RESEARCH/INVESTIGACIÓN

ADDITION OF A NEW INSECT PARASITIC NEMATODE, *OSCHEIUS TIPULAE,* TO IRANIAN FAUNA

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ABSTRACT

Karimi, J., N. Rezaei, and E. Shokoohi. 2018. Addition of a new insect parasitic nematode, *Oscheius tipulae*, to Iranian fauna. Nematropica 48:00-00.

On behalf of an ongoing project on diversity of insect pathogenic and insect parasitic nematodes of Iran, a new species was collected and characterized. This species was collected in soil from the Mashhad, Arak, and Mahalat regions of Iran through 2011-2012 using *Galleria* larvae baits. Based on morphologic and morphometric traits as well as SEM images, the species tentatively has been identified as *Oscheius tipulae*. Phylogenetic analysis based on ITS and 18S rDNA genes confirmed the species delimitation. This is the first record of this species from Iran.

Key words: 18S rDNA, insect parasitic nematode, Iran, ITS, *Oscheius tipulae*, SEM

RESUMEN

Karimi, J., N. Rezaei, y E. Shokoohi. 2018. Adición de un nuevo nematodo parásito de insectos, *Oscheius tipulae*, a la fauna Irán. Nematropica 48:00-00.

En nombre de un proyecto en curso sobre diversidad de patógenos de insectos y nematodos parásitos de insectos de Irán, se recolectó y caracterizó una nueva especie. Esta especie fue recolectada en suelo de las regiones de Mashhad, Arak y Mahalat de Irán durante el período 2011-2012 utilizando cebos Galleria. En base a los rasgos morfológicos y morfométricos, así como a las imágenes SEM, la especie tentativamente ha sido identificada como *Oscheius tipulae*. El análisis filogenético basado en los genes ITS y 18S rDNA confirmó la delimitación de especies. Este es el primer registro de esta especie de Irán.

Palabras claves: 18S rDNA, Irán, ITS, nemátodo parásito de insectos, *Oscheius tipulae*, SEM

INTRODUCTION

Nematodes comprise a tremendously diverse group that have the most abundance in marine and terrestrial habitats. In the latter case, they represent a significant component of the soil community (Kaya and Gaugler, 1993). The interest in the use of nematodes as biocontrol agents for pest control has increased exponentially over last decade (Kaya and Gaugler, 1993; Grewal *et al*., 2005). The most important groups of nematodes for use as biocontrol agents are the families Steinernematidae and Heterorhabditidae (Rhabditia). Entomopathogenic nematodes (EPN) in both families are symbiotically associated with bacteria and are potent biocontrol agents against a variety of insect pests that are widely distributed in soils throughout the world (Kaya and Gaugler, 1993; Gaugler, 2002; Grewal *et al.*, 2005; Ye *et al*., 2010; Torres-Barragan *et al*., 2011). Moreover, other members of the Rhabditida have shown potential to infect insects and are promising as new candidates for biocontrol of insect pests. The Rhabditidae are important in nematological studies because of their great ecological diversity and association with insects. These bacterial feeding nematodes are suitable models for understanding the evolution of parasitism in diverse lineage groups of Secernentea (Sudhaus, 2011; Liu *et al*., 2012).

The broad range of association of members of

the Rhabditidae with insects can be divided in different ways. Some species of Rhabditids have mutualistic association with insects. The Rhabditidae have various feeding behaviours and are usually specialized to feed on bacteria (decaying organic matter) and are often associated with animal hosts or vectors. *Caenorhabditis, Mesorhabditis*, *Pellioditis*, *Rhabditis*, *Phasmarhabditis,* and *Oscheius* are the main genera of this family. Andrássy (2005) described the genus *Oscheius* and subsequently *O. insectivorus* was assigned as type species. Sudhaus (1976) placed *Oscheius* in the Rhabditidae and divided the genus into two groups. "Insectivora-group" with leptoderan bursa, comprising 14 species and "Dolichura-group" with peloderan bursa, comprising 13 species (Sudhaus, 2011). The species of *Oscheius* are common soil nematodes, all reproducing hermaphroditically. *Oscheius tipulae* reproduces through XX hermaphrodites and XO males such as *C. elegans* (Félix *et al*., 2001; Felix, 2006; Pervez *et al*., 2013). Species of *Oscheius* in the "insectivorus" group represent various associations with invertebrate hosts ranging from facultative to obligate parasitism (Liu *et al*., 2012). Torrini *et al*. (2015) described the first EPN from the Dolichura group and later Campos-Herrera *et al.* (2015) provided more data about the *Oscheius* genus. *O. insectivorus* (Andrássy, 1976) and *O. maqbooli* were described as insectivorous species (Tabassum and Shahina., 2010), *O. carlianonsis* as EPN (Ye *et al*., 2010), *O. amsactae* as a necromenic associate (Liu *et al*., 2012) and *O. gingeri* as an EPN (Pervez *et al*., 2013). *Oscheius tipulae* was described for the first time by Lam and Webster (1971) and redescribed by Sudhaus (1993). This species has been associated with leatherjackets, larvae of a tipulid dipteran, *Tipula paludosa*. The nematode specific epithet refers to the etymology of its host name (Sudhaus, 2011). DNA sequence analysis of several loci including 18S and ITS rRNA as well the D2-D3 expansions of 28S rRNA (De Ley *et al*., 2005; Stock, 2003; Stock *et al*., 2009) is widely accepted for nematode identification and assessing phylogenetic relationships (Dorris *et al*., 1999; Kumari and Lišková, 2009; Stock, 2009). The objective of this research was to identify a new Rhabditid species from Irainian fauna using classic approach, as well molecular analysis of two genes. The SEM images of the Iranian population of the new species and their drawings are provided (Fig. 1-4).

MATERIALS AND METHODS

Nematodes isolation

During 2010 to 2013, a project was conducted to identify native EPNs in different locations of Iran. We applied a routine sampling of soil using the *Galleria mellonella* baiting technique (Bedding and Akhurst, 1975) to soil from apple and apricot orchards. This work led to the isolation of several EPN species and a Rabditid nematode. The aforementioned rhabditid was collected from Mashhad (Razavi Khorasan Province, 36°15'N, 59°38'E), Arak (Markazi Province, 34°4'N, 49°46'E), and Mahalat (Markazi Province, 50°41'N, 33°91'E). The killed larvae of *G. mellonella* with the nematode had visible colour.The nematodes emerging in the White trap were collected (Kaya and Stock, 1997). These extracted nematodes were tested for ability to infect wax moth larvae. Mixed stages of nematodes were emerged from the larvae cadavers 10 days after inoculation and were collected for 10 more days.

Light microscopy

For morphological characterization, 50 adults and infective juveniles (IJs) were randomly selected from *G. mellonella* cadavers, fixed using hot (80°C) 4% formaldehyde solution, and processed to anhydrous glycerin for mounting (De Grisse, 1969). Fixed nematodes were mounted on a glass slide using cover glass and glass rod supports to avoid flattening. Measurements and microphotographs assured quality and accuracy. Drawings were made using a drawing tube attached to an Olympus light microscope CH-2.

Scanning electron microscopy (SEM)

First-generation adults were fixed in 3% glutaraldehyde buffered with 0.1 M phosphate buffer then fixed with 2% osmium tetroxide solution for 12 hr at 25°C. The samples were washed with sterile water three times and dehydrated in a graded ethanol series (10, 20, 50, 70 and 100%), mounted on SEM stubs, and coated with gold using CamScan MV2300 microscope operating at 15 mA. Images of the species were obtained with LEO 1450VP scanning electron microscope (LEO Co. Ltd., Oberkochen, Germany).

DNA extraction, PCR, and sequencing

Genomic DNA was extracted from an individual adult nematode. The adult nematode was picked and transferred into a microtube after grinding in 50 µl 5% Chelex-100 (Sigma Aldrich, Germany) and 2 µl proteinase K, incubated at 60°C for 3 hr, followed by 10 min at 95°C. After centrifugation at 13,000 rpm for 3 min, the extracted DNA was stored at –20°C. A molecular approach, including sequences analysis of ITS and

18S genes were considered for molecular characterization of the isolate. The primers for amplification of ITS gene were 5′-TTG ATT ACG TCC CTG CCC TTT-3′ (forward), and 5′-TTT CAC TCG CCG TTA CTA AGG-3′ (reverse) (Vrain *et al*.,1992) and the primers used for 18S rDNA amplification were 5′-GGT GAA ACT GCG AAC GGC TCA-3′ (forward) and 5′-CCG GTT CAA GCC ATT GCG ATT-3′ (reverse) (Blaxter et al.,1998). The PCR mixture was carried out in a reaction volume of 25 µl, containing 2.5 µl of 10× PCR-buffer, 15.7µl of H_2O, 1 µl of $MgCl_2$ (25mM), 0.5 µl of dNTPs (10 mM), 0.3µl *Taq* polymerase (5 units/µl), 1µl of forward primer (10 pmol/µl), 1µl of reverse primer (10 pmol/µl), and 3 µl of template DNA. After DNA amplification, all PCR products were loaded on 1% agarose gel. Aliquots of PCR products were electrophoresed at 80 V for 45 min. The gels stained using Green viewer (SYBR). PCR products were sequenced with Sanger BigDye chemistry on an Applied Biosystems 3730xl DNA sequencer, trimmed using Chromatogram Explorer v.3.2, and species identification was made by best hits to the NCBI using BLASTn.

Multiple alignments and phylogenetic analysis

The sequences chromatograms were checked using BioEdit software. The sequences were compared with those of other nematode species available in GenBank using the BLAST homology search program to confirm the sequences and repeated. The consensus sequence for each gene was assembled in DNA Baser. Phylogenetic trees were generated using the Maximum-Parsimony, Maximum-Likelihood, Minimum-Evolution, and Neighbor-joining method in MEGA 6 (Tamura *et al*., 2013). The Minimum Evolution trees were searched using the Close-Neighbour-Interchange (CNI) algorithm (Nei and Kumar, 2000) and Neighbour-Joining algorithm (Saitou and Nei, 1987) was used to generate the initial trees. The evolutionary distances were computed using the P-distance method (Nei and Kumar, 2000) and were expressed as the number of base differences per site. For phylogenetic analysis, valid and verified sequences of family Rabditidae were retrieved from the GenBank and aligned together with the sequences results from the current study. The sequences were aligned using the ClustalX software with default parameters. The ITS sequences of *Steinernema carpocapsae* (accession numbers EU 122951) and 18S sequence of *Steinernema carpocapsae* isolate Breton (accession numbers GQ421604) were used as outgroup taxon to resolve relationship among the nematode species examined for ITS regions and 18S rDNA, respectively.

PCR based RFLP

We checked genetic diversity among three populations of the species studied. The selected restriction enzymes were *Bgl* II and *Hinc* II. After PCR, the amplicons of ITS gene were subjected to digestion (Felix *et al*., 2001). We also checked the digestion results by *in silico* digestion using Webcutter 2.0.

RESULTS AND DISCUSSION

Three isolates of collected Rhabditid were belonged to *Oscheius* genus. These *Oscheius* nematodes were collected from Arak in 2010, Mashhad in 2011, and Mahallat in 2012 in an apple orchard, apricot orchard, and apple orchard, respectively. A comprehensive approach was applied to characterize the isolated populations as *O. tipulae*. While the three collected populations were from different geographical origins, analysis of ITS region as well morphological and morphometric data of those populations showed high similarity. Therefore, we have selected the Mahallat's population for representative data. Table 1 provides morphological traits of females and larvae. The isolated nematode identified as *O. tipulae.* Morphological traits resembled the original description of *O. tipulae* (Lam and Webster, 1971).

Description

Oscheius tipulae Lam and Webster, 1971. Population from Mahallat, Markazi Province, Iran (11♀♀ five females and four juveniles; in good state of preservation, Figs. 1, 4). Measurements: see Table 1.

Female: Body length slightly curved ventrad after fixation. Cuticle annulated; annuli 1.9 µm. Lateral field not visible. Lip region continuous with body contour, having six rounded lips, bearing small papillae. Stoma rhabditoid, 13 to14 µm long, with distinct cheilo-, gymno- and stegostom. Cheilostom finely cuticularized. Gymnostom longer than cheilostom, having well cuticularized lumen. Stegostom having isomorphic glottoid apparatus with two to three denticles. Pharyngeal collar present, covers half of the stoma. Pharyngeal corpus 1.6 to 1.9 times isthmus length, with procorpus longer than metacorpus. Metacorpus distinct, swollen. Isthmus robust, distinctly separated from metacorpus. Basal bulb ovoid, with valvular apparatus. Cardia conoid, surrounded by intestinal tissue. Nerve ring at isthmus level, at 59 to 74% of neck length. Excretory pore opening at isthmus level, at 65 to 87% of neck length. Deirid not visible. Intestine without distinct

Table 1. Morphometric data of *Oscheius tipulae* Lam and Webster, 1971. Measurements in µm and in the form: mean ± standard deviation (range).

Parameter	Female[y]	Larvae[z]
Body length	689.5±170.3 (524-962)	404.8±59.5 (333-476)
a	19.5±3.6 (17-26)	16.0±1.0 (15-17)
b	7.2± 2.4 (5.2-11.1)	5.3±.09 (4.3-6.2)
c	9.4±1.4 (8.2-11.6)	7.8±1.0 (6.9-9.2)
c'	4.8±.03 (4.5-5.4)	4.1±0.3 (3.8-4.5)
V	50±0.0 (48-52)	-
Lip region diameter	7.7±0.7 (7-9)	5.5±0.5 (5-6)
Stoma	13.5±0.7 (13-14)	13±2.9 (12-17)
Pharyngeal corpus	58.5±5.2 (50-63)	43±2.4 (40-46)
Isthmus	34.8±2.9 (31-39)	26.4±2.3 (24-29)
Bulb	26±2.6 (24-29)	19.2±0.0 (19.0-19.2)
Pharynx length	113.6± 8.9 (104-125)	88.9±7.3 (79-96)
Neck	135.6±1.3 (134-137)	101.8±6.1 (95-110)
Nerve ring to ant. end	93.5±8.1 (80-101)	67.5±5.9 (63-74)
Excretory pore to ant. end	107.3±12.5 (87-118)	82.0±5.2 (77-89)
Deirid to ant.end	93.1±4.4 (87-96)	72.8±2.8 (71-77)
Body diameter: neck base	30.6±4.6 (24-37)	24.3±4.3 (19-29)
Body diameter: midbody	35.4±5.3 (27-40)	25.5±4.7 (19-31)
Body diameter: anus	15.2±2.0 (14-17)	12.7±1.2 (12-14)
Annuli width	1.9±0.0 (1.9-1.9)	1.9±0.0 (1.9-1.9)
Cuticle thickness	2.1±0.4 (2-3)	1.9±0.0 (1.9-1.9)
Vulva- anterior end	322.1±42.1 (277-381)	-
Vagina	12.8±5.6 (10-19)	-
Anterior genital branch	223.1±42.3 (183-269)	-
Posterior genital branch	206.7±11.3 (192-216)	-
Ovary	81.5±9.4 (67-93)	-
Rectum	11.8±3.2 (10-16)	8.9±0.5 (9-10)

[y] n = 5
[z] n = 4

specialization. Reproductive system didelphic-amphidelphic. Vulva slightly protruding, located posterior to middle part of body. Oviduct short. Uterus tubular, with swollen lumen, about 2 times as long as corresponding body diameter. Vagina with fine walls, extending inward one-third of the body width. Rectum 0.7 to 1.2 times anal body diameter. Tail conical, with acute end. Phasmid at 20 to 29% of tail length from the anus.

Male: Not observed.

Remarks: The nematodes examined are similar to the original description of *O. tipulae* provided by Lam and Webster (1971), however, our nematodes have longer bodies (524-962 *vs* 624-780 µm). In comparison with the nematodes examined by Sudhaus (1993), the Iranian specimens have shorter pharynx (104-125 *vs* 129-151 µm). Morphological characteristics are essentially the same as *O. tipulae* characterized by Lam and Webster (1971). No significant differences were observed in other characters among the Iranian specimens and those studied by Sudhaus (1993). Other minor differences among isolates may be expected due to geographical distribution and habitat.

Molecular analysis of O. tipulae

To confirm the morphological identification of the nematode isolate of *O. tipulae*, a selected sample was analyzed by molecular approaches. Complete sequence of the ITS and 18S were determined and deposited in the GenBank under the accession number JF920965 (Mashhad isolate)

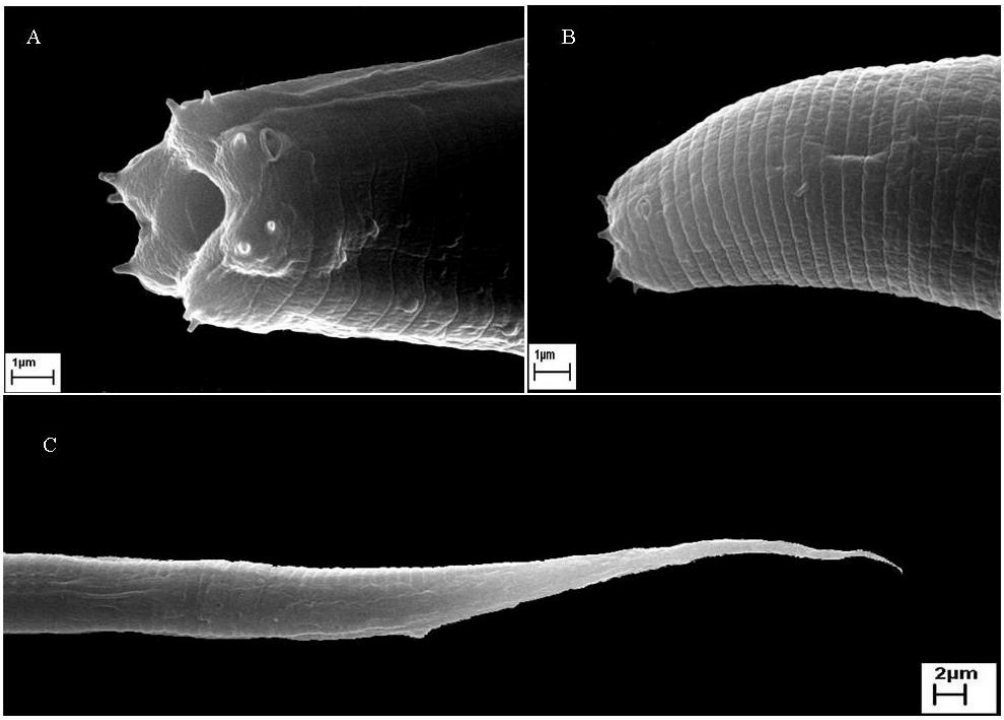

Fig.1. *Oscheius tipulae* Lam and Webster, 1971 (SEM): (A) Anterior end (Frontal view); (B) Anterior end (Lateral view); (C) Female posterior end.

and KM923756 (Mahalat isolate) for ITS and KM923757 for 18S (Mahalat isolate). The BLAST search through GenBank showed that the Iranian isolate has 99% identity with GA4, GT4 and F1 strains of *O. tipulae* with accession numbers KX036752, KT728763 and KT728764, respectively. Analysis of the ITS rDNA region, based on NJ method (Fig. 2) showed three main clades (I, II and III). Phylogenetic analyses of the ITS rDNA region using both Likelihood and distance-based approaches produced almost identical trees. Reconstructed phylogenetic trees placed the Iranian isolate of *O. tipulae* with other isolates of this species in clade I with 99% similarity. The high node support of distance tree was shown by other reconstructed phylogenetic trees using NJ and ML methods (data not showed). Mean interspecific distance of ITS sequences was 0.448% (range 0.00 to 0.999%), which was calculated by the K2P model.

nBLAST analysis based on 18S gene for Iranian population from Mahalat attributed 99% similarities with *Oscheius tipulae* isolate KS599 (HQ130502) and *Oscheius tipulae* strain CEW1 (EU196009). The reconstructed phylogenetic tree based on 18S sequence showed that *O. tipulae* formed a monophyletic group with *O. dolichura*, *Rhabditis* sp., and *Dolichura*-group. Those species, together with the *Oscheius,* were placed in a single clade (Fig. 3). Mean interspecific distance of 18S sequences was 0.156% (range 0.00 to 0.292%), which was calculated by the K2P model.

The results of digestion pattern indicated similar electrophoresis bands for all populations. We did not find any difference between the digested profiles of three populations. This was confirmed by *in silico* digestion and our data from digestion patterns were in line with the data gathered from analysis of ITS sequences with the Webcutter software. Morever, no morphological or morphometric differences were observed between the current population and the original data given by Lam and Webster (1971).

The genus *Oscheius* Andrássy, 1976 is synonymous with *Heterorhabditoides* Zhang *et al.*, 2008 and *Dolichorhabditis* Andrássy, 1983. According to Andrássy (2005), *Oscheius* is similar to *Amphidirhabditis* Andrássy, 1978, distinguished by metastom (bearing warts vs bearing spoon like teeth) and cheilostom (not sclerotized vs heavily sclerotized). On the other hand, *Oscheius* can be distinguished based on key morphological characteristic including a short buccal tube (Andrássy, 2005). *Oscheius* comprises 27 valid species divided into two groups "dloichura; including 13 species" and "insectivorus; including 14 species" (Sudhaus, 2011). The results by ML (18S rDNA) and NJ (ITS rDNA) places *O. tipulae* close to *O. dolichura* and *O. dolichuroides*. Concern to the morphology, all belong to the "dolichura group" by having peloderan bursa with three pre-cloacal papillae (Sudhaus, 2011).

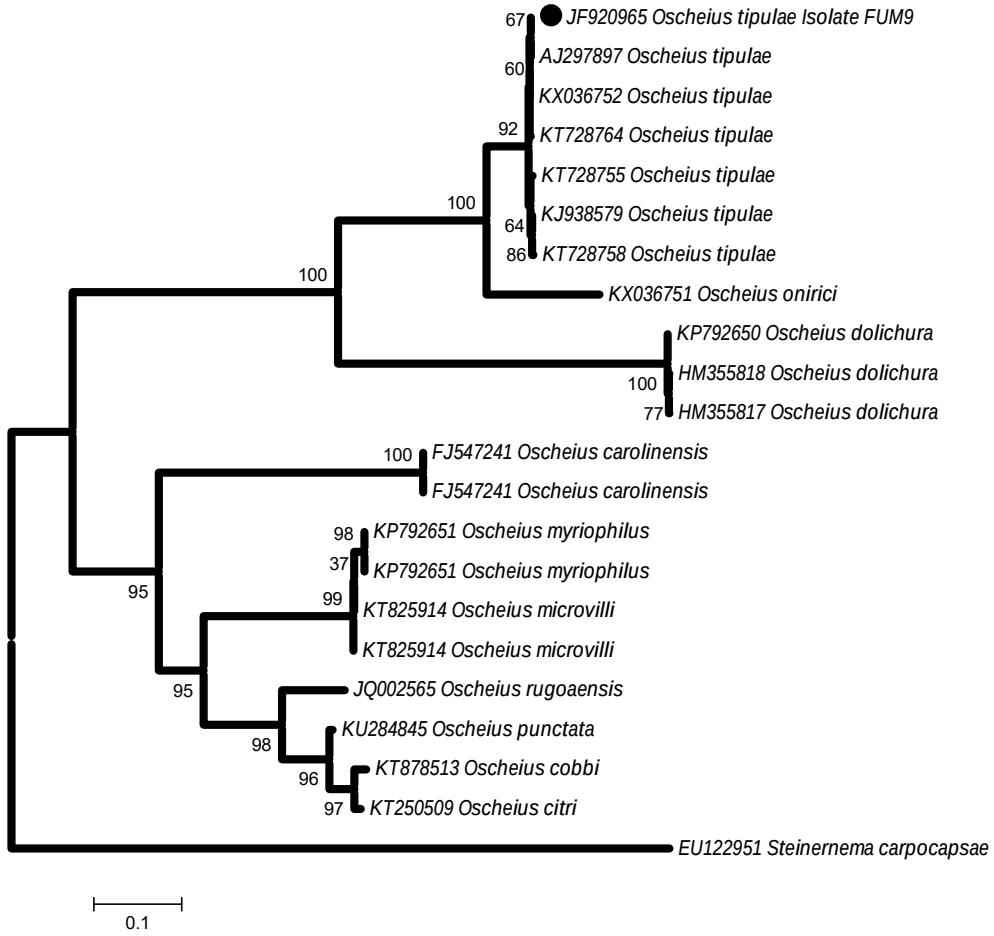

Fig. 2. Phylogenetic relationships of *Oscheius tipulae* and other closely related species reconstructed by Neighbor Joining analysis based on ITS rDNA sequences data.

However, males in *O. tipulae* are rare and not in the original description by Lam and Websrter, 1971. The general morphology in all three species is similar (e.g., stoma, pharynx, weakly developed median bulb, long rectum and female and male tail and genital system). On the other hand, metastom in *O. tipulae* has three denticles (Lam and Webster, 1971), *O. dolichuroides* has a single denticle, and *O. dolichura* bears two denticles (Andrássy, 2005). Pharynx lumen is zipper like in *O. dloichura* and *O. dolichuroides* (Sudhaus and Hooper, 1994), while not described in *O. tipulae* (Lam and Webster, 1971). Finally, more samples belonging to more species to compare against to confirm genus are needed to resolve the real relationship of these two groups of the genus *Oscheius*.

The increasingly used approach to morphological identification of nematodes is based on molecular data, mostly 18S, 28S, and ITS rDNA sequences (Felix *et al*., 2001). The traditional methods of nematode identification are often time consuming and challenging because of overlapping characters as well as problems of interpreting plesiomorphic states and convergent (homoplasious) features (Stock and Reid, 2003; Dorris *et al*., 1999; Stock, 2009; Kumari and Lišková, 2009). In addition, most of the characters that apply for classic identification are only good for diagnostic purposes without phylogenetic value (Dorris *et al*., 1999; Stock and Reid 2003). Therefore, a transition to molecular identification methods have particular value (Dorris *et al*., 1999; Kumari and Lišková, 2009).

While we considered *Oscheius* as a genus, Sudhaus (1993) considered it as subgenus of *Rhabditis* and redescribed *Rhabditis* (*Osceius*) *tipulae*. The original description of the species shows it was found in leatherjackets [larvae of *Tipula paludosa* (Diptera: Tipulidae)] by Lam and Webster (1971). We isolated this species in several orchards in Khorasan Razavi, Arak, and North Khorasan, Iran. In each province, several locations are surveyed for native EPN species.

To date, eight species of the family Rhabditidae has been reported from Iran (Shokoohi and Abolafia, 2011; Darsouei *et al*., 2014). All members (except *O. rugaoensis*) were isolated directly from soil; however, it seems extracting this

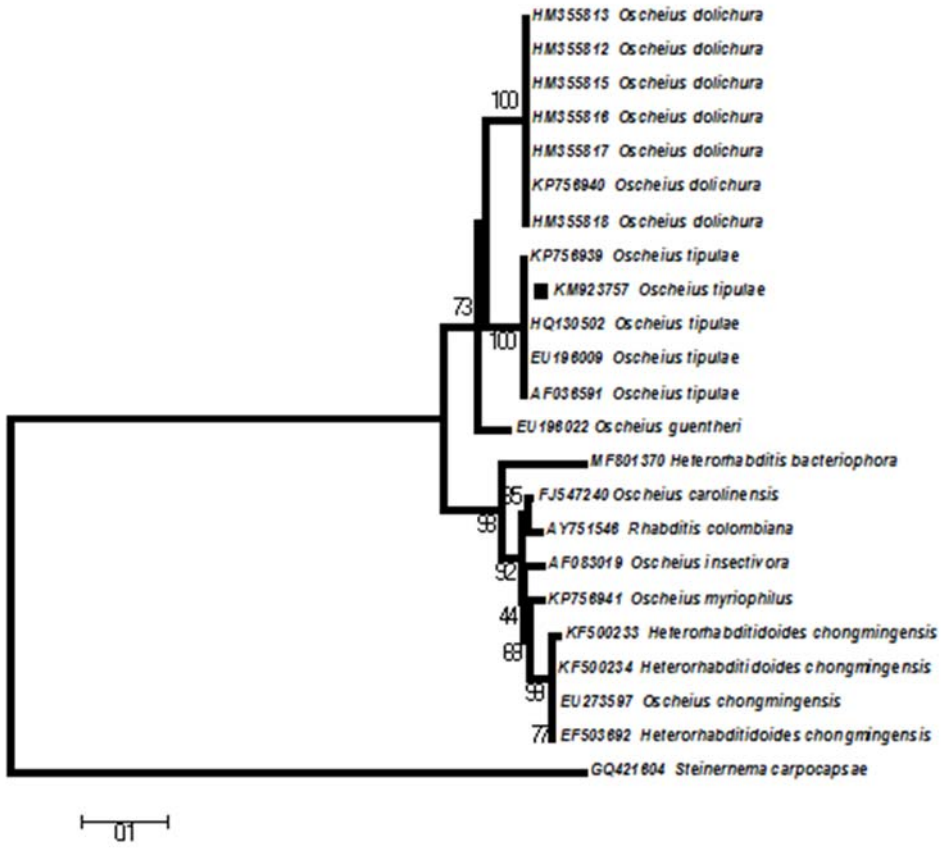

Fig. 3. Phylogenetic relationships of *Oscheius tipulae* and other closely related species reconstructed by Maximum likelihood analysis based on 18S rDNA sequences data.

nematode by *Galleria* baiting can open a new window to consider them as enotomophilic, and considering them as candidate biocontrol agents. Seven species of this genus including, *O. chongmingensis* (Zhang *et al*., 2008; Liu *et al*., 2012), *O. carolinesis* (Ye *et al*., 2009), *O. siddiqii* and *O. naizi* (Tabassum and Shahina, 2010), *O. amsacate* (Li *et al*., 2012), *O. rugaoensis* (Zhang *et al*., 2012; Darsouei *et al*., 2014) and *O. gingeri* (Pervez *et al*., 2013) were previously reported as entomophilic. Interestingly, our results about infectivity of *O. tipulae* against *G. mellonella* larvae and the corresponding data confirmed high pathogenicity of the nematode when assayed in petri dishes. Increasing IJ concentration directly correlated to the mortality of the tested larvae (Karimi, unpublished data).

Recently, other species have been shown to use bacteria to parasitize insect hosts. The *Oscheius chongmingensis* and *O. carolinensis* have been identified as insect pathogens by baiting soil for nematodes (Dillman *et al*., 2012), similar to the method which we used here. Our preliminary attempt indicated that the *O.tipulae* associated with insect pathogenic bacteria of the genus, *Serratia*. On the basis of Dillman *et al*. (2012) and Torini *et al*. (2015), we assigned the *O. tipulae* as an entomopathogenic nematode, due to its potential to reproduce within the the body of the *Galleria* and also its potency to cause the insect death in less than 48 hr, compared to longer time of those for phoretic, necromenic, and other types of associations.

During the last 15 yr, several species and isolates of insect pathogenic nematodes in the Steinernematidae and Heterorhabditidae have been recorded. Parasitic nematodes, including *Oscheius* and *Pristionchus*, are unknown in Iran. Iran has a rich fauna and flora biodiversity resource due to its diverse geographic, climatic, and weather conditions, which resulted in habitats with great diversity of herbivore insects and their natural

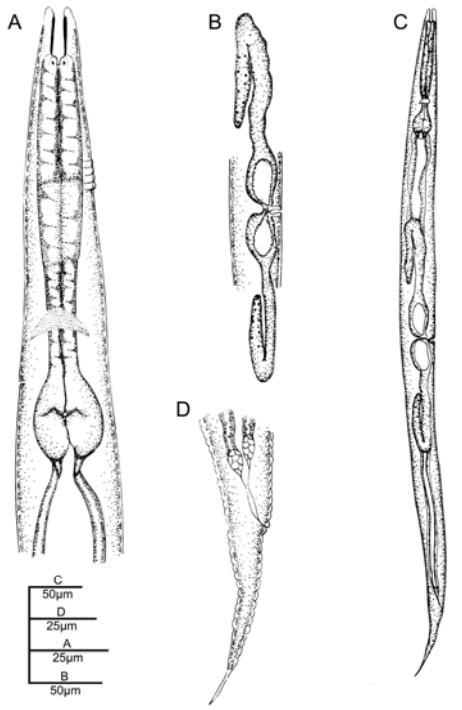

Fig. 4. *Oscheius tipulae* Lam and Webster, 1971. A: Female anterior end. B: Reproductive system. C: Entire female. D: Female posterior end.

enemies. It is assumed that there are diverse routes of relationships between these groups. Therefore, increasing our knowledge about species diversity of insect parasitic nematodes together with insect pathogenic nematodes provides vast opportunities for conducting either fundamental or applied research, ultimately leading to new insights for biological control programs.

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