https://doi.org/10.26682/ajuod.2023.26.1.3
Journal of University of Duhok., Vol. 26, No.1(Agri. and Vet. Sciences), Pp 23-29, 2023

FIRST RECORD OF THE ENTOMOPATHOGENIC NEMATODE *OSCHEIUS TIPULAE* (LAM AND WEBSTER, 1971) IN IRAQ

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(Received: November 9, 2022; Accepted for Publication: January 22, 2023)

ABSTRACT

***Oscheius tipulae*, first described by Lam and Webster 1971, is one of the entomopathogenic, inhabiting soil nematodes and is very common in the soil of many regions of the world. *O. tipulae* was isolated from soil samples collected from olive and grapevine trees (Summel district, Duhok province, Kurdistan Region - Iraq) using galleria bait traps. Molecular identification based on ITS-rDNA gene was done to identify the samples. Using blast search, a homology of (98%) was exhibited to the *O. tipulae* which is the first record of this species in Iraq.**

***KEYWORDS*:** Oscheius tipulae, new record, olive and grapevine soils, Iraq

INTRODUCTION

Entomopathogenic nematodes (EPNs) have been known since the 17th century and include more than 30 families (Kaya and Stock, 1997). The most common entomopathogenic nematodes are belong to the families Steinernematidae and Heterorhabditidae (Kaya and Koppenhöfer, 2004). These two families include more than 16 *Heterorhabditis* species and at least 60 species of *Steinernema* (Hunt, 2007 and Nguyen and Hunt, 2007). There are a lot more EPNs have been described, mainly from Africa and Asia (Khatri-Chhetri, *et al.*, 2011). The genus *Oscheius* was considered as a new member of entomopathogenic nematodes (Torres-Barragan, *et al*., 2011). *Oscheius* spp. placed under Rhabditidae family. Nowadays, *Oscheius* have about 30 identified species; from them, about 13 species are lethal to insect pests (Tabassum, *et al.*, 2016 and Ye, *et al.*, 2018). One of the insect parasitic species is *Oscheius tipulae* (first described by Lam and Webster,1971). It is named *O. tipulae* depending on *Tipula paludosa* name which is found associated with its larvae. This species is very common in the soil of many regions of the world. *O. tipulae* has the potential as a biological control agent against *Galleria mellonella* larvae and Mediterranean fruit fly *Ceratitis capitate* (Loulou *et al.* 2022). A number of studies were carried out in the Middle East about EPNs, but in spite of that, there is no enough data about the diversity and potential of EPNs in Iraq. According to the previous studies of entomopathogenic nematodes, unknown species of *Steinernema* was isolated from the date palm longhorn stem borer *Jebusea hommerschmidtii* and fruit stalk borer *Oryctes elegans* (AL-Jboory and Saleh, 2001) and *Steinernema carpocapsae* was isolated from apricot stem borer's *Chalcophorella bagdadensis* larvae and adults (Al-Jboory and Al-Zubaii, 2006) but their identification depends only on morphological characteristics. Recently, in Iraq *Heterorhabitis bacteriophora* and *Oscheius myriophilus* were reported and identified based on the analysis of ITS and 18s rDNA gene (Al-Zaidawi *et al.*, 2019). This research aims to survey, isolate and identify indigenous EPNs from soil cultivated with olive and grapevine trees in Summel district, Duhok province, Kurdistan Region - Iraq.

MATERIALS AND METHODS

Samples collecting

Soil sampling done during Autumn season of 2021, at different locations in Summel district, Duhok province. Samples were taken from soils cultivated with olive and grapevine trees. Three trees were randomly chosen for each plant species including a minimum distance of 20m between them. Four sub samples each about 250 gm were taken for each tree (1kg/ tree) (producing 12 soil samples/ plant species). Twenty-five soil samples, were collected using a hand shovel at a depth of 0-20 cm around the tree's roots. Afterward samples were kept in

bags and stored in a refrigerator at 4C for later use. In order to isolate the entomopathogenic nematodes, soil samples were processed using the insect-baiting *Galleria mellonella* (*Galleria* trap) techniques (Bedding and Akhurst, 1975). For each sample, 300 grams of soil were placed in 500 ml plastic cans. Then, 10 of *G. mellonella* larvae were added to soil container and moistened using a water sprayer. The containers incubated at 25 °C in the darkness for 5 days. The cadavers were cleaned with water and then transferred to white traps (White, 1927) (Figure 1), wherein after 2–5 days, the larvae killed by nematodes were placed above a water reservoir. The nematode produced by this method is harvested by placing moist filter paper on a concave side up watch glass surrounded with water in a large Petri dish. The progeny IJs migrate from the depleted host cadaver into the water reservoir, where they are trapped and subsequently harvested. The traps were checked every day, emerging nematodes were collected and maintained in the darkness at 25°C.

Morphometric characteristic and measurements

For morphological observations, infective juveniles and adults Picked up randomly from nematode suspension isolated by *G. mellonella* traps. The nematodes were killed in worm water at 60°C, fixed in 4% formalin solution, and process to a modification of glycerin-ethanol series (Seinhorst, 1959). Then fixed nematodes were mounted on a slide and covered with a cover slide to calculate their morphometric characters included: body length, maximum body width or diameter, tail length, distance from the anterior end to excretory pore (EP), distance from the anterior end to the base of the pharynx (ES), distance from the anterior end to the nerve ring. Following morphometrical measurements of body, ratios were calculated as a = body length/ maximum body width, b= body length/ esophageal length, c= body length/ tail length (Machado *et al.*, 2010). Specimens were examined and measured with a Olympus CX22RFS1 microscope (Japan) at up to 1000×magnification with using an ocular micrometer and photographed using Honer X9 camera 64mp.

Fig.(1):- A white trap for *Oscheius tipulae* from infected *Galleria mellonella* larvae

MOLECULAR IDENTIFICATION

DNA extraction

Individuals of adult nematodes were used for extraction of DNA from them. The adult nematodes were added to an eppendorf (1.5 ml) containing 100 ml lysis buffer and 2 ml proteinase k, incubated at 56°C overnight. The other steps of extraction were done according to the protocol of Animal DNA Preparation-solution kit. The extracted DNA was stored at -20°C.

Amplification of DNA using Polymerase Chain Reaction (PCR)

The extracted DNA was used as template for PCR amplification of internal transcribed spacer region (ITS) by using the set primers of TW81

as forword (5'-GTTTCCGTAGGTGAACCTGC-3') and AB28 as reverse (5'-ATATGCTTAAGTTCAGCGGGT-3') (amplification size of 900 bp) (Joyce et al.1994). The final PCR reaction volume were of 40 µl as 20 µl of ready to use 2xTaq PCR Master mix, 1 µl of each forward and reverse primer (10 pml), 10 µl genomic DNA (10-20 µg/µl) and 8 µl sterilized water.

The PCR reaction were conducted using A Gene Amp PCR system 9700 thermocycler for amplification following a program as: 94°C (4 min), 35 cycles (94°C for 1 min), 55°C (1 min), and 72°C (2 min), and the last extension at 72°C (10 min) (Al-Zaidawi, *et al.* 2019) . After Amplification, agarose gel electrophoresis (1%) with 3 ml of Eva Green ® Fluorescent Gel stain (Jena Biosciences Germany) was used to visualize the PCR product. PCR products of ITS region from individual nematodes were purified for sequencing using the protocol of ZR-96 Zymoclean™ Gel DNA Recovery Kit (Figure 2).

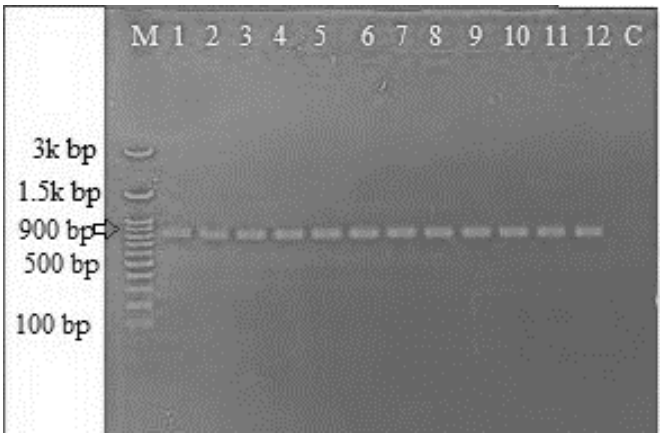

Fig.(2):- Agarose gel electrophoresis of the PCR products

Sequencing and phylogenetic analysis

The sequencing was performed at Microgen Molecular Company Korean. The sequences were aligned, then submitted to GenBank in NCBI, and the accession numbers were determined as OP476678 and OP376148. The sequence similarity homologous sequence deposited in GenBank calculated by BLAST tool on NCBI website. Alignment of sequences was done with Clustal W. The Neighbour- Joining method and Jukes-Cantor model in MEGA-11 was used for phylogenetic tree construction. Branch support was estimated by bootstrap analysis with 1000 replicates.

RESULTS

Eleven isolates of ENPs were recovered from soil cultivated with different plants collected from Summel district. Based on microscopic observations, isolates display the morphological characteristics found in the genus *Oscheius* that described by Sudhaus (1976).

Sequencing of ITS and phylogenetic tree

To confirm the morphological identification of the isolated nematodes, selected specimens were analyzed by molecular approaches. Sequencing results of rDNA-ITS of *Oscheius* isolates showed 742 and 537bp of special DNA fragments sequenced. The resulting sequences were compared with sequences of rDNA accessed in Genbank using BLAST search. Phylogenetic analysis showed that the obtained sequences share 98% homology to *Oscheius tipulae* strains: Italian (KT728760, KT728763), Switzerland (KJ938579, MW667576) and Chinese isolate (MK294848) (Figure 3). Together, analysis of ITS region as well morphometric data and morphological characters showed that *Oscheius* isolates is *Oscheius tipulae* (Genbank accession No. OP476678 and OP376148). According to the blast of NCBI both isolates differ from each other by 1.05% or percent identity between them is 98.95%, thus the DNA dot plot (a graphical method for comparing two biological sequences) (Figure 4) was create to show the real genetic differences between both isolates though they belong to the same species.

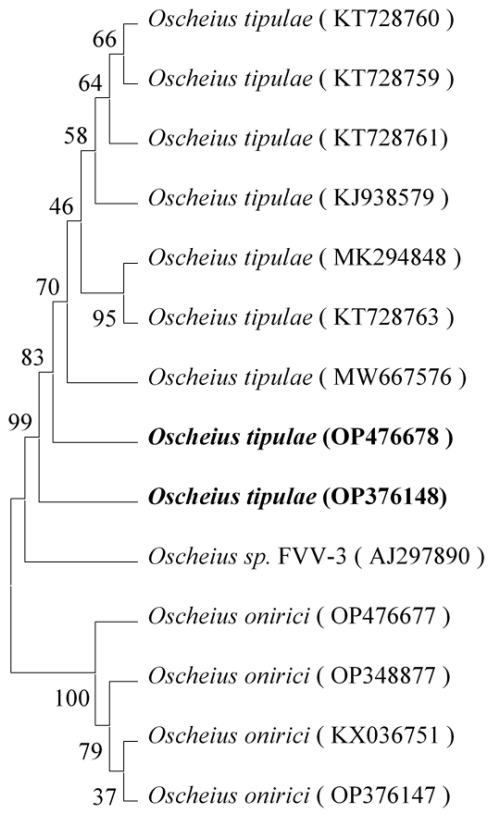

Fig.(3):- Phylogenetic tree of *Oscheius tipulae* conducted in MEGA.11 based on Neighbor-Joining analysis with 1000 bootstrap replicates of ITS-rDNA sequences of the new strains from Iraq (in bold) and related *Oscheius* species from GenBank. GenBank accession numbers provided next to species names.

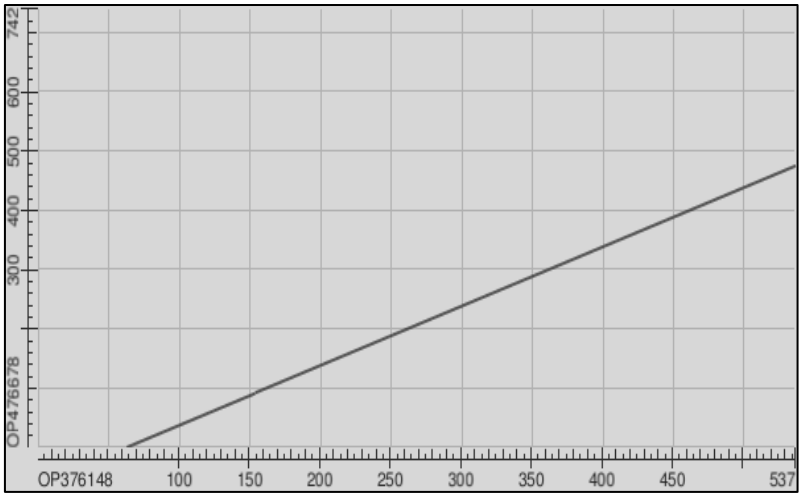

Fig.(4): -DNA Dot Plot between both isolates of *Oscheius tipulae*

Description of *Oscheius tipulae* as a new record from Iraq: *Oscheius tipulae* (Lam and Webster, 1971) (Figure 5)

After fixation, female length slightly curved ventral. Cuticle annulated; Lip region continuous with body contour, having six rounded lips. Body length ranged between 500-1000 µm, maximum body width ranged from 31-51 µm, tail length 66-110 µm. As average of both isolates, **a** value 17.29µm, **b** value 5.35 µm and **c** value 8.22µm. For juvenile stage, body length ranged between 300-452 µm, maximum body width ranged from 14-30 µm, tail length 45-68 µm. As average of both isolates, **a** value 15.94µm, **b** value 4.39 µm and **c** value 6.31µm (Table 1).

Table (1):- Morphometrics data of two isolates of *Oscheius tipulae*.

Morphometric characteristics	Isolate SKO kurd (OP476678)		Isolate SKG kurd (OP376148)	
	Juvenile stage	Female	Juvenile stage	Female
Body length	344 ± 39.08 (300-410)	696 ± 110.47 (500-810)	332 ± 47.71 (300- 452)	720 ± 147.6 (589-1000)
a	16.15 ± 3.24 (12.33-22)	17.57 ± 2.82 (14.75-23.67)	15.73 ± 2.56 (12-19)	17.1 ± 2.83 (14-22.2)
b	4.73 ± 0.67 (3.45-5.82)	5.15 ± 0.77 (4.17- 6.40)	4.05 ± 0.50 (3- 4.52)	5.56 ± 1.16 (4.2- 8)
c	5.93 ± 0.77 (5-7.27)	8.57 ± 1.56 (5.56-10.39)	6.68 ± 1.23 (5-7.6)	7.87 ± 1.39 (6-10)
Body width	22.29 ± 5.64 (16-30)	39.96 ± 6.67 (31-51)	20.83 ± 4.19 (14 - 25)	44 ± 5.79 (35-50)
Tail length	58.5 ± 6.72 (52- 66)	82.2 ± 10.14 (66- 85)	54.10 ± 9.75 (45-68)	92.8 ± 11.72 (75-110)
Distance from anterior end to nerve ring (EN)	64.30 ± 10.84 (55-78)	93.23 ± 9.70 (80-102)	50.25 ± 6.01 (45-60)	104.6 ± 12.76 (90-110)
Distance from anterior end to excretory pore (EP)	60.98 ± 12.33 (50-82)	113.80 ± 8.50 (100-123)	70.3 ± 4.95 (65-76)	121.6 ± 4.65 (115-130)
Distance from anterior end to the base of the esophagus (ES)	73.68 ± 10.05 (65-88)	135.2 ± 10.90 (120-150)	87.7 ± 11.35 (76-100)	129.7 ± 7.54 (124-145)

All measurements are in µm: mean± S.D (range)

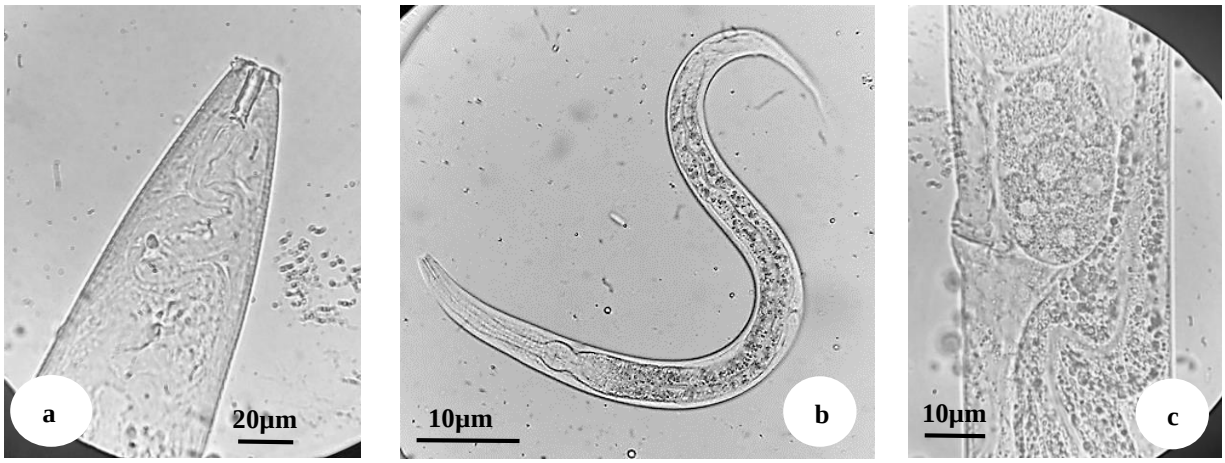

Fig.(5): -Light micrographs of *Oscheius tipulae* female (Isolate SKO kurd); a- Anterior end (lateral view); b- Entire female body; c- Vulval region

DISCUSSION

The isolates were identified and characterized based on ITS-rDNA gene. The molecular analysis was conducted to confirm the identification. The resulting sequences were compared with sequences of rDNA accessed in Genbank using BLAST search. There are a few studies on Entomopathogenic nematodes in Iraq and depends only on morphological characters for identification. This current study findings revealed that the new ENP species *Oscheius tipulae* is recorded in Iraq for the first time and occur in Iraqi soil along with the other entomopathogenic nematodes (EPN) as *Steinernema carpocapsae* (Al-Jboory and Al-Zubaii, 2006), *Heterorhabditis bacteriophora* and *Oscheius myriophilus* (Al-Zaidawi *et al.*, 2019).

Nowadays, *Oscheius* consist of 27 species arranged by two groups "Dolichura; as 13 species" and "Insectivora; as 14 species" (Sudhaus, 2011). The results of ML (18S rDNA) and NJ (ITS rDNA) put *O. tipulae* close to *O. dolichura* and *O. dolichuroides*. While most researchers considered *Oscheius* as a genus, Sudhaus (1993) considered it as subgenus of *Rhabditis* and re-described *Rhabditis* (*Oscheius*) *tipulae*. Recently, the *O. tipulae* has been recorded in Iran as a new insect parasitic nematode (Karimi *et al.*, 2018). We isolated this species in olive and grapevine orchard soils in Summel district, Duhok province, Kurdistan region, Iraq. In each orchard, several locations are surveyed for native EPN species.

Morphological traits resembled the original description of *O. tipulae* (Lam and Webster 1971). However, our specimens had longer bodies (589-1000 µm vs. 624-780 µm). In comparison with nematodes examined by Sudhaus (1993), the Iraqi specimens have the same esophagus length (120-150µm vs. 129-151 µm), and differed with having longer body length (589-1000 vs. 505- 708 µm) and tail length (75-110 µm vs. 70-95 µm). The differences among isolates are due to geographical distribution and habitat. Thus, after investigating the previous studies in Iraq, it was found that identification of this EPN is the first record from Iraq. This study might be confirmed that Iraqi soil, specially Duhok province soil is rich with the different ENP nematodes. This study results indicated that *Oscheius tipulae* is locally abundant in different agroecosystems in Duhok province which is a vast region with a high variation in geographical and ecological indices such as temperature, altitude, vegetation cover, etc.

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