Assiut Journal of Agricultural Sciences 54 (4) 2023 (168-182) Website: http://ajas.journals.ekb.eg/
ISSN: 1110-0486 / EISSN: 2356-9840 E-mail: ajas@aun.edu.eg

(Original Article)

Morphogenetical Studies on Egg Parasitoids *Trichogramma* Released to Control Lepidopteran Sugarcane Pests in Qena Governorate, Egypt

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DOI: 10.21608/AJAS.2023.219324.1270
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Abstract

The egg parasitoid *Trichogramma* was used in Qus, Qena Governorate, Egypt, to control lepidopteran sugarcane insect pests. The primary objective of this study was to identify the egg parasitoid, *Trichogramma*, by morphological and genetic approaches. Light microscope was used to examine the morphology of male and female adults and their measurements. Results showed that females were shorter than males in *Trichogramma*. Also, the external morphology of the antennae, wings, and male genitalia was recorded. The sequence of *ITS2* of *Trichogramma turkestanica* was deposited in GenBank under the accession number of MW459187.1. Its nucleotide length was 434 bp. The nucleotide frequencies of adenine (A), cytosine (C), guanine (G), and thymine (T) were 19.8, 28.3, 26.3, and 25.6 %, respectively. The C+G content was 54.6, which was higher than the A+T content. The tested species can be identified *as Trichogramma turkestanica* through combined morphological and molecular methods.

Keywords: *Trichogramma, Sugarcane pests, Morphological, molecular identification*

Introduction

Sugarcane, *Saccharum officinarum* L., (Poales: Poaceae) is one of the most important economic crops in the world (Ali *et al*., 2021). The global harvested and produced area of sugarcane are 26349551ha and 1859390044.3tonnes, respectively (FAO, 2023).

In Egypt, Sugar factories spread in many governorates. The cultivation area of sugarcane amounts to about 128298 ha and 12360553.27 tonnes (FAO, 2023).

Sugarcane is attacked by several insect pests such as mealy bugs, aphids, and serious lepidopteran insect pests (Kumar *et al*., 2019). In Upper Egypt, sugarcane stem borers are common insect pests in sugarcane fields (Kira and ElSherif ,1974a and 1974b; Elwan *et al*., 2009).

Sesamia cretica Led. (Noctuidae: Lepidoptera) infests sugar cane plants in the early season, while the small sugarcane borer, *Chilo agamemnon* Bles.,

Received: 22 June 2023/ Accepted: 1 October 2023/ Published online: 5 October 2023

(Pyralidae: Lepidoptera) infests the sugar cane during the late stages of plant growth (Ali *et al*., 2021).

Chemical pesticides are usually ineffective against stem borers (Mahesh *et al*., 2018). Also, chemical control harms non-target organisms, the environment, and people (Crowder *et al*., 2010). Biological Control is an alternative pest management strategy that promotes sustainable sugarcane production (Srikanth *et al*., 2016). Egg parasitoids *Trichogramma* have been successfully used to control lepidopteran pests (Zang *et al*., 2021). They are relatively easy to culture, kill the host eggs before the larvae hatch, and can prevent damage Elwan *et al*. (2009).

It is difficult to identify *Trichogramma* spp., morphologically because of their small size and relative few variations between species (Ksentini *et al*., 2010).

Recently, molecular techniques have become one of the most accurate methods of identifying species. Many genes have been used in identification, such as the DNA sequence of internal transcribed spacer 2 (ITS2) regions of nuclear rRNA for species identification (Ercan *et al*., 2011; Kumar *et al*., 2016; Hajjar *et al*., 2018). The species of *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae) were identified genetically in most of the world. In Egypt, they characterized differences between *Trichogramma* wasps by molecular markers (Abdel-Galil *et al*., 2018)

The objective of this study was to assess egg parasitoid morphological features to determine the strain's morphological and molecular identity.

Materials and Methods

1-*Trichogramma* collecting and rearing

Egg parasitoid *Trichogramma* was collected from Qus, Qena Governorate (*T*Qus strain) (26° 09’ 51.05” N, 32° 43’ 36.16” E). *Trichogramma* wasps were collected in glass tubes. It was raised using the host eggs of various moth species, including *Sitotroga cerealella* (Olivier), for two generations under laboratory conditions (23±2°c, 75±5% RH, L 16:D 8) in The Biolo. Cont. Lab., Plant Protect. Dept., Assiut Univ., Assiut, Egypt.

2-Morphogenetical identification of egg parasitoid *T*Qus strain

Light microscopy

Permanent specimens for species identification were used as described by Knutson (1998). Measurement was done using the methods applied by Abdel-Galil *et al*. (2018).

Body morphometric

The characteristics of measurements for *Trichogramma* are body length, antennae criteria flagellar length, flagellar width, scape length, and longest seta for males. Also, club length and antennal club width for females. Forewings length and width in both sexes. Female abdomen measurements of abdominal length and abdominal area. The measurements of male genitalia general length, general width, aedeagus length, apical distance, apical width, and basal distance. Species were

identified using a combination of the following author's taxonomic publications: Pintureau (2008), Polaszek *et al*. (2012), Del Pino *et al*. (2013), Abdel-Galil *et al*. (2018), and Khan *et al*. (2020).

3-Molecular genetic identification

DNA extraction

The genomic DNA was extracted from the preserved samples using the QIAamp DNA Mini kit (Qiagen, Hidden, Germany) by following the manufacturer's guidelines. The molecular genetic experiments were conducted in Central Laboratories, Faculty of Sciences, South Valley University, Qena, Egypt.

PCR Conditions

The internal transcribed spacer 2 (*ITS2*) region in *Trichogramma* was amplified using *ITS2* forward and *ITS2* reverse. These primers were the same as those used by Stouthamer *et al*. (1999) for distinguishing sibling species of *Trichogramma*.

PCR assays were carried out in 50µl reactions containing 25µL PCR master mix, 1µL of each forward and reverse primer, 1µL ng of genomic DNA, and 22µL Nuclease-Free Water. The PCR cycling conditions were performed with an initial denaturation at 94ºC for 4min., followed by 30cycles including denaturation for 60sec. at 94ºC, annealing for 60sec., at 52ºC (*Trichogramma turkestanica*) and an extension for 60sec., at 72°C followed by a final extension at 72ºC for 10min., 1.5% agarose gel containing ethidium bromide, which was used to separate the amplified products. For sizing the amplified PCR fragments, 100bp DNA Ladder RTU (Ready-to-Use), GeneDireX) was used.

The Sequencing of PCR product

All DNA sequencing was achieved by Macrogen (Seoul, South Korea). The sequences were subjected to the National Center for Biotechnology Information (GenBank/NCBI) for obtaining accession numbers.

Sequence alignment was performed using the MUSCLE program (Edgar, 2004) with default settings. MEGA version 7.0 18 (Kumar *et al*., 2016) was used to perform the phylogenetic tree analysis using Neighbour Joining (NJ) and Minimum Evolution (ME) methods of trees construction, with 1000bootstrap iterations (Felsenstein, 1985). Calculation of sequence divergences occurred by utilizing Kimura's two-parameter distances (Kimura, 1980). To determine the similarity of our sequence to those already found in the database, we used BLAST searches of the GenBank NCBI database.

4-Data analysis

Means ± standard deviation (SD) was determined according to Pinto (1999). Values of t-test were determined using Microsoft Excel 2016.

Results and Discussion

1-Morphological and molecular identification of *Trichogramma* Qus strain

Morphological description

Body coloration

The detailed morphological structures of male and female parasitoids were specified by light microscopic photos. Egg parasitoid adults in both sexes are very small and light brown (Fig. 1).

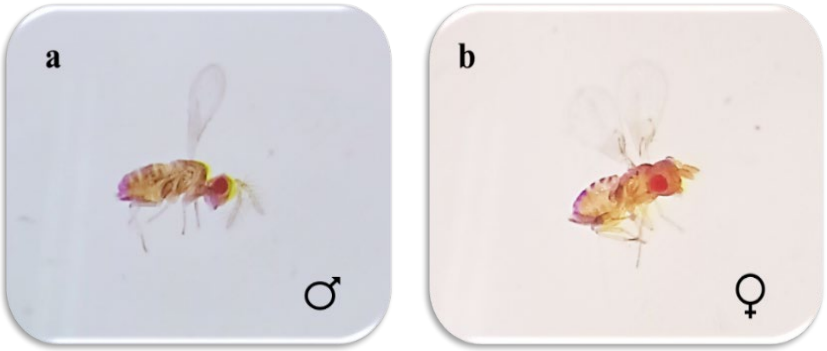

Fig. 1. Light micrograph of the lateral view of *Trichogramma* Qus strain adult (magnification =16 x): a) Male and b) Female

The head

In both sexes, the head is relatively small and round. It contains appendages including compound eyes, antennae, and mouthparts.

Compound eyes

In both sexes, the compound eyes take up a sizable portion of the head capsule. There are three red ocelli on top of the head (tiny, dot-like light sensors) (Fig. 2).

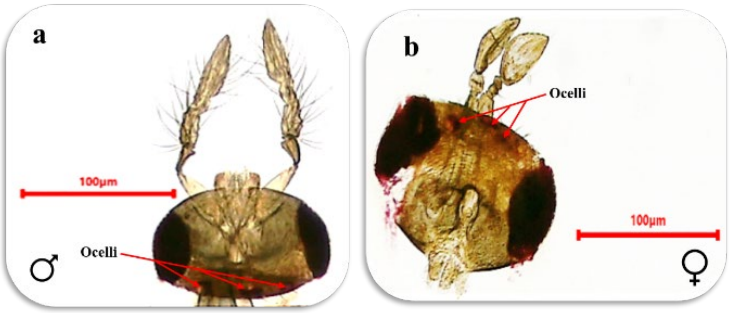

Fig. 2. The compound eyes of adult *Trichogramma* Qus strain: a) Male and b) Female

Antennae

The antenna of the *Trichogramma* Qus strain is geniculate and brown. In males, hairs are more condensed and elongated than in females. Also, the flagellum is fused with the club, each antenna consists of an elongated scape with a basal radical, pedicel, and flagellum. (Fig.3a)

In females, antennae are geniculate at the scape-pedicel joint. The scape is the longest antennomere and pedicel. The flagellum is divided into a funicle, which is composed of several short antennomeres known as anelli, and a terminal large antennomere defined as the club (Fig.3 b).

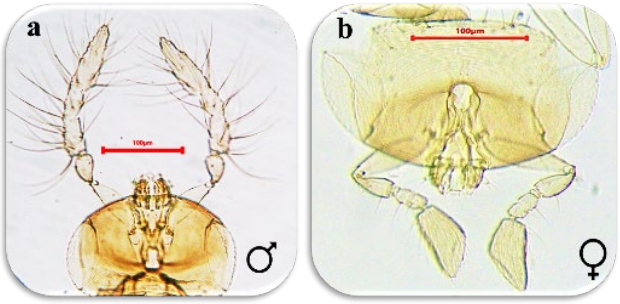

Fig. 3. Antennae of *Trichogramma* Qus strain by light microscope: a) Male and **b)** Female

Mouthparts

The mouthpart is chewing and brown in both sexes of *Trichogramma* Qus strain.

The thorax

Wings

A light micrograph of a *Trichogramma* Qus strain wing with severely diminished venation is depicted in **Fig. 4**. The forewings are wider than the hindwings. The submarginal, marginal, and stigmal veins of the forewing are joined into a single arch close to the forewing's edge. Short hairs cover the broad forelimbs, and longer hairs form a fringe along the wing **(Fig. 4 a)**. Long clusters encircle the hind wing's posterior margin **(Fig. 4 b)**.

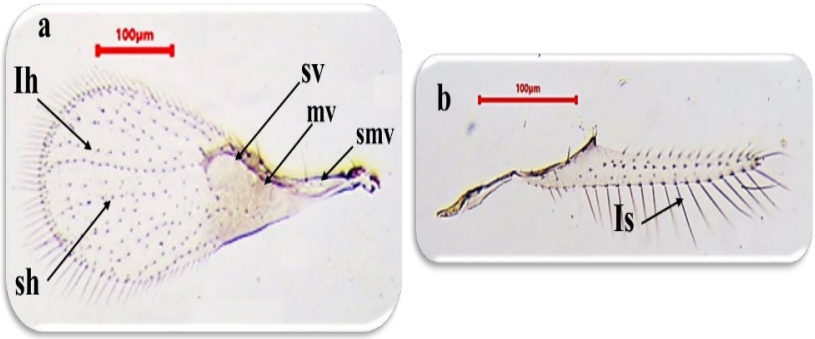

Fig. 4. Light micrograph of *Trichogramma* Qus strain wings: a) Forewing (*sv* stigmal vein, *mv* marginal vein, *smv* submarginal vein, *sh* small hairs and *lh* longer hairs) and b) hindwing (*ls* long setae).

Legs

The legs in *Trichogramma* Qus strain are slender and ambulatory. A leg consists of the coxa, two-segmented trochanter, femur, tibia, and three-segmented tarsus. Tibiae have well-developed, branching spurs. Two claws and a fully formed arolium are present on the apical tarsomere (**Fig. 5**).

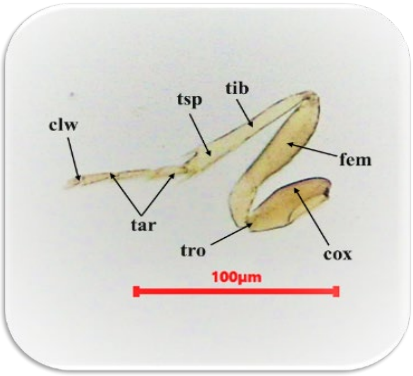

Fig. 5. Light micrograph *Trichogramma* Qus strain Leg: cox coxa, tro trochanter, fem femur, tib tibia, tar Tarsus, clw claw and tsp tibial spur

The abdomen

In the ventral view of the abdominal female in *Trichogramma* Qus strain, the petiole is not pronounced, mesosoma and metasoma are broadly joined. Metasoma consists of seven visible tergites as illustrated (Fig. 6).

Fig. 6. Light micrograph of female metasoma ventral view

External male genitalia

The ventral view of male genitalia in *Trichogramma* Qus strain is represented by simple aedeagus, phallobase, and parameres, as shown in (Fig. 7).

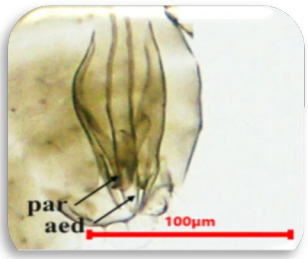

Fig. 7. Light micrograph of *Trichogramma* Qus strain male genitalia: aed aedeagus, par parameres

Ovipositor

The ventral view of the abdominal female in *Trichogramma* Qus strain is represented by the ovipositor with plates and stylet (Fig. 8).

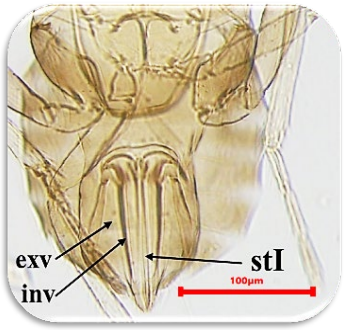

Fig. 8. Light micrograph of female ovipositor ventral view: exv external valves, inv internal valves, stl stylet

Morphometric of *Trichogramma* Qus strain

Body length

Data in Table 1 and Fig. 9 indicate that the body length of adult *Trichogramma* Qus strain males was an average of (537.3±30.24µm). However, female measurements ranged with an average of (525.43±41.80µm). So, the above-mentioned measurements based on ten observations of each collection (n= 10) indicated that the female body length is shorter than the male (t value 0.727, p= 0.238).

Table 1. Body Length of *Trichogramma* Qus strain male and female

Criteria (N= 10)	Body Length measurements (µm)	
	Male	Female
Min.	459.81	463.61
Max.	565.62	571.96
Mean	537.3	525.43
± SD	30.24	41.80

Antennae

One of the most important morphological criteria of *Trichogramma* identification is trichiation on adults' antennae as reported by Dang *et al*. (2005) and Ksentini *et al*. (2010). Data present in Table 2 is based on 10males' antennae of T Qus strain. Measurements of flagellar length/flagellar width (FL/FW), flagellar length/scape length (FL/ScL), and longest seta/flagellar width (LS/FW) averaged 5.10±0.55, 2.04±0.09, and 2.70±0.27µm, respectively based on n=10.

Table 2. Flagellar Length and Width, Scape Length, and Longest Seta characters (µm) of *Trichogramma* Qus strain male antenna

Criteria n=10	Male antennae measurements (µm)						
	Flagellar Length (FL)	Flagellar Width (FW)	Scape Length (ScL)	Longest Seta (LS)	FL/FW	FL/ScL	LS/FW
Min.	158.32	30.08	80.48	83.86	4.26	1.87	2.27
Max.	182.01	39.83	87.01	98.71	5.69	2.2	3.08
Mean	172.122	34.031	84.23	91.287	5.10	2.04	2.7
± SD	8.19	3.43	2.38	3.99	0.55	0.09	0.27

Data present in Table 3 is based on 10 females' antennae of *Trichogramma* Qus strain. Measurements of Antennal Club length (ACL), Antennal Club width

(ACW), and ACL/ACW with an average of 81.67±3.43, 36.47±3.23, and 2.24±0.15µm, respectively.

Table 3. Measurements of female Antennal Club length (ACL), Antennal Club width (ACW), and ACL/ACW of *Trichogramma* Qus strain

Criteria n=10	Female Antennae measurements (µm)		
	Antennal Club length (ACL)	Antennal Club width (ACW)	ACL/ACW
Min.	75.96	32.34	2.02
Max.	87.24	40.61	2.5
Mean	81.67	36.47	2.24
SD±	3.43	3.23	0.15

Wings length

The data present in Table 4 indicate the measurements of forewing length and width for males and females of *Trichogramma* Qus strain. In males, the averages forewing length and width were 484.14±26.59 and 238.44±13.12µm, respectively. However, in females, the averages were 440.41±140.53 and 214.22±3.45µm, respectively. Statistical analysis of the data indicated that the differences in the measurements of forewings length and width for males and females of *Trichogramma* Qus strain are highly significant (t value=4.09, p=0.0004 and 5.64, p=0.0001, respectively).

Table 4. Measurements of forewings length and width of male and female of *Trichogramma* Qus strain

Criteria n=10	Wings measurements (µm)			
	Forewing length (FWL)		Forewing width (FWW)	
	Male	Female	Male	Female
Min.	436.97	410.56	218.49	210.21
Max.	527.98	474.2	254.59	219.32
Mean	484.13	396.37	238.43	214.22
SD ±	26.59	140 .53	13.12	3.45

The measurements of the female abdomen

Measurements of female abdomen length in *Trichogramma* Qus strain from the front to the tip of the abdomen reached an average of 245.98±12.47µm. Also, the abdominal area reached 43274.3±5031.23µm² and the average ovipositor length was 164.52±8.11µm (Table 5).

Table 5. Abdominal length and abdominal area characters (µm) and ovipositor length (µm) of *Trichogramma* Qus strain female abdomen

Criteria n=10	Female Abdomen measurements (µm)		
	Abdomen length (µm)	Abdomenal Area (µm ²)	Ovipositor length (µm)
Min.	225.26	34727.22	156.06
Max.	264.74	48221.04	178.36
Mean	245.98	43274.3	164.52
± SD	12.47	5031.23	8.11

Male genitalia

Measurements of general length/general width (GL/GW), apical distance/general length (AD/GL), and apical width/general width (AW/GW) averaged 2.66±0.19, 0.39±0.04, and 0.85 ±0.05µm, respectively (Table 6).

Table 6. General length and width, aedeagus length, apical distance, basal distance, and apical width characters (µm) of *Trichogramma* Qus strain male genitalia

Criteria n=10	Male Genitalia Measurements (µm)								
	General Length (GL)	General Width (GW)	Apical Distance (AD)	Basal Distance (BD)	Apical Width (AW)	Aedeagus Length (AL)	GL/GW	AD/GL	AW/GW
Min.	112.06	40.24	40.61	64.31	32.72	117.7	2.36	0.33	0.75
Max.	129.35	52.27	54.15	83.86	45.88	139.09	2.98	0.44	0.91
Mean	121.9	46.06	47.19	72.33	39.12	129.92	2.66	0.39	0.85
± SD	6.67	3.94	4.92	5.98	4.54	8.14	0.19	0.04	0.05

Due to the use of *Trichogramma* in controlling many insect pests in Qus, Qena Governorate; it was necessary to accurately know the right species used in controlling certain sugarcane lepidopteran insect pests which was called *Trichogramma evanescens* Westwood. In these results, morphometric tools were used to distinguish among the various species of *Trichogramma*. Our morphometric data were compared with other authors' data for *T. evanescens*.

According to the statistical analysis, the difference between male and female body length is insignificant (t value=0.727, p=0.238). The mean for males was 537.30±30.24µm and for females was 525.432±41.8µm. The average body length of *Trichogramma* Qus strain ranged from 459.81 to 571.96µm (n=20). This finding disagreed with Polilov (2016), who reported that the average body length of *T. evanescens* ranged from 370 to 420µm (n=10).

Antennal trichiation is one of the tools used to distinguish among the various species of *Trichogramma* according to Abdel-Galil *et al*. (2018). Morphometrics of male antennae including flagellar length (FL), flagellar width (FW), scape length (ScL), and longest seta (LS) were measured. The Morphometrics ratio of FL/FW, FL/ScL, and LS/FW for *Trichogramma* Qus strain was 5.10±0.55, 2.04±0.09, and 2.70±0.27µm (n=10), respectively. Comparing findings of *Trichogramma* Qus strain with *T. evanescens* for male antennae measurements (FL, FW, ScL, and LS) studied by Polaszek *et al.* (2012). Measurements of *T. evanescens* include 5.6±0.1µm (n =4), 2.1±0.1µm (n= 3), and 3.1±0.2µm (n=4) for FL / FW, FL / ScL, and LS / FW, respectively. It was inadequate for FL/FW and LS/FW. So, the *Trichogramma* Qus strain cannot be *T. evanescens*.

Female antenna is one of the tools used to compare different species of *Trichogramma* as reported by Khan *et al*., (2020). So, *Trichogramma* Qus strain measurements of Antennal Club length (ACL), and Antennal Club width (ACW) were 81.67±3.43µm and 36.47±3.23µm, respectively.

Khan *et al*., (2020) reported that the forewing length and width of males and females are important morphometric characteristics of different *Trichogramma* species. So, *Trichogramma* Qus strain measurements recorded an average of males' forewings length and width 484.14±26.59 and 238.44±13.12µm. In females, it was 440.41±140.53 and 214.22±3.45µm, respectively.

To differentiate between egg parasitoid *Trichogramma* spp., male genitalia can be used as reported by Polaszek *et al*. (2012), Del Pino *et al*. (2013), Abdel-Galil *et al*. (2018), and Khan *et al*., (2020). Measurements of *Trichogramma* Qus strain concerning general length/general width (GL/GW), apical distance/general length (AD/GL), and apical width/general width (AW/GW) averaged 2.66±0.19, 0.39±0.04, and 0.85±0.05µm, respectively. Measurement by Polaszek *et al*., (2012) GL/GW value of *T. evanescens* was 2.8µm, while *Trichogramma* Qus strain was different with an average of 2.66µm.

Female abdominal measurement submitted taxonomic approaches for the identification of *Trichogramma* spp., as reported by Abdel-Galil *et al*. (2018) and Mousa (2018). Abdominal length (Abdo. Length) µm, abdominal area (Abdo. Area) in µm², and ovipositor length (Ovip. Length) µm of *Trichogramma* Qus strain were measured and recorded an average of 245.98±12.47µm, 43274.3±5031.23µm², and 164.52±8.11µm, respectively.

By discussing the above-mentioned results concerning morphometric features of *Trichogramma* Qus strain, it is clear as expected, they are different species of *Trichogramma.*

It is important to point out herein that, morphometric measurements of *Trichogramma* Qus strain are different species rather than *T. evanescens*, which has been recorded in Egypt by several scientists as a biological control agent.

Morphometric features of *Trichogramma* Qus strain are not enough to differentiate it from *Trichogramma evanescens*. So, it needs molecular tools for precise identification.

Table 7. The understudied *Trichogramma turkestanica* with their related species from the GenBank/ NCBI based on (*ITS2*) sequences

No.	Species	Accession number
1	*Trichogramma turkestanica* strain *T* Qus	MW459187.1
2	*Trichogramma turkestanica*	DQ088061.1
3	*Trichogramma euproctidis*	JF920453.1
4	*Trichogramma brassicae*	HQ143679.1
5	*Trichogramma ostriniae*	MT362642.1
6	*Trichogramma dendrolimi*	AB094398.1
7	*Trichogramma platneri*	MT084466.1
8	*Trichogramma evanescens*	KR148950.1
9	*Trichogramma chilonis*	DQ088053.1
10	*Trichogramma pretiosum*	MH890848.1
11	*Trichogramma cordubensis*	KM279942.1
12	*Trichogramma achaeae*	EU251070.1
13	*Trichogramma japonicu*	KM361745.1

Molecular identification

The sequencing of *ITS2* produced a nucleotide length of 434bp and was deposited in the GenBank under accession number (MW459187.1). The average nucleotide frequencies of adenine (A), cytosine (C), guanine (G), and thymine (T) were 19.8, 28.3, 26.3, and 25.6%, respectively. The average C+G content was 54.6, which was higher than the A+T content. The sequence of (*ITS2*) was subjected to

BLAST/N at (NCBI) and revealed 12 species of the genus *Trichogramma* (Table 7).

To carry out the phylogenetic tree analysis using *ITS2* sequencing, the sequences of the understudied sample of *Trichogramma turkestanica* Meyer were submitted to the analysis together with the 12 related species.

For more expository phylogenetic relations, we used more than one phylogenetic method; Neighbour Joining and Minimum Evolution based on the *ITS2* sequence. The methods showed nearly the same relations with some differences in support values and revealed that the understudied sample with *Trichogramma turkestanica* formed a sister clade, *and Trichogramma euproctidis* Girault was very near this clade (Figs. 9 and 10).

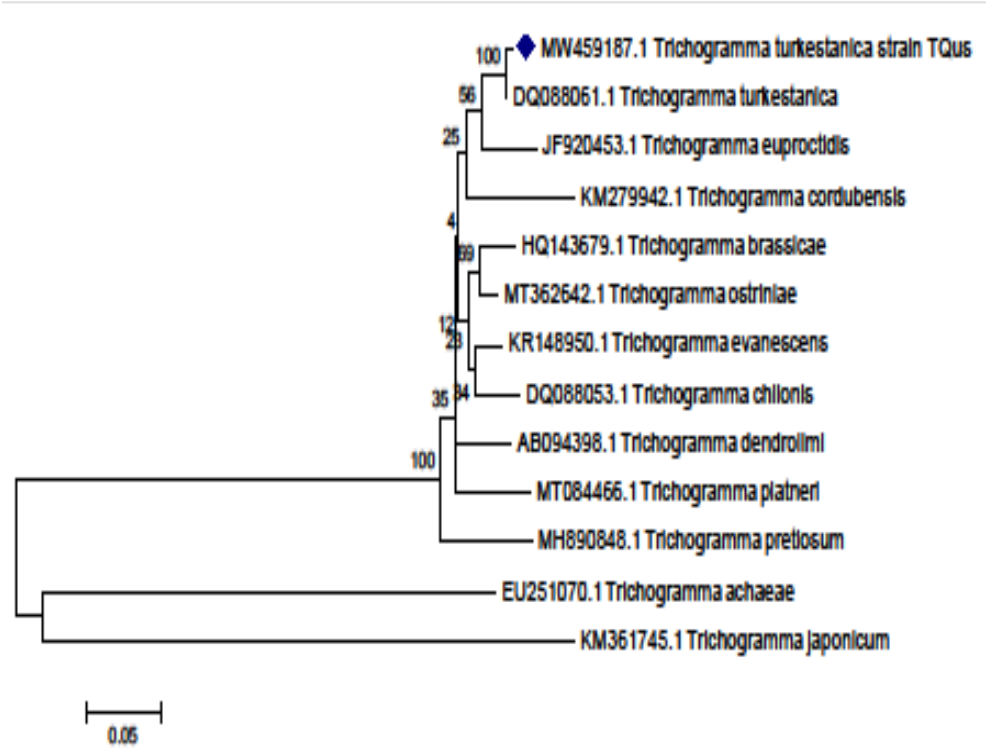

Fig. 9. Phylogenetic tree using the Neighbour Joining method among *Trichogramma turkestanica* with their related species from the GenBank/ NCBI based on *ITS2* sequences.

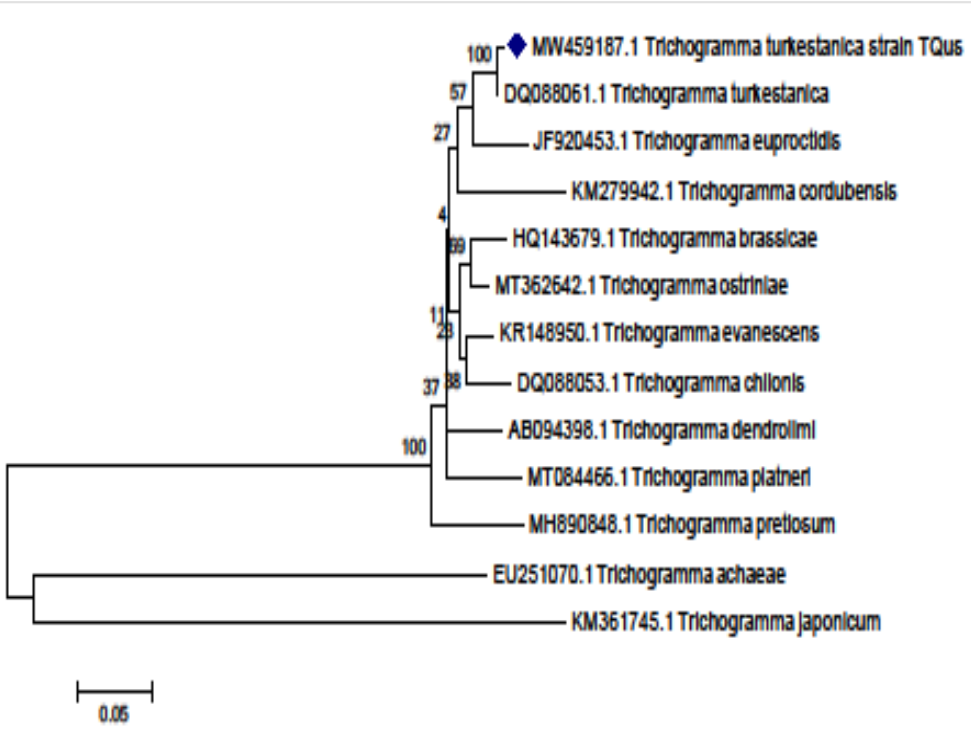

Fig. 10. Phylogenetic tree using the Minimum Evolution method among *Trichogramma turkestanica* with their related Species from the GenBank/ NCBI based on (ITS2) sequences.

Pairwise genetic distances among the understudied *Trichogramma turkestanica* and 12 species` of genus *Trichogramma* ranged from 0.003 to 0.075. The most related species to our sample was *Trichogramma turkestanica*, where the genetic distance was 0.003. Overall, the mean distance value was 0.397.

In conclusion, to have an efficient IPM application, pests, and biological control agents must be accurately identified. The morphometric characteristics and molecular identification proved that *Trichogramma turkestanica* was the species, which used in controlling lepidopterous sugar cane pests in Qus Sugarcane Factory, Qena, Egypt.

Acknowledgment

We are grateful to everyone who contributed to this valuable work to come out this way. I had to thank the Molecular Genetic Laboratory, Faculty of Science, South Valley University, Qena, Egypt, and the Biological Control Unit of the Plant Protection Department, which was established in 2004 by F. A. Abdel-Galil.

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دراسات مورفوجينية على طفيل البيض التريكوجراما الذي يطلق لمكافحة آفات قصب السكر من حرشفية الأجنحة في محافظة قنا، مصر

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الملخص

يطلق طفيل البيض التريكوجراما في قوص محافظة قنا لمكافحة آفات قصب السكر التابعة لرتبة حرشفية الأجنحة.

الهدف الأساسي من هذه الدراسة هو تعريف طفيل البيض، Trichogramma باستخدام الطرق المورفولوجية والوراثية. تم استخدام الميكروسكوب الضوئي لفحص مورفولوجيا الذكور والإناث البالغين واخذ قياساتهم. أظهرت النتائج أن الإناث كانت أقصر من الذكور. بالإضافة إلى ذلك، تم تسجيل الشكل الخارجي لقرون الاستشعار والأجنحة والأعضاء التناسلية الذكرية. بدراسة البيولوجيا الجزيئية للطفيل تم إيداع تسلسل ITS2 الخاص التريكوجراما *T*Qus في بنك الجينات تحت رقم (MW459187.1). كان طول النيوكليوتيدات 434. زوج من القواعد. كان متوسط تكرار نيوكليوتيدات الأدينين (A) والسيتوزين (C) والجوانين (G) والثيمين (T)19.8 و3.28 و26.3 و25.6٪ على التوالي. كانت نسبة القواعد النيتروجينية C+G (54,6) أعلى من نسبة القواعد النيتروجينية A+T. من خلال الطرق المورفولوجية والوراثة الجزيئية، يمكن التأكيد أن نوع طفيل الترايكوجراما المستخدم في قوص محافظة قنا هو *T. turkestanica*.

الكلمات المفتاحية: *قصب السكر، ترايكوجراما التصنيفات المورفولوجية والوراثية.*