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MORPHOLOGICAL AND MOLECULAR DESCRIPTION OF NEW RECORD OF *METARHABDITIS RAINAI* (NEMATODA: RHABDITIDAE) FROM BAGHDAD

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ABSTRACT

Aim of this study was to identify and characterize the entomopathogenic nematode *Metarhabditis rainai,* which was isolated from soil samples carried out from Alrashdya at (33˚25ˈ13.4" N 44˚21ˈ45.3" E) Baghdad- Iraq and was illustrated by both morphological and molecular aspects. All specimens of *M. rainai* were cultured, identified and described using some morphometric criteria based on standard available key. Selected specimens (Zah, IQ2 OR960470 isolate) of this species were characterized by the body length of male was ranged from (377.49 – 563.89 µm), the body length of female was (857.44 – 1111.7 µm) and the body length of juvenile was (419.28 – 757 µm). Using partial 18S rRNA gene sequences, molecular characterization was performed on selected specimens of this species. The 18S-rRNA sequence of the Zah, IQ2 OR960470 isolate showed a sequence homology ranging from (88.50% to 100%) with the 18S rRNA sequence of *M. rainai* found in the NCBI database. The phylogenetic tree has been created to separate *M. rainai* species from closely related genera and species. *Metarhabditis rainai* Zah, IQ2 OR960470 isolate marks the first record of *M. rainai* in Iraq.

Keywords: Entomopathogenic nematode, Iraq, Morphometric, Soil, 18S rRNA gene.
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INTRODUCTION

Nematodes are small-bodied metazoans that are widely distributed and can be found in all types of habitats, including farmed fields, woodlands, and prairies (Fonseca *et al*., 2010). It is estimated that their species richness globally will exceed one million (Lambshead, 2004). Among the different suborders of nematode, the suborder Rhabditina represents a large and important suborder of free-living nematodes. It is composed of species that inhabit a wide range of environments. Despite their predisposition for terrestrial habitats, certain individuals have been found in freshwater or semiaquatic environments. They can survive and procreate in these environments, filling a range of ecological niches (Timm, 1971; Loof, 1973; Gagarin, 2000; Traunspurger, 2000; Wanless and Hunter, 2001; Heyns, 2002) and contributing significantly to the cycling of nutrients. For this reason, they are highly valuable as biological indicators of the environmental condition of lakes and rivers. On the other hand, the species composition of aquatic environments is not dominated by this group (Eyualem-Abebe *et al*., 2001; Beier and Traunspurger, 2003). Some genera belong to this suborder are bacteriophagous which exploit bacterial colonies in animal feces, on carcasses, in frass of wood-inhabiting arthropods, or in dead and decaying matter

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(Trejo-Meléndez *et al*., 2024). Species of *Metarhabditis* and *Oscheius* are considered to be necromantic associates of insects and feed on bacteria decomposing the carcass of the dead insect. Nonetheless, recent research indicates that both genera might be facultative entomopathogens and could serve as biocontrol agents (Ye *et al*., 2010; Dillman *et al*., 2012; Asif, 2013). *Metarhabditis rainai*, first described by Carta & Osbrink, 2005 syn *Rhabditis rainai* Carta & Osbrink, 2005, is one of the entomopathogenic soil nematodes that is widely found in the soil of many different parts of the world. *M. rainai* was initially discovered in Brazil in 2013 after being isolated from soybean crop soil. It had previously been discovered in the stomach and heads of termites in the southern United States (Tomazini *et al*., 2013). This species has been the subject of molecular identification studies to accurately classify entomopathogenic nematodes (Tomazini *et al*., 2013; Bhat *et al*., 2020). The molecular characterization of *M. rainai* has been based on 18S and ITS rDNA gene sequencing, which has allowed for the identification of this species and its potential as a biopesticide. In addition, molecular identification studies have been conducted in various locations such as the USA and Brazil to understand the diversity and distribution of this nematode species (De Brida *et al*., 2017; Bhat *et al*., 2020). Furthermore, integrative taxonomy approaches have been used to associate *M. rainai* with parasitic interactions, highlighting the importance of accurate species identification in studying nematode ecology (Asif *et al*., 2013). *M. rainai* has also been compared to other entomopathogenic nematodes such as *Heterorhabditis indica* and *Oscheius insectivora* through sequence analysis of the 28S rDNA D2D3 segments, revealing high sequence similarity among these species (Bhat *et al*., 2021). Overall, the molecular identification and characterization of *Metarhabditis rainai* have provided valuable insights into the taxonomy, ecology, and potential applications of this nematode species in pest management strategies. Further research in this area is essential to fully understand the role of *M. rainai* in agricultural ecosystems and its interactions with other nematode species.

MATERIALS AND METHODS

Samples collection: Soil samples were collected from three crop fields in Alrashdya (33˚25ˈ13.4" N 44˚21ˈ45.3" E), Aljadrya (33˚16ˈ35.7" N 44˚23ˈ26.3" E), and Abo-Ghareeb (33˚19ˈ15.5" N 44˚11ˈ57.1" E), in total 54 samples were collected within two seasons: Spring (April to May 2023) and Summer (July 2023). Each soil sample consisted of 5-7 subsamples, which were randomly collected around each plant in a square pattern with a hand spade at a depth of 15-20 cm. Sub-samples were included in a plastic bag, which was closed tightly to avoid drying out the contents. The samples were labeled and kept out of direct sunlight, then stored at 8-10 ◦C in a cooler container until sent to the laboratory for extraction and assessment of nematode presence. From various parts of each site, three samples were collected. The weight of every soil sample was between 1.5 and 2 kg. For the purpose of collecting nematodes, three replicates were obtained from each homogenized sub-sample.

Nematodes isolation: Nematodes were extracted from 250 grams of soil using the Baermann funnel technique (Morise *et al*., 2012). From each replicate, 10 ml water suspensions were gathered and specifically screened in a randomly selected 1 ml portion. Nematodes were isolated using a dissecting microscope.

Nematodes cultivation and morphological measurements: Into a Petri dish (9 cm in diameter) along with two filter paper pieces, last stage larvae of *Galleria mellonella* were embedded and killed using a sterilized lancet. The nematodes that had been isolated were put forward to the Petri dish, which was maintained at room temperature (22 ± 2 ºC) for a duration of 5 to 7 days. The reproduced nematodes were collected and transferred to eppendorf tube (1.5 ml) and were kept in a fridge at 8-10 ºC. The morphological identification of preserved specimens (adults and juvenile) was completed using an ocular micrometer (4X, 10X & 40X) in accordance with the standard keys (Stock and Hunt, 2005; Nguyen and Hunt, 2007) after they were fixed

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using different fixation methods (Al-Zaidawi *et al*., 2019). The body parts and other morphological characteristics of the specimens were observed, measured and photographed by camera (opto-Edu image view-2021). The following measurements were considered to described the isolated nematodes: (L) Body length, (a) body length divided by maximum body width, (b) body length divided by oesophageal length, (c) body length divided by tail length, (c') tail length divided by body width at anus, (V%) position of vulva from anterior end as a percentage of body length, (EP) distance from anterior end to excretory pore, (NR) distance from anterior end to nerve ring, (ES) distance from anterior end to end of pharynx, (T) Tail length, (ABD) anal body diameter, (D%) distance from anterior end to excretory pore as a percentage of distance from anterior end to end of pharynx, (E%) distance from anterior end to excretory pore as a percentage of tail length (Nguyen and Hunt, 2007).

Scanning electron microscopy: Morphological features of adults were examined using scanning electron microscopy (SEM). The specimens (adults) were rinsed three times with distilled water for examination. Subsequently, they were affixed to aluminum SEM stubs and coated with gold nanoparticles, plasma spattering coater (China) was used and studied using an inspect f 50 scanning electron microscope (FEI Company, Holland).

DNA extraction, amplification and electrophoresis: For the extraction of total genomic DNA, approximately 50 grams of cultured nematodes was utilized. The extraction process followed the guidelines provided by the manufacturer and used a gSYNC ™ total DNA Extraction kit (Geneaid, Taiwan). A thermal cycler was used to amplify segments of 18S region. The primer set of 26R (5`-CAT TCT TGG CAA ATG CTT TCG-3`) and G18S4 (5`-GCT TGT CTC AAA GAT TAA GCC-3`) was used followed (Trejo-Meléndez *et al*., 2024). The PCR profile for all loci comprised thirty amplification cycles in an eppendorf thermocycler, following a program of 94 °C for 4 minutes for initial denaturation, 35 cycles of 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 2 minutes, with a final extension at 72 °C for 10 minutes. After that, the PCR product was subjected to electrophoresis on 1% agarose gels for 40 minutes with a 10X TBE buffer solution at a concentration of 5%. The gel was then stained using green-viewer (SYBR). At the final step, 3 µl of the PCR product and 2.5 µl of DNA ladder were introduced into each gel well. The size of the amplified products was determined using a 100-bp molecular DNA ladder (Bioneer, Korea).

DNA sequencing and analysis: The PCR products were sent to Macrogen Co. in Korea for sequencing. Subsequently, the quality of the chromatogram was evaluated, and consensus sequences were produced with a DNA Baser Assembler (DNA Sequence Assembler v4 (2013), Heracle Bio Soft, www.DnaBaser.com). Every sequence was subjected to homology searches using the NCBI Blast tool (http://www.ncbi.nlm.nih.gov/).

The phylogenetic analyses and nucleotide distance were calculated using the MEGA.7 program. In addition to this, the evolutionary history was applied using Maximum Likelihood approach based on Kimura 2-parameter model and the phylogenetic tree with highest log likelihood (-3494.49) was documented, the tree percentage for associated taxa clustered for each other also shown next the branch (Kimura, 1980). Additionally, the Neighbor-Join and BioNJ algorithms were automatically applied to a matrix of pairwise distances determined using the Maximum Composite Likelihood (MCL) technique to create the initial tree (s) for the heuristic search, then the topology with the superior log likelihood value was chosen. Using the number of substitutions per site as a measure for branch lengths, the tree is drawn to scale. The analysis included 24 nucleotide sequences, as well as the local isolate and *Caenorhabditis elegans* as an outgroup. The codon positions included first, second, third, and noncoding. Any position that had missing information or gaps was eliminated. There was a total of 743 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

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RESULTS AND DISCUSSION

Among the nematode specimens collected from soil samples (Baghdad, Iraq). *Metarhabditis rainai*, were cultured and identified based on molecular technique and morphological parameters. Morphological examinations of selected individuals (n=21) showed that all identified *M. rainai* in this study exhibited the most characteristic features of *M. rainai*. The specimens of adult *M. rainai* (Figure 1) had the following characteristic features: closed lips that are either slightly apart or closed. Metastegostom isoglottoid stoma with fine warts that are rarely mildly developed. Pairs of female gonads; vulva close to the midbody. Both sexes have conical tails, while females occasionally have cupola-shaped tails. Bursa leptoderan, which can be either open or closed, and infrequently pseudopeloderan, with a short, extremely fine tail filament that is free. Spicules separate or distantly fused. All morphological measurements are illustrated in Table (1) & Fig. (2). (**male**): N=8, (L) the body length ranged from (377.49 – 563.89 µm), a = (19.14 – 23.42), b = (3.62 – 4.48), c = (6.32 – 11.2), c' = (3.69 – 6.29), the maximum body width ranged from (18.95 – 25.19 µm), EP (321.59 – 509.17), NR = (84.33 – 104.05), ES = (99.81 – 132.03), (T) tail length ranged from (45.99 – 64.61 µm), (ABD) Anal body diam. ranged from (8.84 – 14.34 µm), D% = (313.67 – 398.43), E% = (532.5 – 1019.88). (**female**): N=7, (L) the body length ranged from (857.44 – 1111.7 µm), a = (16.12 – 19.5), b = (4.69 – 5.55), c = (9.84 – 11.66), c' = (4.03 – 5.31), V% =(48.95 – 53.89), Greatest body diam. ranged from (44.36 – 67.88 µm), EP = (772.77 – 1013.17), NR = (128.2 – 147.94), ES = (181.47 – 201.67), (T) tail length ranged from (82.43 – 100.75 µm), (ABD) Anal body diam. ranged from (16.17 – 23.79 µm), D% = (423.27 – 505.91), E% = (884.29 – 1065.77). (**Juvenile**): N=6, (L) the body length ranged from (419.28 – 757 µm), a = (18.99 – 21.53), b = (3.91 – 4.39), c = (7.94 – 12.68), c' = (4.04 – 9.4), Greatest body diam. ranged from (21.21 – 36.75 µm), EP = (370.15 – 690.47), NR = (77.7 – 133.32), ES = (107.7 – 172.7), (T) tail length ranged from (43.83 – 71.83 µm), (ABD) Anal body diam. ranged from (5.02 – 15.26 µm), D% = (344.74 - 402.24), E% = (694.36 – 1167.8). The lengths and measurements of the *M. rainai* appeared in this study were consistent with the study of (Carta and Osbrink, 2005). Even though *M. rainai* and *M. amsactae* share a lot of morphological similarities, they were mistakenly identified (Khanum *et al*., 2019; Bhat *et al*., 2020).

Table 1. Morphometric measurements (Mean ± SEM) of adults (female, male) and juvenile of *Metarhabditis rainai* isolate (in µm).

Measurement	Male (n=8)	Female (n=7)	Juvenile (n=6)
L	470.69 ± 93.20 (377.49 – 563.89)	984.57 ± 127.13 (857.44 – 1111.7)	588.14 ± 168.86 (419.28 – 757)
a	21.28 ± 2.14 (19.14 – 23.42)	17.81 ± 1.69 (16.12 – 19.5)	20.26 ± 1.27 (18.99 – 21.53)
b	4.05 ± 0.43 (3.62 – 4.48)	5.12 ± 0.43 (4.69 – 5.55)	4.15 ± 0.24 (3.91 – 4.39)
c	8.76 ± 2.44 (6.32 – 11.2)	10.75 ± 0.91 (9.84 – 11.66)	10.31 ± 2.37 (7.94 – 12.68)
c'	4.99 ± 1.30 (3.69 – 6.29)	4.67 ± 0.64 (4.03 – 5.31)	6.72 ± 2.68 (4.04 – 9.4)
V%	—	51.42 ± 2.47 (48.95 – 53.89)	—
Greatest body diam.	22.07 ± 3.12 (18.95 – 25.19)	56.12 ± 11.76 (44.36 – 67.88)	28.98 ± 7.77 (21.21 – 36.75)
EP	415.38 ± 93.79 (321.59 – 509.17)	892.97 ± 120.20 (772.77 – 1013.17)	530.31 ± 160.16 (370.15 – 690.47)
NR	94.19 ± 9.86 (84.33 – 104.05)	138.07 ± 9.87 (128.2 – 147.94)	105.51 ± 27.81 (77.7 – 133.32)
ES	115.92 ± 16.11 (99.81 – 132.03)	191.57 ± 10.10 (181.47 – 201.67)	140.20 ± 32.50 (107.7 – 172.7)
Tail length (T)	55.30 ± 9.31 (45.99 – 64.61)	91.59 ± 9.16 (82.43 – 100.75)	57.83 ± 14 (43.83 – 71.83)
Anal body diam. (ABD)	11.59 ± 2.75 (8.84 – 14.34)	19.98 ± 3.81 (16.17 – 23.79)	10.14 ± 5.12 (5.02 – 15.26)
D%	356.05 ± 42.38 (313.67 – 398.43)	464.59 ± 41.32 (423.27 – 505.91)	373.49 ± 28.75 (344.74 - 402.24)
E%	776.19 ± 243.69 (532.5 – 1019.88)	975.03 ± 90.74 (884.29 – 1065.77)	931.08 ± 236.72 (694.36 – 1167.8)

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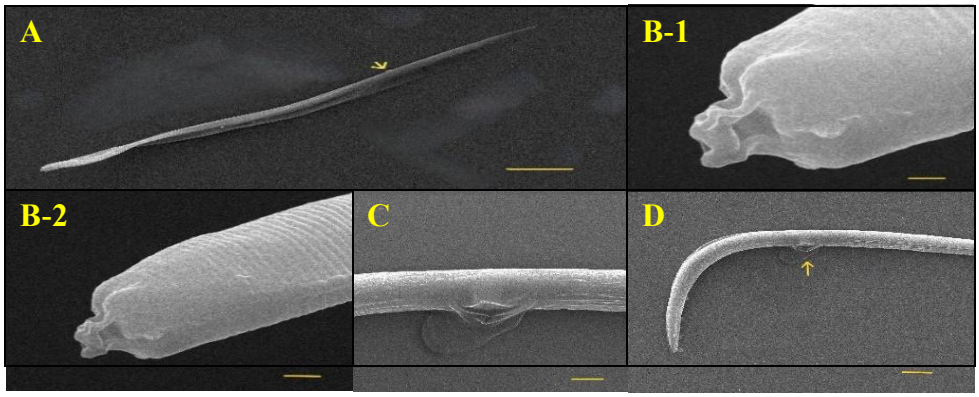

Figure 1. Scanning electron micrographs (SEM) of *Metarhabditis rainai* (Female), (A) whole body of female, (B) Anterior region of female, (C) Vulval region of female showing double flapped epiptygma, (D)Posterior region showing vulva and double flapped epiptygma. Scale bars: A= 100 µm, B1= 5µm, B2= 10 µm, C= 40 µm, D= 100 µm

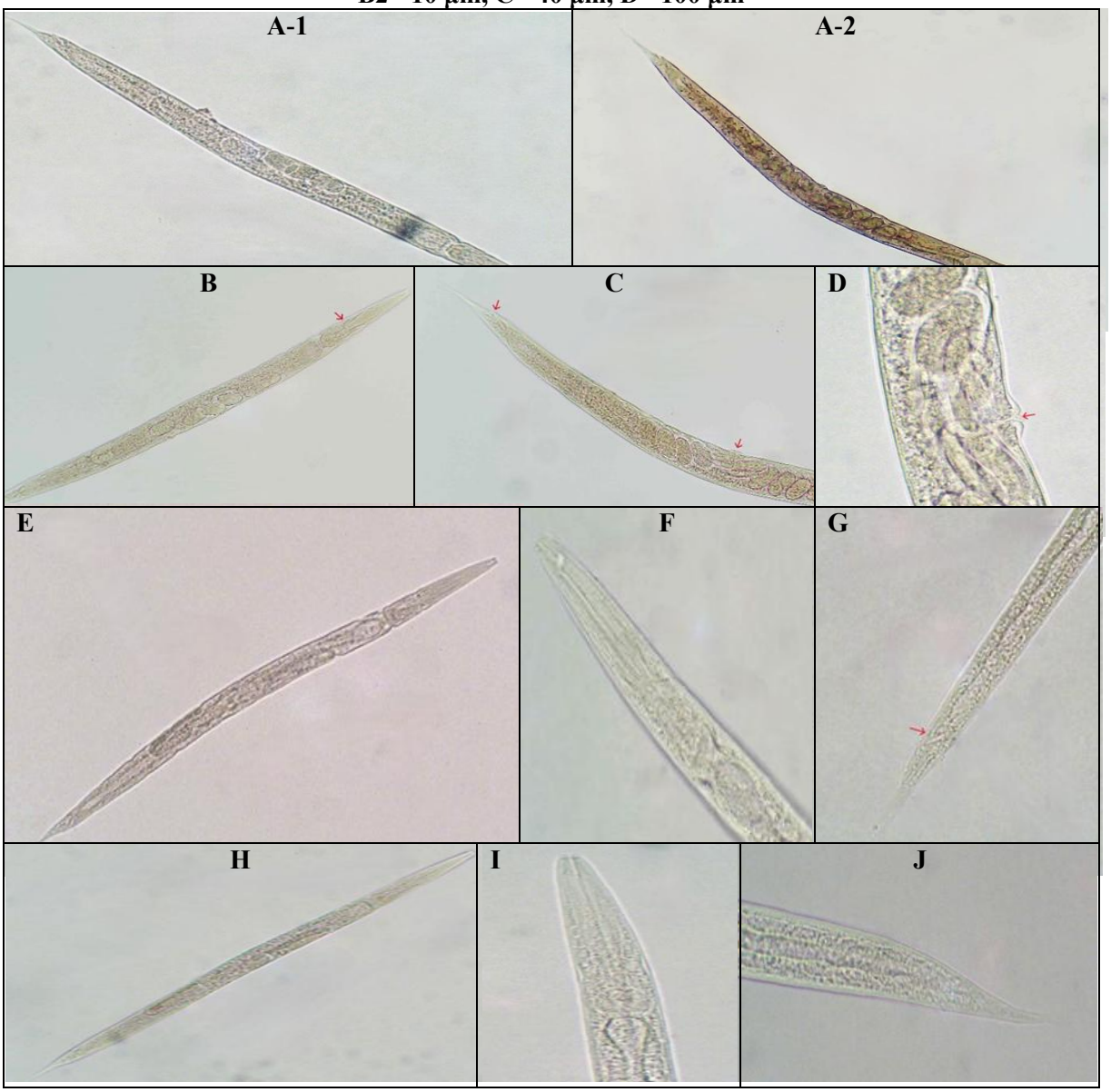

Figure 2. Light microphotographs of *Metarhabditis rainai*, (A) whole body of female (A-1, 10X & A-2, 4X), (B) Anterior region of female NR (10X), (C) Female posterior end (40X), (D) Vulval region of female (40X), (E) whole body of male (10X), (F) Anterior region of male (40X), (G) Tail region of male (40X), (H) whole body of juvenile (10X), (I) Anterior region of juvenile (40X), (J) Tail region of juvenile (40X).

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Other selected specimens of *M. rainai* passed through molecular tactics using DNA coding to confirm the morphological identification of the isolated nematodes. The amplicon of 18S-rRNA (828 bp) from the chosen individual showed single bands on agarose gels. The nucleotide sequence data from this isolate is reported in the GenBank database. Nucleotide sequence data of 18S-rRNA can be found with the accession numbers OR960470. The 18S-rRNA sequences of this isolate had 100% sequence homology with 18S-rRNA sequence of *M. rainai* (Accession number MT012135), (Accession number MT012133) and (Accession number JQ237848). Moreover, it had 99.76% sequence homology with *M. rainai* (Accession #AF083008). It also had 99.28% sequence homology with *M. rainai* (Accession #MT012153). While it had sequence homology of 88.86%, 88.63% and 88.50% with 18S-rRNA sequence of *M. rainai* (Accession number OQ513931), (Accession number MT872504) and (Accession number MT872503.1) respectively. Results in Fig. (3) showed that the sequence of the selected *M. rainai* isolate (Accession number OR960470) as sister groups with (Accession number MT012135), (Accession number MT012133) and (Accession number AF083008) with bootstrap value of 100% and having a same node ancestor with the other previous recorded sequences of *M. amsacate* under the accession number (MT87250, OQ513931 and OQ51390) and *Rhabditis blumi* under the accession number (MN453373). This result agreed with the result reported by Bhat *et al*. (2020), who showed that *M. blumi* and *M. amsactae* were identified as sister or associated species of *M. rainai*. Similar results were also obtained by De Brida *et al*. (2017) who suggested that *M. amsactae*, *M. rainai* and *M. blumi* are sister species. The mean inter-specific distance among *Metarhabditis rainai* ZahIQ2 (Accession number OR960470) isolate and other isolates of *Metarhabditis* were 0.251% (range 0.00 – 1.02 %), which have been calculated using the Tamura 3-parameter model based on the 18S gene. Nucleotide distance between the *Metarhabditis rainai* isolates from Iraq and *Rahabiditis blumi* ISCP01 (Accession number MT012150) was 0.12% (Table 2).

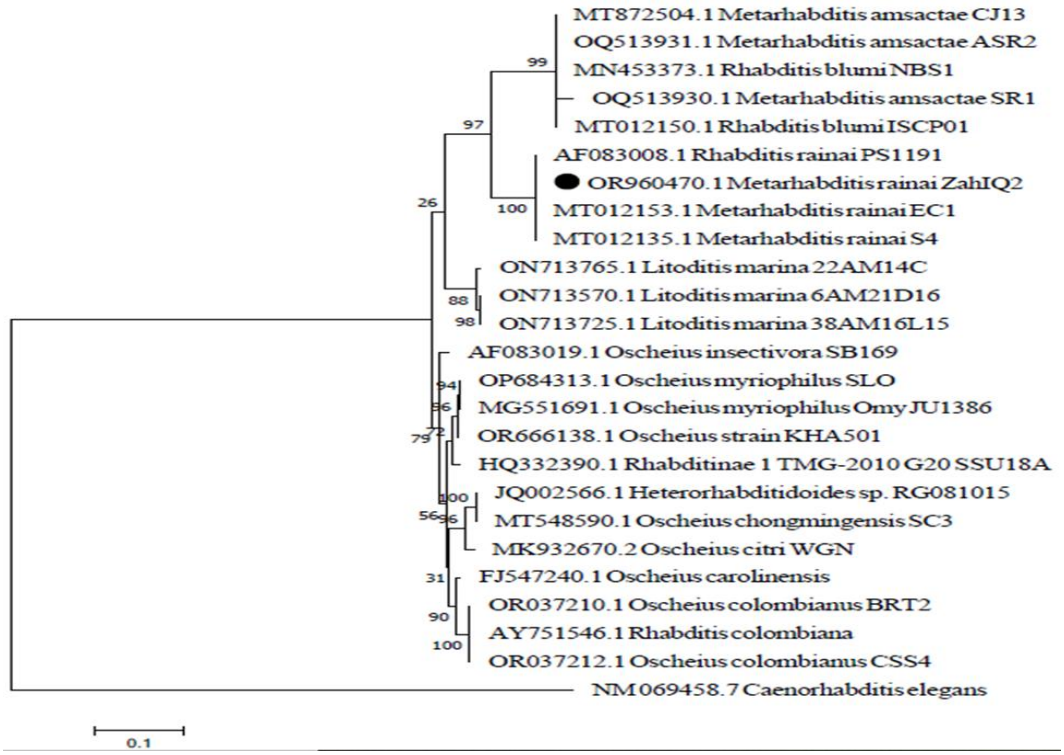

Figure 3. Phylogenetic relationship of *M. rainai* isolate with 23 isolates of other species related to *Metarhabditis* genus based on 18S-rRNA gene sequences as inferred from neighbour joining (NJ) analysis, Caenorhabditis elegans (NM069458) was used as outgroup, Support values are presented near the notes in the form: bootstrap in ML.

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Table 2. Conducting pairwise comparisons of various *Metarhabditis* species and isolates according to their nucleotide differences from *Metarhabditis rainai* ZahIQ2 using 18S sequences.

	Metarhabditis species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	OR960470 *Metarhabditis rainai* ZahIQ2														
2	MT012153 *Metarhabditis rainai* EC1	0.00													
3	AF083008 *Rhabditis rainai* PS1191	0.00	0.00												
4	OP684313 *Oscheius myriophilus* SLO	0.14	0.14	0.14											
5	AY751546 *Rhabditis colombiana*	0.14	0.14	0.14	0.04										
6	MT548590 *Oscheius chongmingensis* SC3	0.14	0.14	0.14	0.04	0.05									
7	FJ547240 *Oscheius carolinensis*	0.14	0.14	0.14	0.03	0.02	0.05								
8	MG551691 *Oscheius myriophilus* Omy JU1386	0.14	0.14	0.14	0.00	0.04	0.04	0.03							
9	OQ513930 *Metarhabditis amsactae* SR1	0.14	0.14	0.14	0.16	0.17	0.18	0.17	0.16						
10	ON713765 *Litoditis marina* 22AM14C	0.13	0.13	0.13	0.08	0.08	0.08	0.08	0.08	0.17					
11	AF083019 *Oscheius insectivore* SB169	0.13	0.13	0.13	0.04	0.04	0.05	0.04	0.04	0.15	0.08				
12	ON713570 *Litoditis marina* 6AM21D16	0.12	0.12	0.12	0.07	0.07	0.08	0.07	0.07	0.17	0.01	0.07			
13	MT012150 *Rhabditis blumi* ISCP01	0.12	0.12	0.12	0.15	0.15	0.16	0.15	0.15	0.02	0.16	0.13	0.16		
14	OQ513931.1 *Metarhabditis amsactae* ASR2	0.12	0.12	0.12	0.15	0.15	0.16	0.15	0.15	0.02	0.15	0.13	0.16	0.00	
15	NM 069458 *Caenorhabditis elegans*	1.28	1.28	1.28	1.13	1.16	1.09	1.14	1.13	1.33	1.10	1.15	1.12	1.28	1.29

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

DECLARATION OF FUND

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الوصف المظهري والجزيئي للتسجيل الجديد (Nematoda: Rhabditidae) *Metarhabditis Rainai* في بغداد

*زهراء يحيى كاظم حارث سعيد الورد جواد بلبل الزيداوي

المستخلص

هدفت الدراسة الى تحديد ووصف احد الديدان الخيطية المسببة للامراض الحشرية *Metarhabditis rainai*، الذي تم عزله من عينات التربة التي جمعت من منطقة الراشدية عند (33°25'13.4" شمالاً 44°21'45.3" شرقاً) بغداد– العراق حيث وصفت مورفولوجياً و شخصت جزيئياً. تم تربية جميع عينات *M. rainai* وتحديدها ووصفها باستخدام بعض المعايير المورفولوجية بناءاً على المفتاح التصنيفي المتوفر. تميزت العينات المختارةعزلة Zah, IQ2 (OR960470) من هذا النوع بأن طول جسم الذكر تراوح بين (377.49 – 563.89 ميكرومتر) وطول جسم الأنثى (857.44 – 1111.7 ميكرومتر) وطول جسم اليافع هو (419.28 – 757 ميكرومتر). شخصت العينات المختارة من هذا النوع جزيئيًا باستخدام تسلسلات جين 18S rRNA الجزيئية. كان لتسلسل 18S rRNA لعزلةOR960470 Zah, IQ2 نطاق من التماثل التسلسلي (88.50%–100%) مع تسلسل 18S rRNA لـ *M. rainai* المتوفر في قاعدة بياناتNCBI . أُنشائت شجرة النشوء والتطور لفصل أنواع *M. rainai* عن الأجناس والأنواع ذات الصلة الوثيقة. تمثل عزلة *M. rainai* Zah, IQ2 OR960470 أول تسجيل ل *M. rainai* في العراق.

الكلمات المفتاحية: النيماتودا المسببة للامراض الحشرية، العراق، القياسات المظهرية، التربة، جين 18S rRNA.

*جزء من اطروحة دكتوراه للباحث الاول.