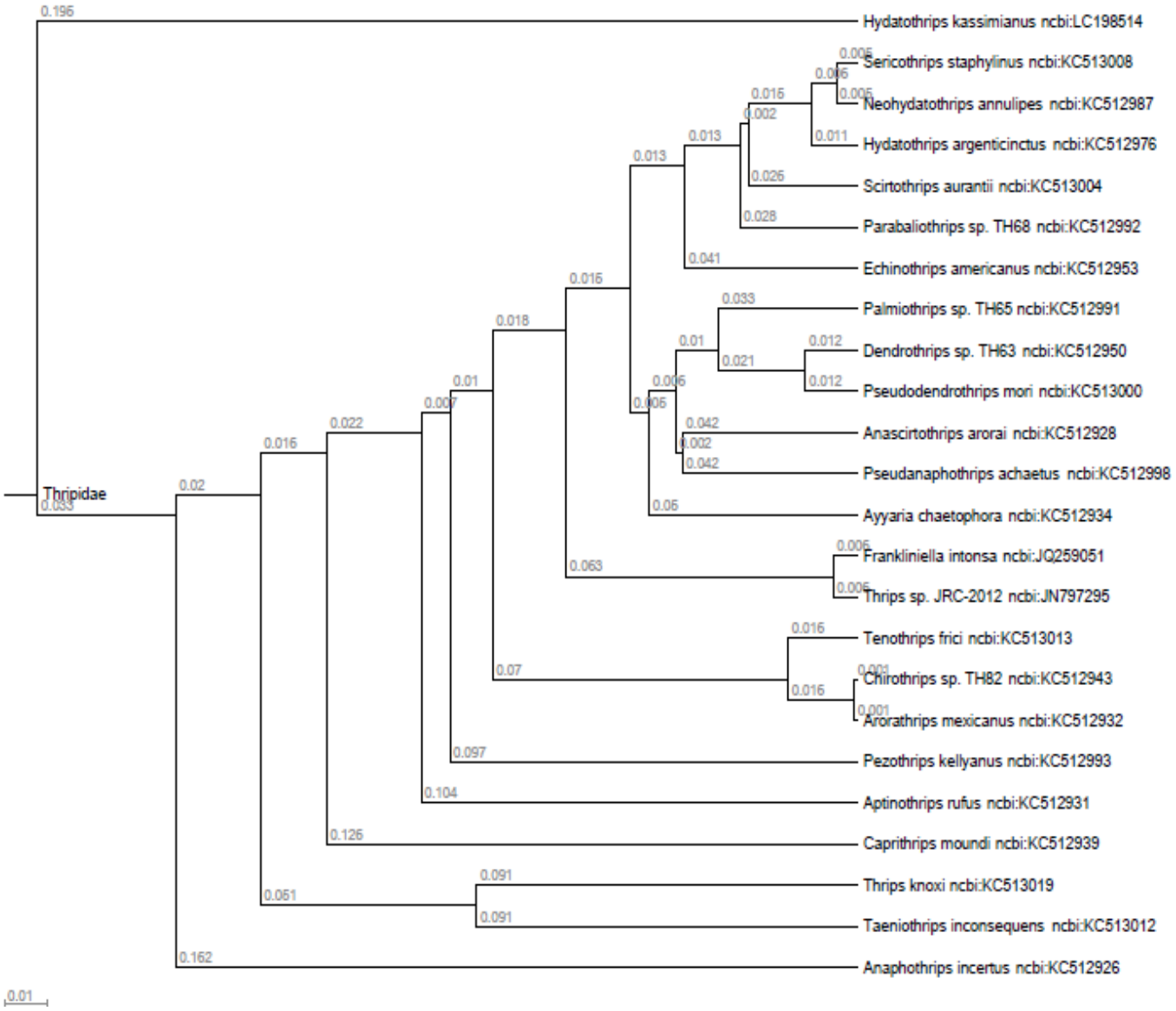


Fig. (7) Phylogenetic tree analysis for the relationships between *Hy. Kassimanus* and other related species recorded in NCBI based on18S r RNA gene

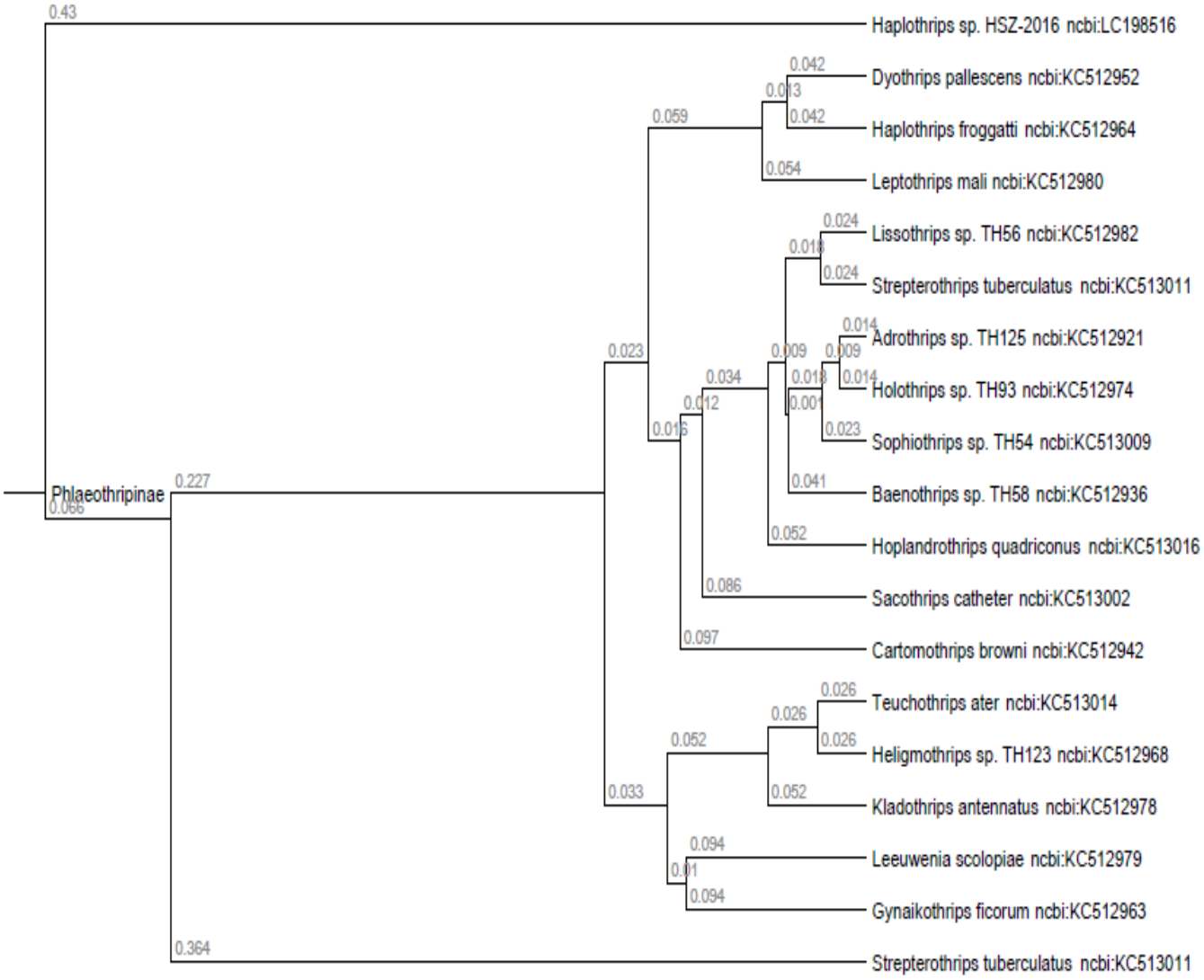


Fig. (8) Phylogenetic tree analysis for the relationships between *H. cahirensis* and other related species recorded in NCBI based on18S r RNA gene

CONCLUSION

The advantage of molecular identification trend over morphology-based taxonomic methods and other techniques is that it is quick, specific, requires only basic laboratory skills, and can be performed with DNA extracted from single individuals, which can be preserved, mounted on slides and used for future reference. On balance, molecular methods for identification of thrips species represent an important possibility to confirm the classical morphological methods. Phylogenetic analysis of 18S rDNA strongly supports the morphological studies of Thysanoptera. Whereas, it emphasis the relation between species based on morphological characters obtained perversely by Agamy *et al*. (2017).

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ARABIC SUMMERY

التعريف الجزيئى للتربس المهاجم بمزراع الزيتون فى محافظه الاسماعيليه، مصر

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لاقى التعريف الجزيئى للتربس قليل من الاهتمام بمصر. وهكذا، تم استخدام الكواشف ITS4A، , 28S,الريبوسومي، 18S الريبوسومي والكواشف المزدوجه COI-3 + COI-2 COI-1 لتعريف الأنواع الثلاثة السائده من التربس (هابلوثريبس كاهيرنسيس كارني، ديندروثريبس إرميكولا بريزينر و هيداتوثريبس كاسيمانوس بريزينر). تم جمع العينات من مزرعه زيتون خاصه خلال عامي ٢٠١٣ و ٢٠١٤ في محافظة الإسماعيلية بمصر. وأظهرت بيانات تسلسل الحمض النووي للجين الرنا الريبوسومي 28S استجابة إيجابية للنوع إتش. كاهيرنسيس و دى. إرميكولا فقط، في حين اظهر 18S الريبوسومي رد فعل مع جميع الأنواع. وأظهرت كل من ITS4A و COI-3 + COI-2 + COI-1 استجابة سلبية للأنواع المدروسة. تم تسجيل تسلسل الحمض النووي للأنواع على أساس 18S الريبوسومي في بنك الجينات. وقد مثلت شجرة الانساب للثلاثه أنواع من التربس أيضاً.