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Isolation, Morphological, and Molecular Characterizations of Nematodes from Soil of South Baghdad

Ghofran Wael Abdulrazzaq Al-Waeely[1], Mysoon Hassan Meshjel Al-Seria[2] and Jawad Bulbul Humud Al-Zaidawi[3]
[1,2]Department of Biology, College of Science for Woman, University of Baghdad, Baghdad, Iraq.
[3]Agricultural Research Directorate, Ministry of Science and Technology, Baghdad, Iraq.

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KEYWORDS
Survey, Entomopathogenic nematodes, *Rahabitis, Oscheius,* Molecular dentification.

ABSTRACT:
A recent survey was conducted in Al-Madaen region, which is in south of Baghdad, a capital city of Iraq. The aim of this study was to isolate and identify nematodes from soil in this region. Nematode detection was performed on samples gathered from 4 locations across the region, including alfalfa fields, eggplant greenhouses, citrus groves, and fruit tree orchards. Two nematode isolates were found out of a total of thirty samples. Morphological descriptions were made of the samples, and a molecular approach based on the ITS gene was used to validate the identification. Soil samples from nearby alfalfa fields and citrus groves yielded the two isolates. Analysis of sequencing data was followed by a blast of DNA sequences against the GenBank database at the National Center for Biotechnology Information (NCBI). Both isolates were tested, and Mad.1 was found to be Rhabditis, while Mad.2 was found to be Oscheius. Using the DNA sequences of 2 loci, we performed a molecular analysis and phylogenetic analysis of the relationship between the two genera. Both species of Rahbitis and Oscheius have been named; Rahabiditis blumi for the former and Oscheius macrovilli for the latter. The first records of these species in Baghdad. Pathogenicity assays and additional testing will build on the findings of this first study.

1. Introduction

The potential of entomopathogenic nematodes as biological control agents has made them one of the most studied of all nematode species (Akhurst R &Smith K, 2002). Soil-dwelling EPN are necessary parasites of insects and work in tandem with disease-causing bacteria. Symbiotic relationships exist between the nematodes of the genera Heterorhabditis, Steinernema, and Oscheius and the bacterium species Photorhabdus, Xenorhabdus, and Serratia, which cause disease in insects (Akhurst R & Smith K, 2002; Lacey L *et al*., 2015). EPNs can be found in a wide variety of soil environments, and they are highly diverse in terms of host range, infectivity, symptomatology, reproduction, distribution, and survival conditions (Darsouei R *et al*., 2014). In order to effectively manage some significant lepidopteran, dipteran, and coleopteran insects of commercial crops, EPNs have been used (Hazir S *et al* ., 2004). Insects lay eggs on susceptible plants or animals, and the success of the nematodes that hatch from those eggs depends on the nematodes' ability to locate, recognize, and enter a host (Kaya HK& Gaugler R, 1993). EPNs infect susceptible insect hosts when they are in the free-living, non-feeding, infectious juvenile arrested developmental stage. Within 24 to 48 hours, the host develops septicemia or toxemia when they penetrate the intestine and release the accompanying harmful bacteria into the hemocoel (Shapiro-Ilan D *et al*., 2017). After contracting *Steinernema*, juvenile either become feeding males or amphimictic females (Dillman AR *et al*., 2012). The infectious juveniles multiply inside the insect host for 2 to 3 generations while feeding on the infected insect corpse (Ye W *et al*., 2010). Three- stage infectious juveniles are produced and emerge from the corpse after the EPNs have used up all of their resources. They remain in the soil while looking for a new host to infect (Torres-Barragan *et al*., 2011). The ecological compatibility of different regions determines which EPN

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species are selected for use as biological control (Lephoto TE *et al*., 2015). There is a vast range of edaphic environments that are home to EPN species. They are resilient in many different climates and environments (Thomas GM & Poinar GO Jr, 1983). In order to evaluate the efficacy of potential biological control agents against problematic insect pests in specific biogeographical regions, it is required to identify and categorize locally adapted species or isolates from natural settings. In order to determine which, if any, nematodes were present in the area, we set out to isolate and identify EPNs from Iraq to use as biological control agents for insect pest control (Zhou G *et al*., 2017, & Stock *et al*., 1999).

2. Materials and Methods

2.1. Soil Samples

Between 2021 and 2022, soil samples were collected from a wide variety of sites and habitats in the Al-Madaen area, south of Baghdad, Iraq (table 1). Soil was collected using a manual shovel and scooped out to a depth of 15 cm before being placed in 20 separate plastic containers and stored at 22 to 25 °C for transfer to the lab. Soil samples were hydrated and stored at room temperature overnight (Bedding R& Akhurst R, 1975). Fourth stage of wax moths, *Galleria mellonella* (Lepidoptera: Phyralidae) were then used as a host in soil samples (Felsenstein J, 1985 & GlazerI *et al* .,1991). each soil container had 10 Galleria larvae placed on top of it, the soil containers were inverted and placed in a 25 ^{o}C incubator, where they were checked for signs of disease and mortality every day. Larval mortality signs were recorded and used to identify whether or not EPNs were the cause of death (Joyce *et al* ., 1994).

Table 1. Locality, habitat, physical, chemical factors, and soil characteristics of samples.

Origin	Site 1	Site 2	Site 3	Site 4
Coordinates: Positive site	N 33°9’5.583” E 44°33’9.2952”	——	——	——
Coordinates: Negative site	——	N 33°9’4.8204” E 44°33’11.721”	N 33°9’25.5456” E 44°33’22.539”	N 33°9’32.6304” E 44°33’32.277”
habitat	Different Cultivated Fields			
pH	6.54	7.34	7.28	7.06
Salinity	0.15	0.15	0.26	0.27
Organic matter	1.92	3.57	4.08	2.44
Calcium	178	167	170	79
moisture	24.5	24.9	9.26	7.17
Temperature	17.5	17	17.4	17

2.2. Collecting of Nematodes

Larvae that had died were placed on white traps and left at room temperature for another 48 to 72 hours to see if any nematodes would emerge. During the warm summer months, white traps were kept on a bench at room temperature to catch infectious juveniles emerging from dead larvae thought to be contaminated with EPNs, and during the cold winter months, a heater was used to keep the room at 25°C. Infectious juveniles were more likely to emerge from decomposing bodies into the water at room temperatures between 25 and 30 degrees Celsius (Kumar *et al* ., 2016 , Kaya HK & Stock SP,1997). There was precaution taken to prevent the whatman filter paper disc from becoming too wet, which could introduce too much water into the insect cadaver while it was on the trap and cause the death of EPNs before they could emerge. Nematodes from white traps were surface-sterilized and utilized for morphological and molecular identification (figure 1) (Stock SP *et al* ., 1999).

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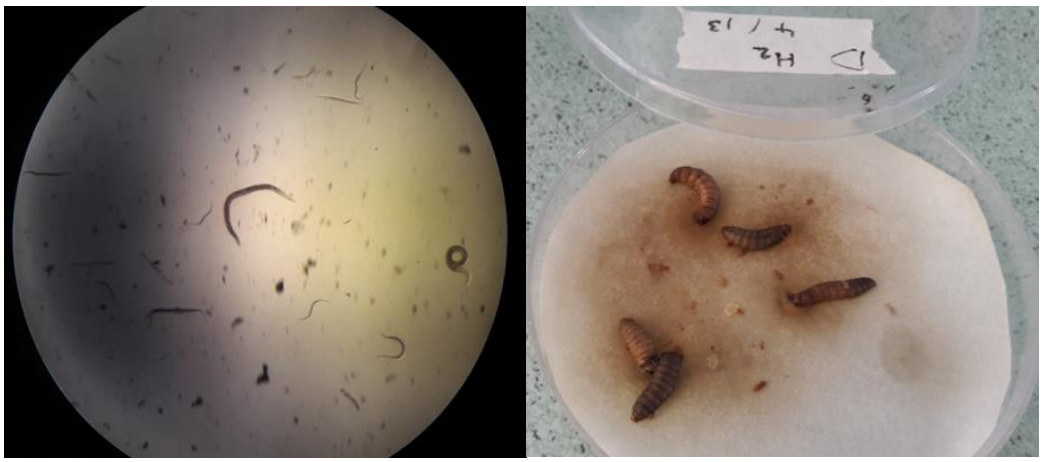

Figure 1. White trap and microscopic view showing infected juveniles and adults emerging from the insect cadaver.

2.3. Identification by Morphology

After 24 hours in 100°C triethanolamine formaldehyde (TAF), 20 nematodes from the first generation were fixed. After a minimum of 24 hours of fixative penetration, the nematodes were placed in a 4-degree Celsius environment with double-strength TAF and kept relaxing for up to an hour. After being fixed in 95% ethanol, 1% glycerin, and 79% water, 0.5 ml of Seinhorst I solution was applied to a petri plate containing worms. The nematodes were placed in a desiccator after 5 ml of 95% ethanol was added to the watch glass containing them. The desiccator was heated for 12 hours in an oven preheated to 35 degrees Celsius. The nematodes were removed from the desiccator and placed in a glass petri plate with Seinhorst II solution (95 parts 95% ethanol, 5 parts glycerine). The petri dish was kept slightly ajar to allow the ethanol to slowly evaporate. The glass from the watch was baked in an oven at 40 degrees Celsius for three hours, after which nematodes were carefully mounted on glass slides and viewed under a microscope. The morphological study employed a number of magnifications on an Olympus microscope. The abbreviations and their definitions for the samples in tables are as follows: n = number of specimens which were measured, L = overall body length, V = % distance of vulva from anterior relative to body length, a = body length/greatest body diameter, b = body length/distance from anterior to esophago-intestinal valve, c = body length/tail length, c´ = tail length/tail diameter at anus or cloaca, VA = distance from vulva to anus The presence of the isolated DNA was verified using a 0.5% agarose gel, 50 ml of 1× TBE (Tris-borate-EDTA) buffer and 1 L of agarose power were combined to take 0.25 g, ethidium bromide was added, and the mixture was gently stirred. The well comb was inserted, and the gel was allowed to set. Only 5 ml of each 10 ml sample were placed into the wells for the 30 minutes separation on the gel at 90 V immersed in 1 × TBE with constant current.

3. Results

The region of ITS gene of rDNA was used for identification and characterization of studied isolates. According to the shape of the infected larvae and their color, they were sorted. After that, genetic methods were conducted to the whole samples which isolated from soil. Then, Blast in National Center for Biotechnology Information (NCBI) database in GenBank site. The all collected isolates were *Oscheius* or *Rahabitis.*

3.1. Analysis of Target Sequence for Oscheius Species

The collected isolate showed 915 bp as a length of target gene (ITS). By using BLAST software according to ITS gene, the population attributes displayed that 97% of query coverage and 95% of parallels with Oscheius microvilli (Mad.2). An 813-bps segment of the gene was the multiple alignments which was compared with 22 taxa. This showed that 513 sites variable and 201 sites were conserved. Based on ITS sequences, the phylogenetic analysis using neighbor joining model presented that the Mad.1 isolate procedures a monophyletic cluster with Oscheius microvilli isolates (figure 1). Also, the main distance of inter specific of this gene sequences was 0.075(0.21-0.98), which was recorded using (Kumar S *et al.,* 2018). The difference was 0.2% between Mad.2 isolate and recorded isolate in GenBank (table 2).

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3.2. Analysis of Target Sequence for Rahabitis Species

The length of fragment sequences of this gene was 815 bp, BLAST site was utilized to compare these sequences with recorded fragments of same gene in GenBank. according to ITS gene, the population attributes displayed that 97% of query coverage and 95% of parallels with Oscheius microvilli (Mad.2). An 813-bps segment of the gene was the multiple alignments which was compared with 22 taxa. This showed that 513 sites variable and 201 sites were conserved. Based on ITS sequences, the phylogenetic analysis using neighbor joining model presented that the Mad.1 isolate procedures a monophyletic cluster with Oscheius microvilli isolates (figure 1). Also, the main distance of inter specific of this gene sequences was 0.075(0.21-0.98), which was recorded using (Kumar S *et al*., 2018). The difference was 0.2% between Mad.2 isolate and recorded isolate in GenBank (table 2). Morphometric analysis of isolates from infecting juvenile and male nematodes indicates that the strain (TEL) is conspecific with Heterorhabditis and Oscheius.Body length, tail length, and pharynx length all appear to be shorter in TEL infected juveniles compared to those documented for Rahabiditis blumi and Oscheius. Males of R. blumi are taller than the 1032m-long Oscheius macrovilli. The length of the infected juveniles overall infectious juveniles of Rahabitis TEL have a total body length 522µm which is taller than O. *microvilli.* The male TEL specular in O. microvilli are taller than the Rahabitis, the TEL pharynx length is the same in both species which are 186.9µm, the male O. *myriophilus* has a tail length of 48.9µm which is taller than Rahabitis. The most constant morphometric data for the Rahabitis and Oscheius microvilli described by Jawad Al- Zaidawi are those shown in (table 2). The surface structure of nematodes can be understood by scanning electron microscope.

Table 2. Comparative morphometric data of Oscheius microvilli and *Rahabiditis blumi* isolated from Iraq.

	***Oscheius macrovilli* (µm)**	***Rahabiditis blumi* (µm)**
	Infected Juveniles	
Body length (L.)	879	1032
Tail length	48.9	24.6
Specular length	52.5	43.5
Pharynx length	186.9	186.9
	Male	
Body length	420	522
Tail length	40.3	50.4
Specular length	-	-
Pharynx length	99.8	110.1
	Female	
Body length (L.)	998	1061
Tail length	122.3	104.7
Specular length	_	_
Pharynx length	198.7	173.4

Figure 2. *Oscheius* male length.

Figure3. *Oscheius* female length.

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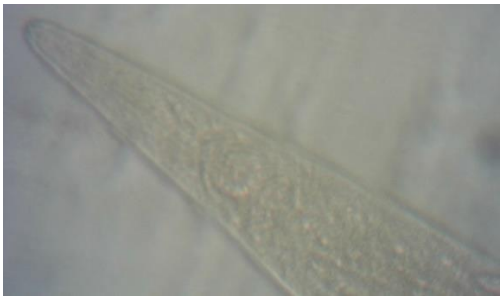

Figure 4. *Oscheius* Pharynx.

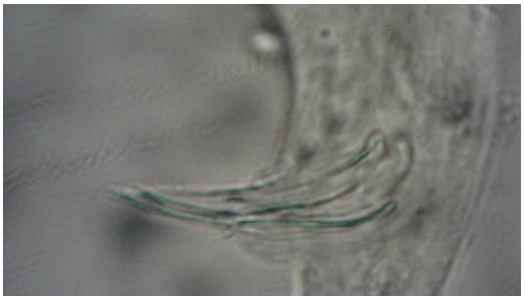

Figure 5. *Oscheius* Male Specular.

Figure 6. Heterorhabditis body.

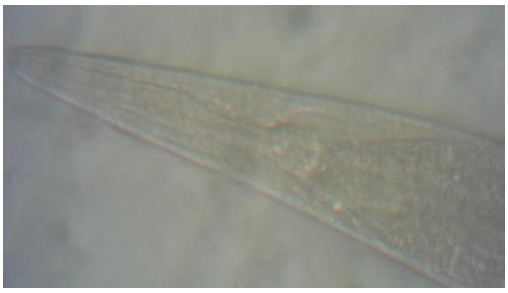

Figure 7. Heterorhabditis Pharynx.

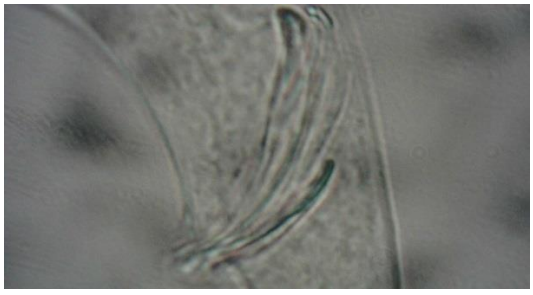

Figure 8. *Heterorhabditis* Spicular.

Figure 9. *Heterorhabditis* Female.

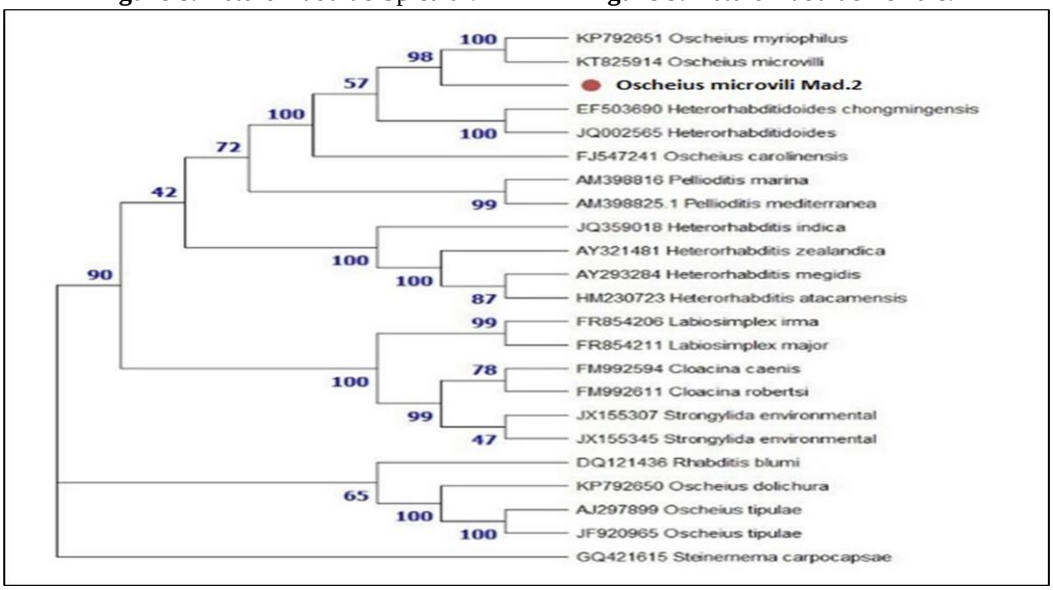

Figure 10. Phylogenetic analysis of the *Oscheius microvilli* isolate under investigation and homologous sequence from GenBank for the same genus. According to the evolutionary tree, the isolate described in the study is *Oscheius microvilli.*

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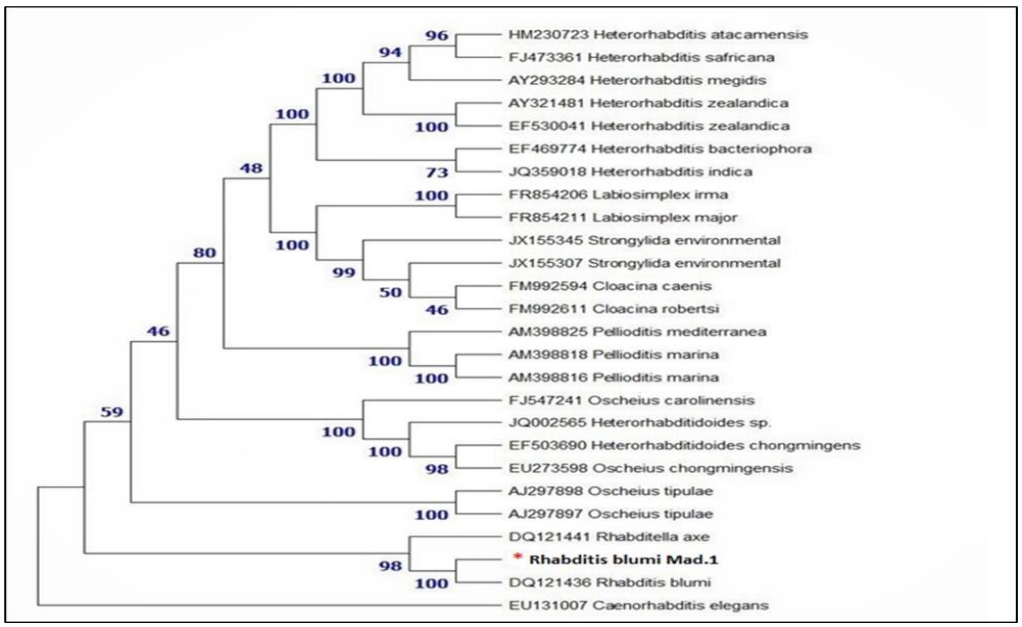

Figure 11. Phylogenetic analysis of the *Rhabditis blumi* isolate under investigation and homologous sequence from GenBank for the same genus. According to the evolutionary tree, the isolate described in the study is *Rhabditis blumi*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Rhabditis_blumi_Mad.1																							
DQ121436_Rhabditis_blumi	0.26																						
FM992594_Cloacina_caenis	0.89	0.92																					
EF469774_Heterorhabditis_bacteriophora	0.90	0.83	0.70																				
JQ359018_Heterorhabditis_indica	0.86	0.85	0.68	0.28																			
AY293284_Heterorhabditis_megidis	0.86	0.80	0.60	0.27	0.33																		
FM992611_Cloacina_robertsi	0.91	0.93	0.02	0.69	0.67	0.62																	
HM230723_Heterorhabditis_atacamensis	0.84	0.77	0.56	0.23	0.29	0.07	0.56																
FJ473361_Heterorhabditis_safricana	0.85	0.78	0.57	0.23	0.29	0.07	0.57	0.01															
AY321481_Heterorhabditis_zealandica	0.83	0.78	0.59	0.29	0.35	0.14	0.59	0.11	0.11														
EU131007_Caenorhabditis_elegans	1.17	1.26	1.28	1.28	1.22	1.26	1.28	1.27	1.24	1.32													
EF530041_Heterorhabditis_zealandica	0.83	0.78	0.59	0.29	0.35	0.14	0.59	0.11	0.11	0.00	1.32												
FR854206_Labiosimplex_irma	0.94	0.95	0.12	0.72	0.68	0.63	0.13	0.60	0.61	0.64	1.31	0.64											
FR854211_Labiosimplex_major	0.91	0.94	0.10	0.71	0.67	0.61	0.11	0.58	0.59	0.61	1.32	0.61	0.06										
FJ547241_Oscheius_carolinensis	0.84	0.82	0.74	0.80	0.76	0.74	0.75	0.77	0.77	0.77	1.41	0.77	0.73	0.72									
EF503690_Heterorhabditidoides_chongmingens	0.87	0.79	0.86	0.81	0.83	0.78	0.87	0.77	0.77	0.79	1.46	0.79	0.88	0.88	0.43								
EU273598_Oscheius_chongmingensis	0.87	0.79	0.86	0.81	0.84	0.79	0.87	0.78	0.77	0.79	1.48	0.79	0.88	0.88	0.44	0.00							
JQ002565_Heterorhabditidoides_sp.	0.87	0.80	0.86	0.80	0.84	0.77	0.87	0.76	0.75	0.77	1.45	0.77	0.88	0.88	0.43	0.01	0.01						
AJ297898_Oscheius_tipulae	0.88	0.96	0.89	0.92	0.91	0.88	0.87	0.85	0.84	0.86	1.27	0.86	0.86	0.85	0.90	0.91	0.91	0.93					
AJ297897_Oscheius_tipulae	0.89	0.96	0.88	0.93	0.90	0.89	0.86	0.85	0.85	0.86	1.28	0.86	0.86	0.85	0.88	0.91	0.91	0.93	0.01				
AM398818_Pellioditis_marina	0.75	0.76	0.58	0.57	0.59	0.60	0.59	0.55	0.56	0.57	1.15	0.57	0.64	0.65	0.64	0.70	0.70	0.68	0.90	0.89			
AM398816_Pellioditis_marina	0.77	0.74	0.58	0.55	0.60	0.57	0.59	0.53	0.54	0.56	1.20	0.56	0.63	0.66	0.65	0.70	0.70	0.69	0.91	0.92	0.04		
AM398825_Pellioditis_mediterranea	0.79	0.75	0.57	0.58	0.59	0.54	0.58	0.52	0.52	0.54	1.17	0.54	0.64	0.64	0.71	0.75	0.75	0.74	0.87	0.86	0.28	0.26	
DQ121441_Rhabditella_axe	0.50	0.48	0.80	0.80	0.83	0.83	0.84	0.84	0.83	0.84	1.15	0.84	0.85	0.82	0.80	0.79	0.79	0.80	1.08	1.09	0.75	0.75	0.75
JX155307_Strongylida_environmental	0.88	0.94	0.03	0.71	0.69	0.63	0.04	0.58	0.59	0.61	1.24	0.61	0.13	0.12	0.75	0.86	0.86	0.86	0.89	0.89	0.58	0.58	0.57
JX155345_Strongylida_environmental	0.92	0.98	0.04	0.69	0.66	0.61	0.05	0.57	0.58	0.59	1.26	0.59	0.15	0.13	0.77	0.88	0.88	0.88	0.89	0.89	0.60	0.60	0.59

Figure 12. Estimates of evolutionary Divergence between Sequences.

The number of base sustiturion per site from between sequences are shown.Analysis were conducted using the (Tamura K, 1992). model. There were 26 nucleotide sequences used in this study. First-plus-second-plus-third-plus noncoding codons were also included. Each sequencing pair has all ambiguous positions eliminated (using the pairwise deletion method). The completed database had 918 different jobs. MEGA X was used for the evolutionary analyses (Kumar S *et al* ., 2018).

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		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Oscheius microvilli Mad 2																						
AJ297899_Oscheius_tipulae	0.93																					
AM398816_Pellioditis_marina	0.60	0.88																				
AM398825.1_Pellioditis_mediterranea	0.56	0.83	0.26																			
AY293284_Heterorhabditis_megidis	0.73	0.99	0.61	0.59																		
AY321481_Heterorhabditis_zealandica	0.76	1.02	0.61	0.61	0.13																	
DQ121436_Rhabditis_blumi	1.00	0.85	0.87	0.88	1.02	0.99								Overall avarage =0.75								
EF503690_Heterorhabditidoides_chongmingensis	0.34	0.85	0.60	0.52	0.66	0.68	0.95															
FJ547241_Oscheius_carolinensis	0.40	0.94	0.62	0.56	0.75	0.83	1.10	0.47														
FM992594_Cloacina_caenis	0.79	0.99	0.61	0.57	0.70	0.69	0.95	0.81	0.82													
FM992611_Cloacina_robertsi	0.79	1.01	0.63	0.58	0.70	0.68	0.97	0.80	0.83	0.02												
FR854206_Labiosimplex_irma	0.83	1.04	0.67	0.64	0.69	0.67	0.94	0.85	0.88	0.12	0.13											
FR854211_Labiosimplex_major	0.81	1.04	0.69	0.62	0.69	0.66	0.95	0.79	0.85	0.10	0.12	0.06										
GQ421615_Steinernema_carpocapsae	1.22	1.34	1.35	1.34	1.23	1.26	1.18	1.30	1.32	1.24	1.23	1.23	1.25									
HM230723_Heterorhabditis_atacamensis	0.71	0.96	0.56	0.59	0.07	0.10	1.01	0.65	0.79	0.68	0.68	0.66	0.66	1.26								
JF920965_Oscheius_tipulae	0.92	0.01	0.90	0.82	0.98	1.01	0.86	0.86	0.92	0.99	0.99	1.02	1.02	1.33	0.95							
JQ002565_Heterorhabditidoides	0.35	0.84	0.61	0.53	0.67	0.69	0.95	0.01	0.47	0.82	0.82	0.87	0.81	1.29	0.65	0.85						
JQ359018_Heterorhabditis_indica	0.76	1.04	0.64	0.65	0.30	0.33	1.07	0.75	0.81	0.77	0.77	0.75	0.75	1.29	0.25	1.03	0.76					
JX155307_Strongylida_environmental	0.78	0.99	0.62	0.57	0.71	0.69	0.94	0.80	0.82	0.03	0.04	0.13	0.12	1.21	0.68	0.99	0.82	0.77				
JX155345_Strongylida_environmental	0.77	1.00	0.63	0.57	0.70	0.67	0.99	0.82	0.85	0.04	0.05	0.15	0.13	1.23	0.67	0.98	0.84	0.76	0.04			
KP792650_Oscheius_dolichura	1.15	0.57	0.93	0.83	1.00	1.01	0.93	1.04	1.08	1.14	1.16	1.11	1.14	1.48	0.94	0.57	1.01	1.04	1.16	1.14		
KP792651_Oscheius_myriophilus	0.21	0.90	0.55	0.52	0.69	0.70	0.93	0.33	0.38	0.73	0.74	0.77	0.75	1.19	0.67	0.89	0.34	0.71	0.76	0.73	1.00	
KT825914_Oscheius_microvilli	0.20	0.89	0.54	0.51	0.68	0.69	0.90	0.33	0.39	0.73	0.74	0.76	0.75	1.17	0.66	0.88	0.33	0.70	0.75	0.73	0.98	0.01

Figure 13. Estimates of evolutionary Divergence between Sequences.

The number of base sustiturion per site from between sequences are shown. Analysis were conducted using the (Tamura K, 1992). model. Twenty-three nucleotide sequences were used in this study. First-plus-second-plus-third-plus noncoding codons were also included. Each sequencing pair has all ambiguous locations eliminated (using the pairwise deletion option). The final database included 945 positions. MEGA X was used for the evolutionary analyses (Kumar S *et al* .,2018).

4. Discussion

Different species and isolates of EPNs could be distributed into diverse regions and areas and these need to exhibit an essential and significance in both behavioral and physiological variations. Consequently, it is important and necessary to achieve a successful and effective use of these biological control agents, this is extensive for identification and characterization of the locally and native adapted isolates or species that probably will be isolated and collected from a particular area or specific region (Hominick WM, 2002). Therefore, it is essential and crucial to find a precise identification approach for understanding biodiversity, behavioral system and topographical or geographical distribution and dispersal of different species of *Rahabiditis* and *Oscheius* (Campos-HerreraR *et al* ., 2015).

Isolation and Recovery of the nematode's species of these two genera (*Rahabiditis* and *Oscheius*) that collected and isolated from south Baghdad showed a huge organism's diversity in these target regions. This requires further investigations and extensive sampling. The probable of some of these isolated and endemic entomopathogenic nematodes could apply and form as an infective agent using in controlling and management endemic agricultural pests. This kind of evaluation could be another issue that should be tested in different conditions. Appling and adapting endemic entomopathogenic strains of nematodes. In the current paper, the species *O. microvilli* was identified and characterized using both morphological and molecular data; the successful characterization has been done by using molecular sequences of ITS genomic fragments. The advantage of molecular investigation and data were important to distinguish among the closely species that are follow same or close genus. These linked and closed forms have already been documented (Hominick WM, 2002).

Similarity, *Rahabiditis* species were characterized following the same procedure above. The tree of phylogeny for the received sequence analyzed and confirmed grouping after comparison with population investigated in the recent study as *R. blumi*. In addition to this, morphological characterizations confirm this relationship. According to results that were reported by

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(Powers TO *et al* ., 1997). they documented those molecular approaches considered as reliable and easier tool to diagnosis and even characterization of new species of nematodes and other organisms especially during the last two decades. The recovery of different species of EPNs could be very essential criteria for efficacy and success of entomopathogens in long term, cropland soil such as the soil conditions were the samples collected has more nematodes existence as compared with other soil (Castro-Ortega, 2020).

Conclusion

The nematode species Rahabiditis blumi and Oscheius microvilli were isolated and described here for the first time from the central areas of Iraq. These two isolates add significantly to our understanding of the biogeography of EPNs, and their discovery in arid southern Baghdad suggests that EPNs could be useful for the biological control of insect pests in tropical zones. This publication equips us with foundational knowledge about these two crucial genera and may aid future efforts to evaluate them as local and native biological control agents against various insect pests.

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