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Molecular description of the native nematodes, Heterorhabditis indica and Heterorhabditis megidis in Wasit province, Iraq

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ABSTRACT
The study continued in Iraq / Wasit province during the period from October 2022 to October 2023. Samples were collected from different regions in this province (north, south, center, east, and west). The soil samples were 100 samples from these areas, from which soil samples were collected randomly from orchards (citrus and palm trees) and wheat fields. Entomopathogenic nematodes were isolated using insect white trap. Then, entomopathogenic nematodes were found in 60 samples, and entomopathogenic nematodes were identified based on a molecular approach. ITS rDNA genes were selected to characterize the isolated insect pathogenic nematodes. The isolates were contrasted with other nematode isolates and species in GenBank and described using nucleotide BLAST in NCBI. There were two distinct categories of isolates. These are Heterorhabditis indica (IRQ.1 (PP869212 Iraq/Wasit)), which was found to be, and Heterorhabditis megidis (IRQ.2 (PP869236) Iraq/Wasit), which was found to be the first isolate and diagnosis of Heterorhabditis. Sp in Iraq.

Keywords: Entomopathogenic nematodes, Heterorhabditis indica, Heterorhabditis megidis, H, molecular description, National Center for Biotechnology Information (NCBI), Wasit, Iraq**.**

INTRODUCTION

In the world, a variety of insect pests are infested by the deadly parasites known as EPNs, which are members of the families Heterorhabdidae and Steinernimatidae.Many countries, including the US and Europe, have successfully managed and controlled major pests of economic crops using species of EPNs as biological management agents, particularly plant-boring insects and insect pests in soil. (Haziret al.,2004). According to Kaya and Gaugler (1993), EPNs are obligate insect parasites that can eliminate their prey within 48 hours. According to Poinar (1990) and Lewis and Clarke (2012), Xenorhabdus. Bacteria are linked to Steinernema nematodes, while Heterorhabditids are linked to Photorhabdus. The symbiotic bacteria are housed in a specialized bacterial receptacle in the anterior section of the colon for Steinernemaitis and in the gut mucosa for Heterochorditids in the dauer juvenile, also known as the infective juvenile (IJ). (Ciche& Ensign, 2003; Martens & Goodrich-Blair, 2005; Cicheet al., 2008; Bird &Akhurst, 1983). The nematodes can enter the target insect pest through openings that occur naturally, such as moth, anus, and occasionally through the cuticle of insects, and they can result in pest mortality (Grewal et al., 2005a). These biocontrol programs have drawn a lot of attention from governments around the world due to the unique characteristics of the agents used, including their capacity for massive reproduction, ease of mass production in the lab, and safety for humans and other vertebrates. (Akhurst& Smith 2002).Over 21 kinds of Heterorhabditis around 100 genuine species of Steinernema have been determined from various nations to date. In order to identify employ regionally appropriate EPN species to manage foliar insect pests and soil-dwelling insect pests in agricultural fields., extensive surveys have been carried out all over the world.While descriptions of the majority of the new species have come from Asia, certain Asian nations are still in the early stages of research. Australia has discovered another species, also Throughout Central, North, and South America, there are species of local nematodes that have been documented, employed as biological control agents, and even used for commercial purposes.

Additionally, research has been done on the EPN variety in European countries, leading to the discovery, utilizati on, and commercialization of novel species..Numerous previously unreported species as well as numerous new ones have been reported from Africa(Bhat et al., 2020). The Heterorhabditidae family is home to the most prosperous EPN strains. There are now 19 recognized species in the genus Heterorhabditis (Nematoda: Rhabditida),that exists naturally in the world. (Machado et al., 2021).H. bacteriophora and H. indicaspecies appear to be dispersed globally, with a focus on the warm and sub-warm(Bhat et al. 2020).The type strain of H. indica was first discovered in southern India (Poinaret al., 1992). Following its discovery, it was found in

numerous different parts of the world; the most recent reports were from eastern Australia (Aryalet al., 2022) and the Fiji Islands (Kouret al., 2020).

The study's objectives

This research aimed to locate, isolate, and recognize genetically native EPNs from the Wasit province in Iraq.

MATERIALS AND METHODS

Collecting Soil Samples and Separating Nematodes from Them

The study continued in Iraq / Wasit province during the period from October 2022 to October 2023. To determine whether EPNs are present in Iraqi soil or not, several soil samples were collected from the north, south, central, west and east of Wasit Governorate. After collecting about 100 samples from soil, citrus fruits, palm groves, and lands planted with wheat, the presence of entomopathogenic nematodes was identified in 60 (three sites). The fourth and fifth instar larvae of the waxworm were reared to obtain numbers of the infective stage of the nematode by applying bioassay technology (Orozco, R et al., 2014).

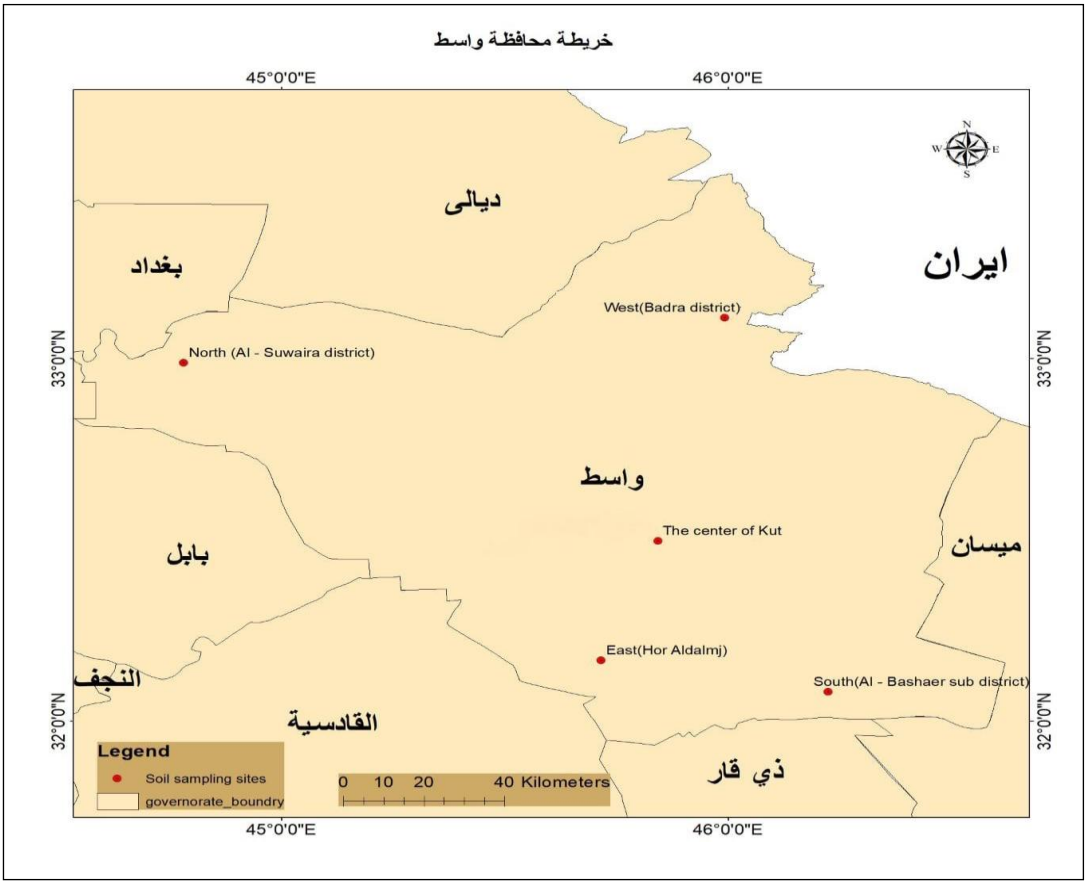

Figure 1. A map displaying the sampling sites.

Molecular identification of isolated nematodes

DNA Extraction

Individual nematode (female) was transferred to 1.5ml micro-tube using pipit with eyelashes modified for this purpose before washing it three times by sterilized water.The micro-tube with live nematode was placed inside liquid nitrogen for someminutes and mix products together using modified tip of sample.Chelex 5% (20 µl) and Proteinase K (2 µl) were added to the tube that had the grindedsample and then the product mixed together for 30 sec. using vortex device modelMSV-3500, Multi Speed Vortex. The tube with the products was placed into water bath at 64 ºC for 3 h, the tube wascentrifuged each one hour using simple spin. Thetube transferred to thermo-black at 95 ºC for 10 min, following by centrifuge with13000 rpm for 3 min.40.The supernatant was removed and transferred to new and sterilized micro-tube(0.1ml), the tube then was kept in fridge at -20 ºC for the rest of steps. (Al-Zaidawi, J. B. et al.,2019).

Amplification of ITS

TW81 (5-GTT TCC GTA GGT GAA CCT GC-3) as a forward primer and AB28(5- ATA TGC TTA AGT TCA GCG GGT-3)as a reverse primer. (Joyce et al.1994) was utilized. The PCR solution was 6.25 µl of sterilized water, 12.5 µl of 2X TaqPreMix, 1 µl of every primer, 1.25 µl of dimethyl sulfoxide, and 3 µl of genomic DNA in its final volume of 25 µl. A thermocycler (A300 Fast Thermal Cycler, Hangzhou Longgene Scientific Instruments Co., Ltd., China) was used to conduct the PCR reactions. The PCR run was as follows: a 4-minute initial denaturation at 94 °C; 35 cycles of 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 2 minutes; and a 10-minute final extension at 72 °C.Subsequently, the PCR product was electrophoresed on 1% agarose gels for 40 minutes with 10X TBE buffer 5%, and the gel was stained with green-viewer (SYBR). Lastly, 3 µl of the PCR product and 2.5 µl of DNA ladder were added to each gel well.The size of amplified products was estimated by matching to 100-bp molecular DNA ladder (Agricultural Research Directorate \ Ministry of Science and Technology, Baghdad).

Analysis and sequencing of DNA

Duplicate PCR products from both strains were sent to Macrogen Co. (Korea) for sequencing. Next,utilizing the DNA Sequence Assembler v4 (2013) as a DNA Baser
Assembler Heracle BioSoft, www.DnaBaser.com), the quality of the chromatograms was examined, and consensus sequences were generated. The DNA sequence was then cross-referenced with the NCBI database.

Phylogenetic analyses of nematode

The evolutionary research of the genus Heterorhabditis isolate included nucleotide sequences from 60 valid and validated DNA sequences in addition to our own, using the MEGA 7 software tool (Kumar et al., 2016). The maximum likelihood approach based on K2 + G was used to derive the evolutionary history (Kimura 1980).DNA sequences from Caenorhabditis elegans were employed as an outgroup taxon for Heterorhabditis. (KX572972). (Spiridonov et al., 2004). To ascertain branch support, 1100 repetitions of bootstrap analysis were employed. (Nguyen &Hunt 2007). Additionally, the neighbor-joining approach was used in this program for phylogenetic analysis (Saitou & Nei 1987). Using 10,000 bootstrap replications (Felsenstein 1985).Next, Clustal and Clustal X version 2 were used to compute the nucleotide distance (Larkin et al. 2007).

RESULTS AND DISCUSSION

Entomopathogenic nematodes isolated from the soil

Five locations in the Wasit province of Iraq (north, south, west, and east) provided soil samples about 100 samples from these regions and then demonstrated the presence of entomopathogenic nematodes in the60 regions (north, center and east of province) and showed entomopathogenicnematodes virulence against Galleria larvae, enabling the production of IJ.The isolated IJ shared traits with the Heterorhabditidae family members .Two species of local nematodesH. indica and H. migidis were found in several provinces. It shows the worms' virulence against Galleria larvae when they are insect-pathogenic. To achieve high yields of the infective stage (IJS), waxworm larvae were employed for the fourth and fifth stages by applying the bioassay technique (Orozco, R, et al ,2014).

Molecular description

The ITS gene in rDNA was used to identify and characterize the isolates that were the subject of the study. Diagnoses based on color and form are used to identify and separate diseased larvae. After that, each isolated species found in each soil sample was examined using the molecular diagnostic approach. After examination, the genus Heterorhabditis was confirmed to be the home of each isolated species by comparing DNA sequences and dates with the NCBI database in Gen Bank.

Analysis using ITS sequence for Heterorhabditis indica

The IRQ.Wasit.1(PP869212) isolate's ITS gene measured 850 bp in length. Based on the ITS gene, the BLAST analysis for this population shows 100% query coverage and 99% similarities with Heterorhabditis indica (MK271288). A 620-bps section of the ITS gene was multiplely aligned for 30 taxa, revealing 221 conserved, 395 variable, 184 singleton, and 207 parsimony informative sites. The IRQ.Wasit.1 strain represents a monophyletic group with other Heterorhabditis indica samples, according to the phylogenetic tree erected using ITS sequences and neighbor-joining technique (Figure 2). The Tamura 3-parameter model yielded an inter-specific distance mean of 0.217% (range 0.02 – 0.1) for ITS sequences. A 0.002% was present. (Table 1).

Table 1: Comparing the number of nucleotide differences between Heterorhabditis indica isolate IRQ .Wasit.1 and many other Heterorhabditis species and isolates pairwise. Based on ITS rDNA sequences

Heterorhabditis_indica_IRQ.Wast.1																
MW365745_Heterorhabditis_indica_IISR	0.002															
EF043445_Heterorhabditis_indica_N2	0.004	0.006														
JN620538_Heterorhabditis_noenieputensis_SF66	0.017	0.015	0.021													
EU099032_Heterorhabditis_georgiana	0.204	0.201	0.206	0.212												
KU573057_Heterorhabditis_downesi_pur1	0.227	0.225	0.230	0.233	0.160											
EF488006_Heterorhabditis_safricana	0.222	0.220	0.224	0.228	0.161	0.055										
HM230723_Heterorhabditis_atacamensis_D099	0.204	0.202	0.207	0.210	0.152	0.047	0.025									
AY321479_Heterorhabditis_marelatus	0.225	0.222	0.227	0.230	0.158	0.053	0.037	0.031								
AY321480_Heterorhabditis_megidis_MD-05	0.249	0.247	0.249	0.255	0.191	0.059	0.087	0.081	0.087							
EF530041_Heterorhabditis_zealandica_NZH3	0.282	0.280	0.285	0.288	0.229	0.132	0.141	0.130	0.125	0.156						
AY321477_Heterorhabditis_bacteriophora	0.204	0.202	0.209	0.212	0.019	0.163	0.164	0.152	0.159	0.191	0.224					
EF043443_Heterorhabditis_taysearae	0.101	0.099	0.106	0.104	0.254	0.256	0.265	0.246	0.256	0.268	0.312	0.255				
AY321478_Heterorhabditis_mexicana	0.095	0.093	0.099	0.097	0.251	0.265	0.274	0.255	0.260	0.277	0.316	0.244	0.013			
DQ372922_Heterorhabditis_floridensis_Fl-332	0.090	0.088	0.095	0.088	0.251	0.265	0.271	0.252	0.257	0.277	0.313	0.244	0.017	0.011		
AF548768_Heterorhabditis_baujardi	0.086	0.084	0.091	0.089	0.236	0.252	0.264	0.245	0.249	0.272	0.308	0.228	0.035	0.029	0.021	
DQ665222_Heterorhabditis_amazonensis	0.080	0.078	0.084	0.082	0.227	0.243	0.249	0.231	0.235	0.264	0.292	0.223	0.035	0.033	0.025	0.017

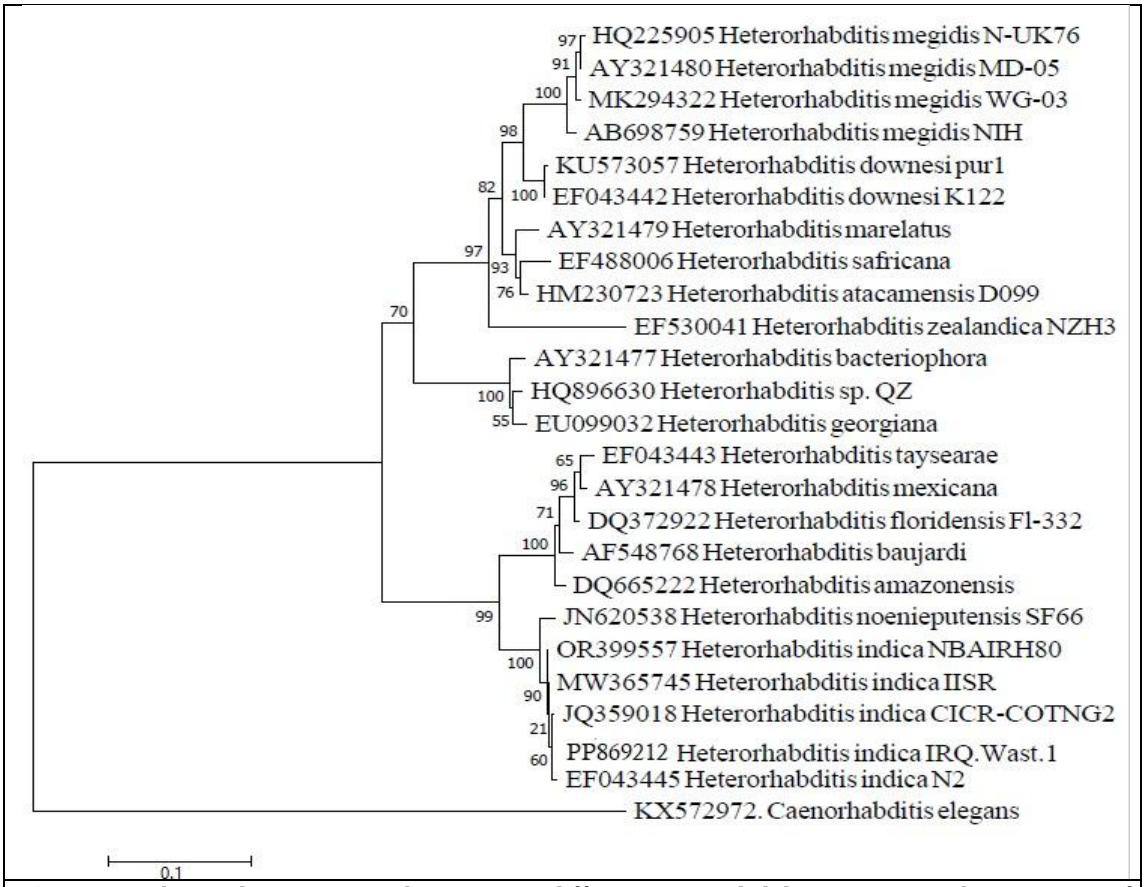

Figure 2: The evolutionary tree between 25 different Heterorhabditis species and one strain of Heterorhabditis, Heterorhabditis indica (PP869212). Based on sequences of ITS Rdna, as the out-group taxon, Caenorhabditis elegans (KX572972) was employed. The proportion of duplicate trees where related taxa were clustered together in the bootstrap (1100) in ML.

Characterization of Heterorhabditis megidis utilizing the ITS sequence

Heterorhabditis megidis (PP869236) was identified for the first time in Wasit Province, Iraq. The Was it isolate's ITS gene measured 870 base pair in length.

Based on the ITS gene, the BLAST analysis indicates that this population has 100% similarity with Heterorhabdi tis megidis (MK294322) and 100% query coverage. An ITS gene fragment spanning 650 bps was repeatedly aligned across 30 species, yielding 233 conserved, 392 variable, 194 singleton, and 215 parsimony informative sites. The evolutionary tree constructed using ITS sequences and the neighbor-joining approach places the IRQ. Wasit.2 strain in a monophyletic group along side other seclusions of Heterorhabditis megidis (Figure 3). For ITS sequences, the Tamura 3-parameter model produced an inter-specific distance mean of 0.231% (range 0.01–0.7). Figure 2 (table 2).

Table 2: ITS rDNA sequences were used to perform a pairwise analysis of the number of nucleotide differences between Heterorhabditis megidis strain IRQ. Wasit.2 and several other Heterorhabditis species and isolates.

Heterorhabditis_megidis_IRQ_Wast.2															
AB698759_Heterorhabditis_megidis_NIH	0.013														
AY321480_Heterorhabditis_megidis_MD-05	0.004	0.017													
EU099032_Heterorhabditis_georgiana	0.206	0.209	0.201												
EF043442_Heterorhabditis_downesi_K122	0.054	0.047	0.058	0.188											
MW365745_Heterorhabditis_indica_IISR	0.243	0.235	0.238	0.211	0.219										
EF488006_Heterorhabditis_safricana	0.087	0.083	0.088	0.186	0.053	0.217									
HM230723_Heterorhabditis_atacamensis_D099	0.079	0.075	0.079	0.179	0.043	0.197	0.023								
AY321479_Heterorhabditis_marelatus	0.090	0.086	0.090	0.181	0.053	0.219	0.039	0.031							
EF530041_Heterorhabditis_zealandica_NZH3	0.154	0.155	0.157	0.269	0.131	0.284	0.145	0.131	0.128						
AY321477_Heterorhabditis_bacteriophora	0.204	0.201	0.202	0.021	0.181	0.209	0.179	0.172	0.174	0.261					
EF043443_Heterorhabditis_taysearae	0.231	0.225	0.228	0.225	0.211	0.109	0.220	0.200	0.212	0.281	0.215				
AY321478_Heterorhabditis_mexicana	0.239	0.234	0.236	0.220	0.220	0.102	0.229	0.208	0.215	0.284	0.209	0.013			
DQ372922_Heterorhabditis_floridensis_FI-332	0.242	0.236	0.239	0.214	0.222	0.098	0.232	0.211	0.218	0.287	0.204	0.017	0.012		
AF548768_Heterorhabditis_baujardi	0.236	0.231	0.234	0.209	0.217	0.102	0.226	0.209	0.213	0.282	0.201	0.029	0.023	0.015	
DQ665222_Heterorhabditis_amazonensis	0.233	0.228	0.231	0.211	0.214	0.102	0.223	0.203	0.209	0.278	0.204	0.025	0.023	0.015	0.011

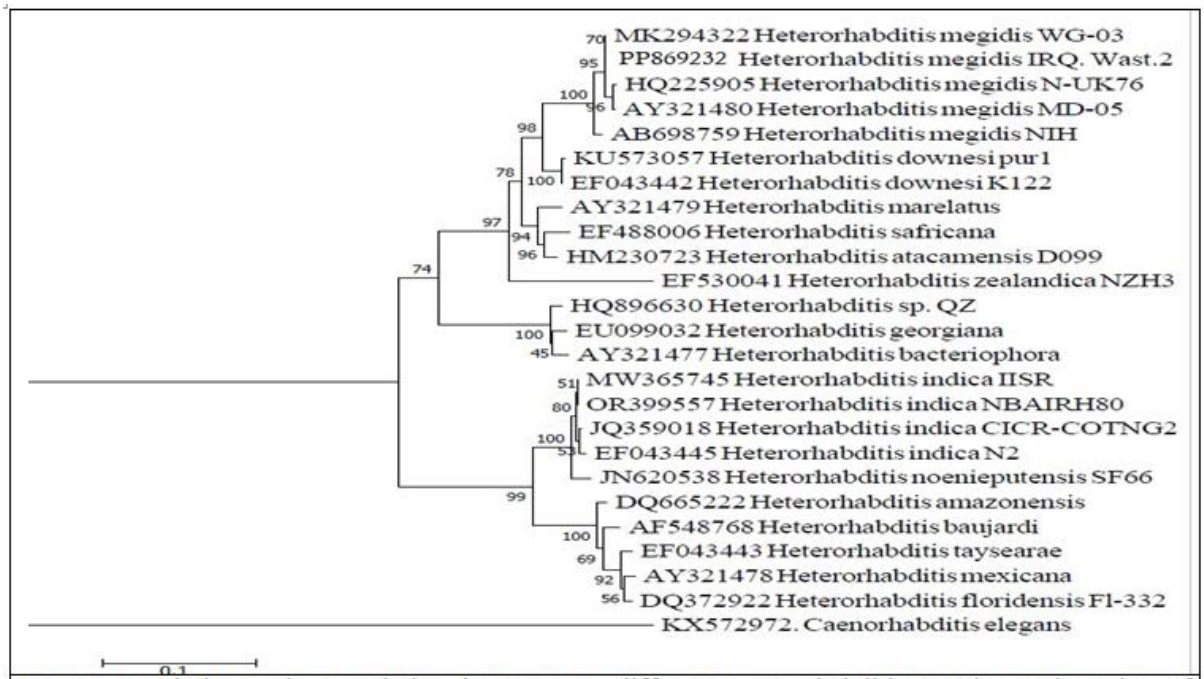

Figure 3: Phylogenetic correlation between 25 different Heterorhabditis species and strains of Heterorhabditis. including Heterorhabditis megidis (PP869232). based on sequences of ITS rDNA. As the out-group taxon. Caenorhabditis elegans (KX572972) was employed. The proportion of duplicate trees where related taxa were clustered together in the bootstrap (1100) in ML

Based the ITS rDNA region as a base, phylogenetic analysis of Heterorhabditidae species showed that the two isolates (H. indica PP869212 and H. megidis PP869232) and a few more likely discovered isolates formed a unique monophyletic group. (Figures 2 and 3). This species of the genus Heterorhabditis found in Wasit indicates a greater species diversity in Iraq. As for H. megidis, which was diagnosed for the first time in Iraq, it could be studied using endemic strains that cause insect diseases. The current research uses molecular data to characterize the two species, Heterorhabditis indica, and Heterorhabditis megidis. However, molecular sequences of a single gene segment (ITS gene) can effectively represent both. The distribution of EPN isolates and species in different regions may influence their physiological and behavioural adaptations. Thus, identifying and recording types or strains that have adapted locally in a certain location is essential for effectively applying EPNs as biological control agents. (Stock et al. 1999). Sequence data from the internal ITS gene is usually used to characterize nematodes molecularly(Reid et al.,1997). Females were used for molecular research because their size allowed for an adequate amount of DNA to be present. Because organisms' nucleotides have been sequenced, it is now possible to identify and confirm new species using sequence variations across EPN isolates (Szalanski et al., 2000; Spiridonov et al., 2004). According to Spiridonov et al. (2004), utilizing nucleotide sequence data in conjunction with evolutionary species concepts considerably facilitates the discovery and emergence of novel species. It has previously been shown that molecular information can be used to differentiate these species from their closely related forms (Campos-Herrera et al. 2015). In Mexico's state of Veracruz, the molecular characterization showed that there have been reports of H. indica isolated from sugar cane crops. Similarly, the morph type and morphology of the original description or the molecular studies of the SU rDNA do not differ from the study's findings assigned to the features of IJS and amphitic males and females. (Grifaldo Alcantara et al .,2020). Despite having a global range, H. indica has been found to have morphological differences in some research compared to the original species' description (Abdel-Razek et al., 2018), despite a near molecular match (Nikdel et al., 2012).According to a study conducted in India, the 12 nematode isolates currently in isolation belong to a monophyletic clade that includes the previously described H. indica, indicating their taxonomic identity is confirmed. This is based on a phylogenetic analysis of all Heterorhabditis species using ITS rRNA gene sequences (Bhat at el.,2021). In a few northern regions of Iraq in 2021 and 2022, A survey was carried out to ascertain the diversity of ENPs in these regions. These isolates were found to be members of the entomopathogenic nematode genera Heterorhabditis and Steinernema using molecular identification. Nematodes of Heterorhabditis were isolated from Baghdad's Salah al-Din, Samarra, and al-Jadriyah regions. Some collected soil samples were negative and had no nematode species such as Dohuk, Sulaymaniyah, Erbil, and Anbar cities. The nematodes that were isolated were identified as Heterorhabditis indica (OQ653109.1), Heterorhabditis bacteriophora (OR243201.1), and Steinernema carpocapsae (OR243196.1) using molecular analyses utilizing 28S genes (Khalid, et al.,2023).

The survey concentrated on three central Baghdad, Iraq, areas to identify and isolate entomopathogenic and insect parasite nematodes. After the obtained specimens were morphologically characterized, ITS and 18S rDNA gene analyses were carried out to identify and describe the isolates. Heterorhabditis, or IRQ.1, is the only gene found thus far. The DNA sequences from the molecular study were utilized to look into the two taxa's evolutionary ties. Heterorhabditis bacteriophora is one of the species designations for Heterorhabditis (Al-Zaidawi et al.,2019). Heterorhabditis species is the Middle Eastern nations' most common EPN genus (Glazer et al., 1991; Iraki et al., 2000; Salama & Abd-Elgawad, 2001).

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CONCLUSION

In this study, I showed how to isolate and obtain nematodes from the local soil in Wasit province, Iraq, and how they were molecularly diagnosed to identify this native species and compare it with international species through genetic analysis of the phylogenetic tree of these native species.

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