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Molecular characterizations of the entomopathogenic nematodes, *Heterorhabditis bacteriophora* and *Oscheius myriophilus* from Iraq

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Abstract

A survey was conducted in three main regions of Iraq to isolate and identify entomopathogenic and insect parasitic nematodes. Soil samples were collected from 15 sites across 3 provinces from grassland, date palm, and citrus fields, and tested for the presence of nematodes. Out of 100 samples collected, 2 isolates of nematodes were recognized. The collected specimens were characterized first morphologically and then by molecular studies to characterize and identify the isolates based on ITS and 18S rDNA genes. Both isolates were recovered from soil samples of citrus and palm orchards located in 3 regions in the city of Baghdad. The isolates were identified by analyzing sequenced results and then by DNA sequences that were blasted against the NCBI database in GenBank. The 2 isolates were separated into 2 genera, assigned as IRQ.1 and were identified as *Heterorhabditis* and IRQ.2, that was identified as *Oscheius*. Molecular analysis and phylogenetic relationship of both genera were analyzed, using the DNA sequences of 2 loci. The *Heterorhabditis* species was assigned as *Heterorhabditis bacteriophora*, while the *Oscheius* species was identified as *Oscheius myriophilus*. These species were the first to be reported in Iraq.

Keywords: Survey, Entomopathogenic nematodes, *Heterorhabditis*, *Oscheius*, Molecular identification, Iraq

Background

Entomopathogenic nematodes (EPNs) from families Steinernematidae and Heterorhabdidae are lethal parasites of a wide range of insect pests worldwide. Species of EPNs have effectively been used as biological control agents to control and manage significant pests of commercial crops, especially soil-dwelling insect pests and plant-boring pests in some countries such as the USA and European countries (Hazir et al. 2004). The EPNs can kill target insects within 48 h and are considered as an obligate insect's parasite (Kaya and Gaugler 1993). *Photorhabdus* (Boemare 2002) and *Xenorhabdus* (Thomas and Poinar 1983) are symbiotically associated bacteria with *Heterorhabditis* and *Steinernema*, respectively. These nematodes could be used effectively to control insect pests, especially when these originated from

the same area of target pests as indigenous agents (Stock et al. 1999). These biocontrol programs have received huge attention in many countries globally, as these agents have special traits such as searching for the insect pests in cryptic habitats, massive reproductive ability, easy mass-production laboratory potential, safety to humans, and other vertebrates as well non-target insects (Akhurst and Smith 2002). More than 100 species of *Steinernema* and 16 species of *Heterorhabditis* have been recorded (Shapiro-Ilan et al. 2017). Some of these nematodes are produced commercially in some countries and have been used successfully as biological control agents. These include *Heterorhabditis bacteriophora*, *H. indica*, *H. marelata*, *H. megidis*, *H. zealandica*, *Steinernema carpocapsae*, *S. feltiae*, *S. glaseri*, *S. kushidai*, *S. kraussei*, *S. longicaudum*, *S. riobrave*, and *S.scapterisci* (Kaya et al. 2006; and Lacey et al. 2015).

Recently, the genus *Oscheius* was discovered, as a new member of EPNs (Torres-Barragan et al. 2011). Some *Oscheius* spp. have a symbiotic relationship with

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symbiotic bacteria. *Oscheius* species are parasitic and lethal to some insect pests and some are also effectually associated with *Serratia* bacteria (Lephoto et al. 2015; and Torrini et al. 2015). According to the criteria of EPNs, their entomopathogenicity has not yet been examined (Dillman et al. 2012). Darsouei et al. (2014) reported two new species, *Oscheius rugaoensis* and *Pristionchus maupasi*, isolated from the white grub larvae *Polyphylla adspersa*. These were the first reported of the *Oscheius* genus from Iran. Additionally, the *Oscheius* species were isolated from vermicompost in the USA. Preliminary experiments showed that these nematodes had the potential to infect and kill the two insect species, *Pieris rapae* and *Tenebrio molitor*, under laboratory conditions. This potentially makes these species viable biological control agents (Ye et al. 2010). Furthermore, *Oscheius microvilli* was recorded in Chongming Island, China, and showed a high level of effectiveness (Zhou et al. 2017).

Several studies have been carried out in the Middle East, but there is no available data about the diversity and activity of EPNs from Iraq. Therefore, the aims of this study were to survey, isolate, and identify indigenous EPNs that present in the Iraqi habitats.

Materials and methods

Soil sampling and isolation of entomopathogenic nematodes

Soil samples were collected from different sites of grassland, citrus, and date palms in three provinces of Baghdad, Iraq. Sub-samples were taken from a depth of up to 20 cm, using a hand spade, from underneath the canopy of the trees. The insect-baiting technique of (Bedding and Akhurst 1975), using the last instar larvae of the greater wax moth, *Galleria mellonella* L., was used. The soil samples were transferred into plastic containers (300 ml) with lids. A 7–10 last instar larvae were used for each container, 7 to 10 replicates were used for each soil sample. Then, these containers with the larvae were placed under lab conditions for 5–7 days. To encourage movement of potential nematodes in the soil, every day the containers were shaken and appropriate amount of sterilized water were added to avoid drying of the soil. The containers were checked every 3–4 days to remove infected larvae with EPNs. The infected larvae, based on color and shape, were transferred to the white trap individually (White 1927). Infective juveniles (IJs) emerged from the cadavers after 8–10 days (Kaya and Stock 1997). To sum up, the emerged IJs were maintained and stored inside a falcon tube (15 ml) that had sterilized pieces of sponges at 8–10 °C. To confirm the pathogenicity of collected nematodes, again last instar larvae of *G. mellonella* were inoculated by 100 emerged IJs/larva to observe same previous symptoms (Koch's postulates) (Kaya and Stock 1997).

Molecular characterization and phylogeny

DNA extraction

The nematodes' DNA, as a single adult, was extracted using 5% Chelex® 100 solution (SIGMA, Bio-Rad Laboratories, Inc., USA) along with a tissue DNA extraction kit (Parstous Biotechnology, Mashhad, Iran). Individual nematodes were collected and transferred into a 1.5-ml micro-centrifuge tube. The sample was crushed, using a micro-pestle and subsequently 20 µl of Chelex and 1 µl of Proteinase K (Parstous, Iran) (www.parstous.com) were added. The tubes were then incubated at 64 °C for 3 h, followed by 10 min at 95 °C onto a hotplate (thermo-block) for heating; then they were centrifuged at 13,000 rpm for 3 min. Finally, the supernatants were transferred to a 0.1-ml micro-tube and stored at – 20 °C for use in the next step. In addition to this method, the Tissue DNA extraction kit was used for extraction of the DNA by following the procedure and protocol given by the manufacturer.

Amplification of ITS and 18S genes

For internal transcribed spacer (ITS) region amplification, the primer set of TW81 and AB28 (Joyce et al. 1994) was used. The PCR solution had a final volume of 25 µl comprising of 6.25 µl sterilized water, 12.5 µl 2X Taq PreMix, 1 µl of each primer, 1.25 µl dimethyl sulfoxide, and 3 µl genomic DNA. The PCR reactions were conducted in a thermo-cycle (A300 Fast Thermal Cycler, Hangzhou Longgene Scientific Instruments Co., Ltd. China). The PCR run consisted of initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min; and a final extension at 72 °C for 10 min. Finally, the PCR product was electrophoresed for 40 min on 1% agarose gels with 10X TBE buffer 5% and green-viewer (SYBR) was added to stain the gel. Finally, 2.5 µl of DNA ladder and 3 µl of the PCR product were loaded into each well of the gel. The size of amplified products was identified by comparing to 100-bp molecular DNA ladder (Parstous, Iran).

DNA sequencing and analysis

The PCR products of both strains with replicates were sent to Macrogen Co. (Korea) for sequencing. Then, the quality of chromatograms was checked and created the consensus sequences by using a DNA Baser Assembler (DNA Sequence Assembler v4 (2013), HeracleBioSoft, www.DnaBaser.com). Then, the DNA sequence was blasted against the NCBI database.

Phylogenetic analysis

Molecular analysis of ITS

The evolutionary analysis of the genus *Heterorhabditis* isolate included nucleotide sequences from 30 valid and verified DNA sequences along with our sequence carried

out, using the MEGA 7 software program (Kumar et al. 2016). The evolutionary history was inferred by using a maximum likelihood method, based on the K2 + G (Kimura 1980). For *Heterorhabditis*, DNA sequences of *Caenorhabditis elegans* was used as an outgroup taxon (accession number EU131007) (Spiridonov et al. 2004). Branch support was determined by bootstrap analysis with 1100 replicates (Nguyen and Hunt 2007). Also, this program was performed for phylogenetic analyses, using the neighbor-joining method (Saitou and Nei 1987) with 10,000 replications of bootstrap (Felsenstein 1985). Then, the nucleotide distance was calculated by Clustal W and Clustal X version 2 (Larkin et al. 2007).

Molecular analysis of 18S

The 18S region was amplified, using 26R and 28A primers (Nguyen and Hunt 2007). The previous procedures were followed for electrophoresis, sequencing, and analysis the PCR products. For phylogenetic analysis of the *Heterorhabditis* isolate, 27 valid sequences of the 18S gene from species of the Heterorhabditidae family were retrieved from GenBank (NCBI) and analyzed together with the sequences of the current study. Clustal X (ver. 2) was used to align the sequences. For calculation of nucleotide distance and phylogenetic analyses, the MEGA.7 program was used.

Results and discussion

The studied isolates were identified and characterized based on both ITS and 18S genes of the rDNA. Based on color and shape, the infected larvae were sorted. Then, the molecular diagnostic method was conducted for all isolated species from all soil samples. After analysis, the sequences date and DNA sequences were blasted against the NCBI database in GenBank, all isolated species were found belonged to *Heterorhabditis* or *Oscheius*.

Analysis using ITS sequence for *Heterorhabditis* species

The length of the ITS gene for the IRQ isolate was 825 bp. The BLAST analysis on the basis of the ITS gene for this population attributes to 99% of similarities and 96% of query coverage with *H. bacteriophora* (EF469774). The multiple alignments of a 799 bps segment of ITS gene for 30 taxa showed that 221 sites were conserved, 553 sites were variable, 239 sites were singleton and 309 sites were parsimony informative. The phylogenetic tree reconstructed based on ITS sequences, using neighbor-joining analysis showed that the IRQ.1 isolate forms a monophyletic group with other *Heterorhabditis bacteriophora* isolates (Fig. 1). The mean inter-specific distance of ITS sequences was 0.027% (range 0.00–0.403), which was calculated by the Tamura 3-parameter model. There was a 0.002% difference between the Iraqi

isolates and *Heterorhabditis bacteriophora* (EF469774) (Table 1).

Analysis of the 18S sequence of *Heterorhabditis* species

BLAST analysis, based on the 18S gene for IRQ.1 isolate, showed that the similarity was 99% and the query coverage was 96% with *H. bacteriophora* (EF469774). The length of the 18S gene for this isolate was 863 bp. In addition, the multiple alignments of a 737 bps segment of 18S region for 28 taxa, illustrated that 283 sites were conserved, 390 sites were variable, 314 sites were singleton and 75 sites were parsimony informative. The obtained results of the 18S regions were similar to those that resulted from the ITS gene. In the phylogenetic analysis, IRQ.1 isolate of *H. bacteriophora* was placed in the same clade with isolates of *Heterorhabditis*. This was supported with a 100% bootstrap support value (Fig. 2).

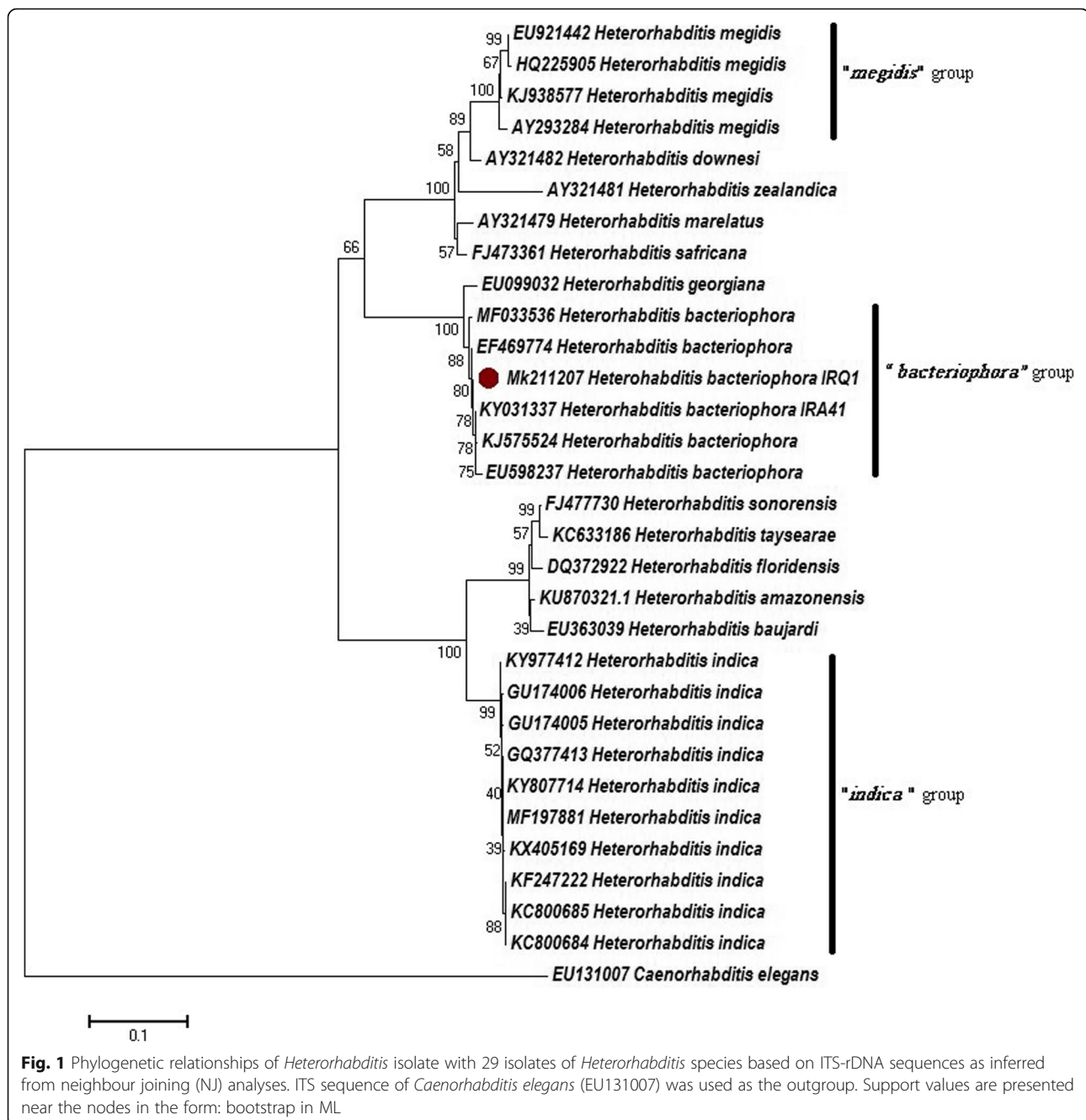
The mean inter-specific distance among *H. bacteriophora* IRQ.1 isolate and other isolates of *H. bacteriophora* were 0.095% (range 0.00–0.97%), which were calculated from the 18S gene using the Tamura 3-parameter model. The nucleotide distance between the Iraqi population of *H. bacteriophora* and *H. bacteriophora* (EF469774) was 0.002% (Table 2).

Analysis of ITS region for *Oscheius* species

The length of the ITS gene for IRQ.2 isolate was 855 bp. Then BLAST analysis of the ITS gene for the Iraqi population attributes to 98% similarities and 95% of query coverage with the ITS sequences of *Oscheius myriophilus* (MG551691). The phylogenetic tree based on ITS showed that *O. myriophilus* IRQ.2 forms a monophyletic group with other *O. myriophilus* isolates, and this group of *Oscheius* was placed in a single clade with other isolates of *O. myriophilus* (Fig. 3). Mean inter-specific distance of the ITS sequences was 0.59% (range 0.00–1.06). There was a 0.161% difference between the Iraqi isolate and *O. myriophilus* (KP792651).

Analysis of 18S region for *Oscheius* species

The length of the 18S gene for IRQ isolate was 911 bps and the BLAST analysis based on this gene attributes to 98% similarities and 95% of query coverage that identity with the 18S sequences of *O. myriophilus* (MG551691.1). For reconstructing the 18S tree, the multiple alignments of a 709 bps segment of 18S gene for 32 taxa and an outgroup taxon (*Steinernema carpocapsae*, accession number: KJ950291) were used. The result showed that 378 sites were conserved, 321 sites were variable, 48 sites were singleton, and 273 sites were parsimony informative. The phylogenetic tree reconstructed based on 18S sequences, using neighbor-joining analysis showed *Oscheius* IRQ.2 forms a monophyletic group with other isolates of *O.*



myriophilus. Figure 4 shows that the bootstrap value of support for the corresponding clade countering *Oscheius* IRQ.2 and other isolates was 99%. Mean inter-specific distance of the 18S sequences was 0.151% (range 0.00–0.27), which was calculated by the Tamura 3-parameter model. There was a 0.005% difference between the Iraqi isolate and *O. myriophilus* (MG551691).

Distribution of EPN species and isolates in different areas could exhibit a significance in behavioral and

physiological adaptation. Therefore, it is crucial for the successful use of EPNs, as biological control agents, to identify and document the locally adapted species or isolates of a particular region (Stock et al. 1999). Therefore, it is important to find an accurate identification method to understand the geographical distribution, behavior, and biodiversity of the *Heterorhabditis* species (Homnick 2002).

Recovery of the species of the *Heterorhabditis* and *Oscheius* genus from Baghdad suggests a higher species

Table 1 Pairwise comparison on the number of nucleotide differences among some of *Heterorhabditis* species and isolates with *Heterorhabditis bacteriophora* isolate IRQ.1 based on ITS rDNA sequences

<i>Heterorhabditis</i> species	1	2	3	4	5	6	7
1 MK21446 <i>Heterorhabditis bacteriophora</i> IRQ1							
2 KY031337 <i>Heterorhabditis bacteriophora</i>	0.002						
3 EF469774 <i>Heterorhabditis bacteriophora</i>	0.002	0.003					
4 KJ575524 <i>Heterorhabditis bacteriophora</i>	0.003	0.002	0.005				
5 MF033536 <i>Heterorhabditis bacteriophora</i>	0.006	0.008	0.005	0.009			
6 EU598237 <i>Heterorhabditis bacteriophora</i>	0.006	0.005	0.008	0.006	0.012		
7 MF197881 <i>Heterorhabditis indica</i>	0.309	0.311	0.306	0.314	0.309	0.319	
8 KY977412 <i>Heterorhabditis indica</i>	0.311	0.314	0.309	0.316	0.307	0.321	0.002

diversity in Iraq and that needs further research and extensive sampling. The potential of some tentative endemic entomopathogenic forms in controlling endemic agricultural pests is another issue and could be tested, using endemic entomopathogenic strains. In the present study, the species *O. myriophilus* was characterized by

its molecular data; however, it was successfully characterized by molecular sequences of two genomic fragments (ITS and 18S genes). The usefulness of molecular data to distinguish these species from its closely related forms has already been documented (Campos-Herrera et al. 2015).

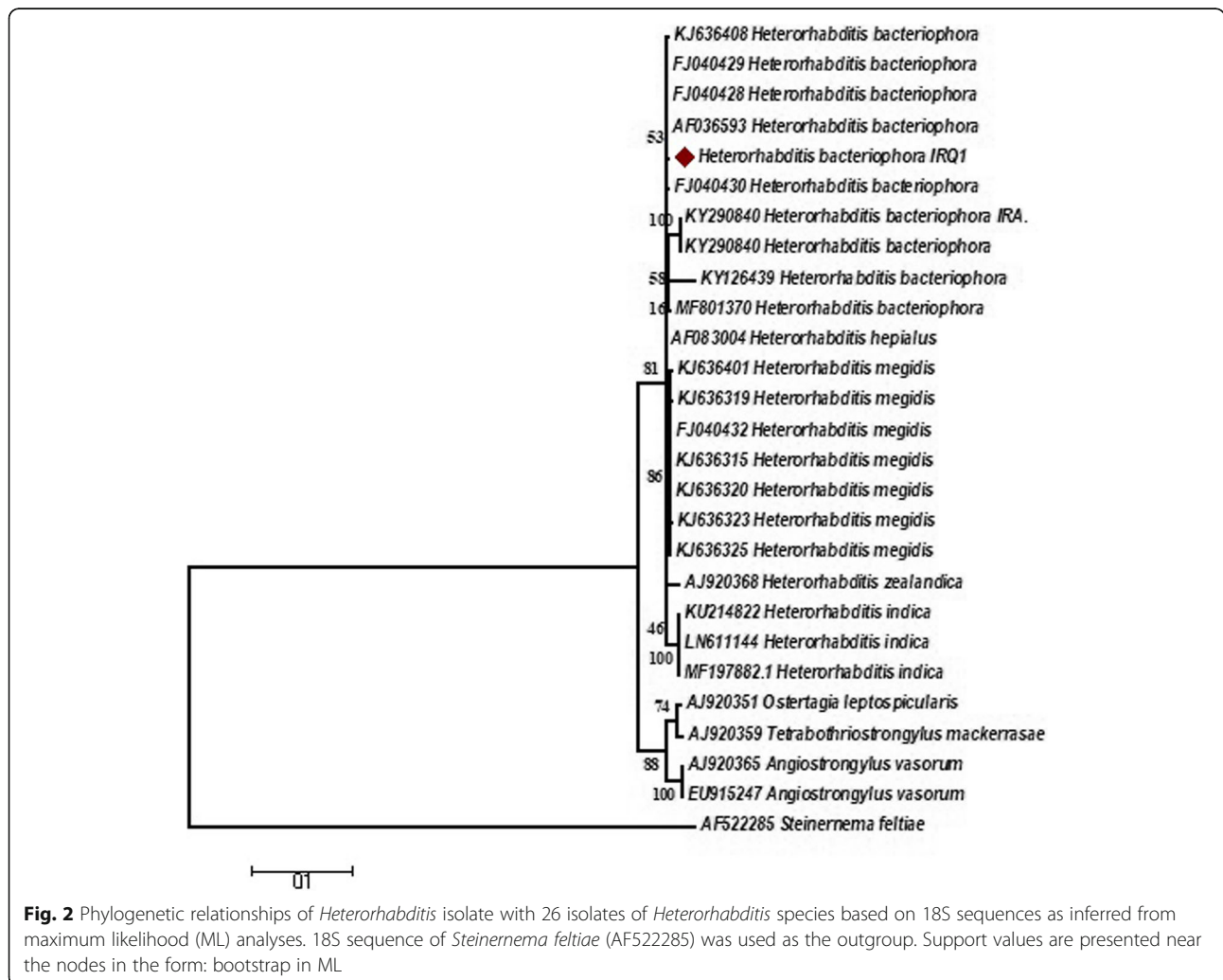


Fig. 2 Phylogenetic relationships of *Heterorhabditis* isolate with 26 isolates of *Heterorhabditis* species based on 18S sequences as inferred from maximum likelihood (ML) analyses. 18S sequence of *Steinerinema feltiae* (AF522285) was used as the outgroup. Support values are presented near the nodes in the form: bootstrap in ML

Table 2 Pairwise comparison on the number of nucleotide differences among some of *Heterorhabditis* species and isolates with *Heterorhabditis bacteriophora* isolate IRQ.1 based on 18S sequences

Heterorhabditis species	1	2	3	4	5	6	7	8	9
1 <i>Heterorhabditis bacteriophora</i> IRQ.1									
2 KY290840 <i>Heterorhabditis bacteriophora</i>	0.014								
3 AF036593 <i>Heterorhabditis bacteriophora</i>	0.002	0.012							
4 KJ636408 <i>Heterorhabditis bacteriophora</i>	0.003	0.014	0.002						
5 KY126439 <i>Heterorhabditis bacteriophora</i>	0.029	0.034	0.028	0.029					
6 KY290840 <i>Heterorhabditis bacteriophora</i>	0.014	0.000	0.012	0.014	0.034				
7 MF801370 <i>Heterorhabditis bacteriophora</i>	0.005	0.014	0.003	0.005	0.028	0.014			
8 AF083004 <i>Heterorhabditis hepialus</i>	0.003	0.014	0.002	0.003	0.029	0.014	0.005		
9 KJ636315 <i>Heterorhabditis megidis</i>	0.008	0.019	0.006	0.008	0.034	0.019	0.009	0.005	
10 KU214822 <i>Heterorhabditis indica</i>	0.017	0.028	0.015	0.017	0.044	0.028	0.019	0.014	0.961

Obviously, phylogenetic analysis of the 18S rDNA and ITS rDNA sequence data to infer the relationships of *Osccheius* with other rhabditids, it was found that *H. chongmingensis* was in a monophyletic clade with 100% support together with *O. colombianus*, *O. insectivorus*, and *O. myriophilus* (Zhang et al. 2008).

H. bacteriophora is one of the most widely distributed EPNs in the world. (Adams et al. 2006; Hominick et al. 1996). This species was the most common and widely distributed EPNs in the north-west of Iran, followed by *S. feltiae* (Kary et al. 2009). The same species was isolated from neighboring countries such

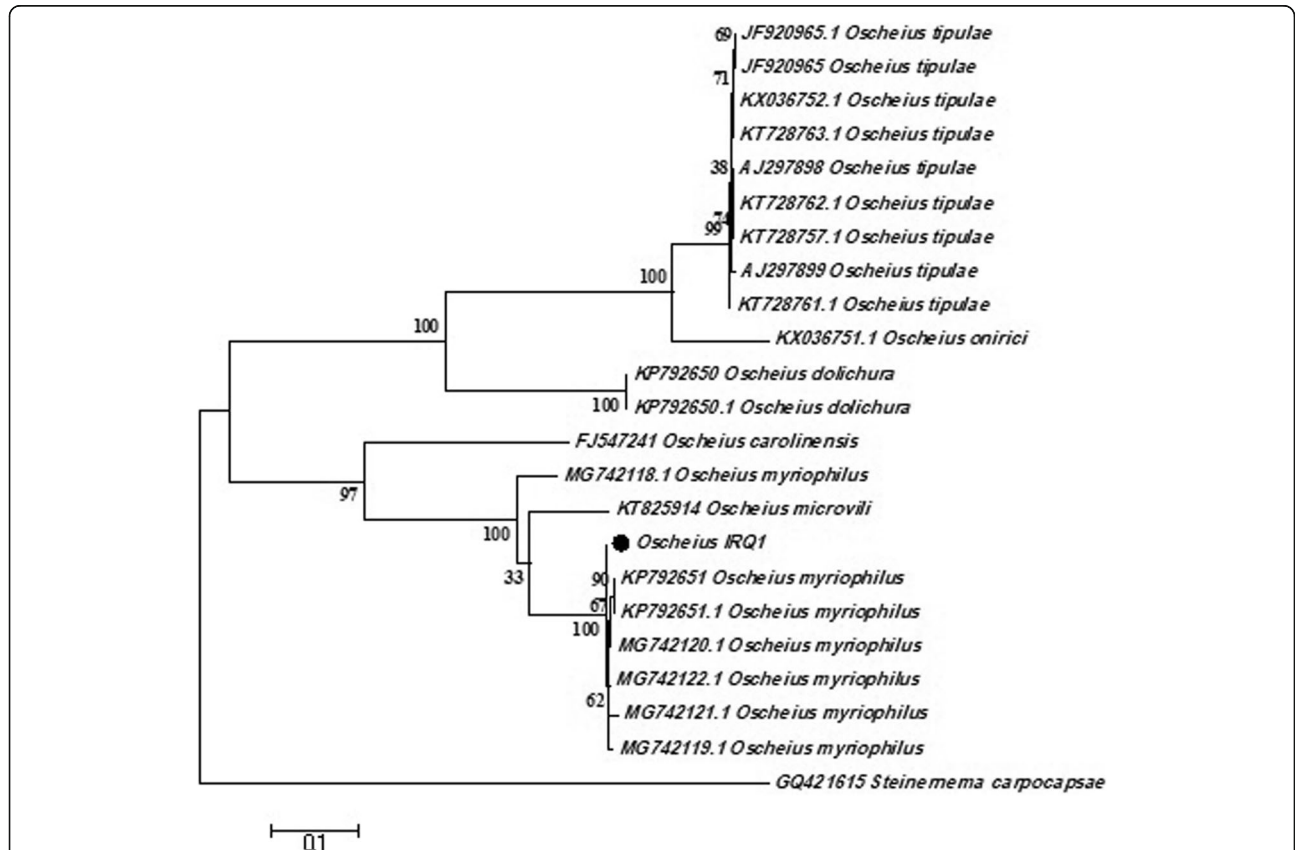


Fig. 3 Phylogenetic relationships of *Osccheius* isolate with 28 taxa based on ITS-rDNA sequences as inferred from maximum likelihood (ML) analyses. *Steinemema carpocapsae* (GQ421615) was used as the outgroup. Support values are presented near the nodes in the form: bootstrap in ML

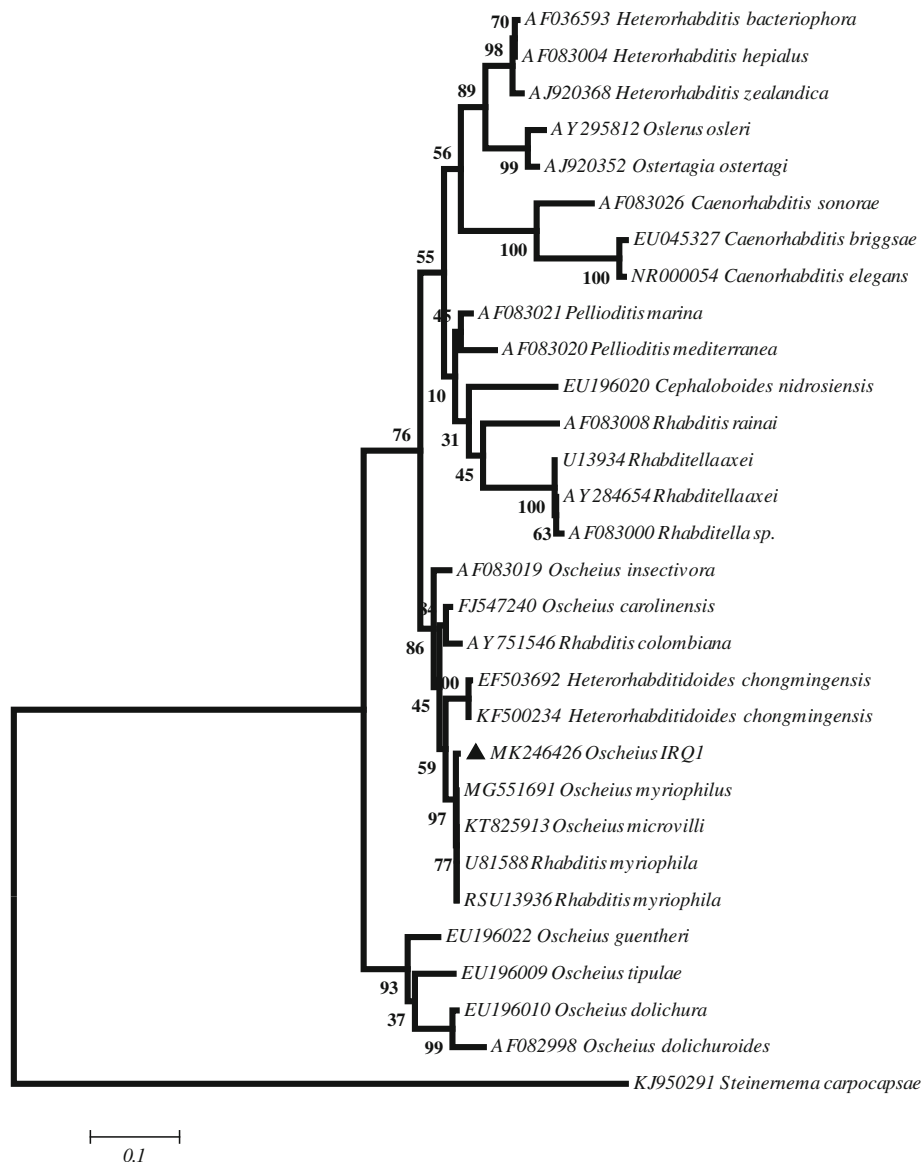


Fig. 4 Phylogenetic relationships of *Oscheius* isolates with 32 taxa based on 18S sequences as inferred from maximum likelihood (ML) analyses. *Steinernema carpocapsae* (accession number: KJ950291) was used as the outgroup. Support values are presented near the nodes in the form: bootstrap in ML

as Iran and the Mediterranean region (Karimi and Salari 2015). *Heterorhabditis* appears to be the dominant EPN genus in the Middle Eastern countries (Glazer et al. 1991; Iraki et al. 2000; Salama and Abd-Elgawad 2001).

Molecular characterization of nematodes is typically based on sequence data from the internal ITS gene (Reid et al. 1997). Molecular analysis was performed on females because their large size provided an appropriate amount of DNA. Sequencing of the nucleotides of organisms enables the use of the sequence differences among EPN isolates for identification and affirmation of

new species (Szalanski et al. 2000; Spiridonov et al. 2004). The applications of nucleotide sequence data together with evolutionary species concepts are very supportive in the discovery and unfolding of new species (Spiridonov et al. 2004).

In the present study, the phylogenetic analysis based on the ITS gene showed a close relationship between *H. bacteriophora* and *H. georgiana*, which is similar to the results of Nguyen et al. (2008). Furthermore, in one clade, there is one isolate of *H. georgiana* placed within the isolates of *H. bacteriophora* (Malan et al. 2014; Karimi and Salari 2015).

Conclusion

This is the first documented record of two species of nematodes isolated from the middle part of Iraq, and these are *Heterorhabditis bacteriophora* and *Oscheius myriophilus*. The presence of *H. bacteriophora* isolate in a dry hot habitat like the middle of Iraq is a significant contribution to the biogeography of these species, and this may lead to use EPNs in the biological control of insect pests of tropical regions.

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Availability of data and materials

All data are available in the GenBank.

Authors' contributions

JK and JBZ designed the work. JBZ collected the samples and did the experiments. JBZ and JK analyzed the data. JBZ, JK, and EMM were involved in the MS preparation and revised the paper. All authors read and approved the final manuscript.

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JBZ is a researcher from Ministry of Higher Education and Scientific Research which conduct his PhD in FUM. JK is faculty of FUM, which involves teaching and researching of insect pathology. EMM is nematologist at FUM.

Ethics approval and consent to participate

NA

Consent for publication

This is to state that I give permission for the publication.

Competing interests

The authors declare that they have no competing interests.

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