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ISOLATION AND DIAGNOSIS ENTOMOPATHOGENIC NEMATODE *RHABDITIS BLUMI* (RHABDTIDA: RHABDITIDAE) FROM LARVAE OF *JEBUSAEA HUMMERSCHMIDTI* (COLEOPTERA: CEARMBYCIDAE)

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Abstract

The collected nematodes were characterized at the first by morphological properties, then molecular studies were conducted to characterize and identify the isolates based on the following; Internal Transcribed Spacer (ITS) regions. Among these isolates, an isolated Rhabditidae family nematode assigned as isolate IRQ.1 which belonged to the Heterorhabditis genus. The phylogenetic relations of Heterorhabditis species were analyzed using the maximum likelihood method. The BLAST analysis base on ITS gene for Iraqi population of nematodes attributes 99% similarities and 96% of query coverage with *Rhabditis blumi* (DQ121436) which were the highest match in ITS. The identified species were an isolate of as *Rhabditis blumi* which was first reported in Iraq, on larvae of *J. hummerschmidti.*

***Key words*:** Entomopathogenic nematode, *Rhabditis blumi*, Date palm stem borer, iTS.

Introduction

These nematodes could be used effectively to control wide range of insect pest and considered as one of the most successful agents within biological control since 1970s, especially these are isolated from the same area of target pests as indigenous agents (Stock *et al*., 1999). These nematodes are group of biological control agents and received huge attention in many countries around the world since 1990s (Bedding *et al*., 1993). They have especial traits like search insect pests in cryptic habitats, massive reproductive ability, mass producing them in laboratory easily and safety to humans and other vertebrates (Akhurst and Smith, 2002, Ehlers *et al.,* 2005).

The nematode have used against some insect pests such as Artichoke plume moth (*Platyptilia carduidactyla*), Banana moth (*Opogona sachari*), Codling moth (*Cydia pomonella* L.), Leafminers *Liriomyza spp*. (Diptera: Agromyzidae), Scarab grubs (Coleoptera: Scarabaeidae and so on (Shapiro-Ilan *et al*., 2017). In addition the first time of isolated nematode from long horn date palm stem borer Jebusaea hummerschmidti in date palm cultivation area (Al-Jabory, 2007).

The study study aimed to diagnosis the nematode which isolated from infected larvae of *J. hummerschmidti* this infested date palm trees in Iraq.

Materials and methods

Larva sampling and isolation of entomopathogenic nematode

Larvae samples were collected from orchards date palm from stem in the depth of placements that appeared of the infestation, indicated by extraction fluid, having a brown color and soft area. White trap technique (White 1927, Kaya and Stock, 1997) were used to collect nematodes from dead larvae of the long horn date palm stem borer, nematodes suspension were reared in the laboratory on the last instar larvae of *Galleria mellonella* at 25±2°C, as described by Woodring and Kaya (1988). After 6-7 days, the samples were checked periodically and the larvae that resembled infected nematodes in the term of shape and color were transferred to White trap again.

The emerged nematodes were harvested with 9-10 days on sponge pieces falcon tube containing then maintain

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in 10°C to until use in diagnosis by the following morphological characteristics and Polymerase Chain Reaction (PCR) then to use in programs of biological control.

Morphological characteristics

The morphological diagnosis was performed in two stages:

First stage: preamble the slides by Al-Zaidawi (2019):

Fixed 1: the components are exposed to temperature at 80 and after the temperature drop at 70 the nematodes collected in the suspension are transferred into a petri dish and after the identity, incubation on a 37°C for 24 hours in a glass container containing ethanol alcohol.

Fixed 2: Add glycerol and ethyl alcohol (Table 1) for 4 hours and the same degree of incubation.

Fixed 3: Nematode treated is taken by nidal of a medical syringe and placed on a glass slide, with the covered of the slide and left to dry from the ethyl alcohol and then fixed using Canada balsam.

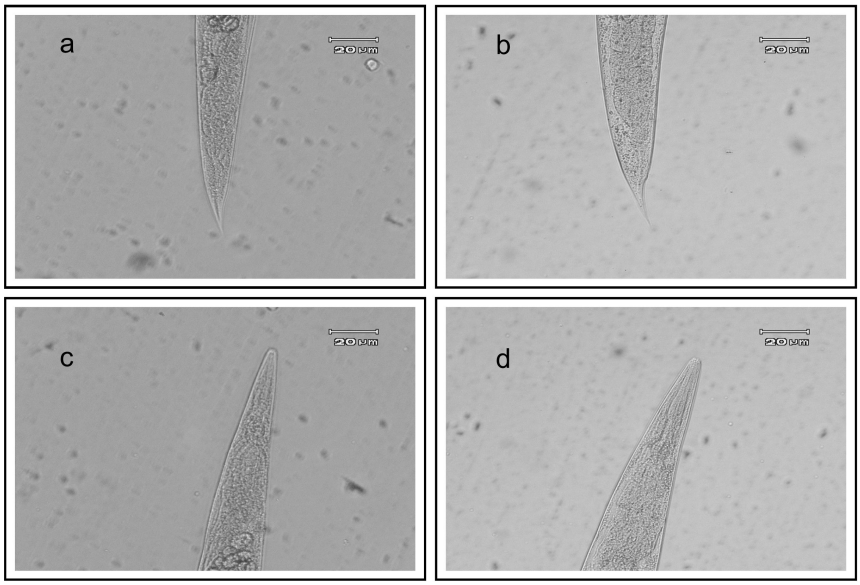
First stage: Inspection and several degrees imaging by using Optika Vision Lite 2.0 of head and tail of body.

Molecular characterization and phylogeny

The DNA of nematodes was extracted by using 5% Chelex ®100 solution (SIGMA, Bio-Rad Laboratories, Inc. USA) and kit (Parstous) (Mashhad, Iran) individual adults were collected and transferred into 1.5 ml microtubule. Then, the sample was crushed using micro-pestle and 20 µl Chelex and 1 µl Proteinase K (Parstous) (Mashhad, Iran). Then, the sample was incubated at 56°C for 3 h, following by 10 min. at 95°C into a thermo-cycler, then it was centrifuged at 13,000 rpm for 3 min. Finally, supernatant was transferred to 0.1 ml microtubule and stored at -20°C for using in the next step. The same procedure was repeated by using Kit instead of Chelex and Proteinase K as following the procedure was given by Parstous Company.

Table 1: Materials used in the preparation of slides for pathogenic nematodes.

No. of treatment	Concentration	Materials	Time of treatment
1	10%	Formalin 40%	24 hours
	1%	Glycerin	
	1%	Acedic acid	
	88%	Sterlized water	
2	5%	Glycerin	4 hours
	95%	Ethyl alcohol96%	
3	50%	Glycerin	4 hours
	50%	Ethyl alcohol 96%	



Pic. 1: entomopathogen nematodes *Rhabditis blumi* a) tail of female, b) tail of male, c) anterior female, d) anterior male

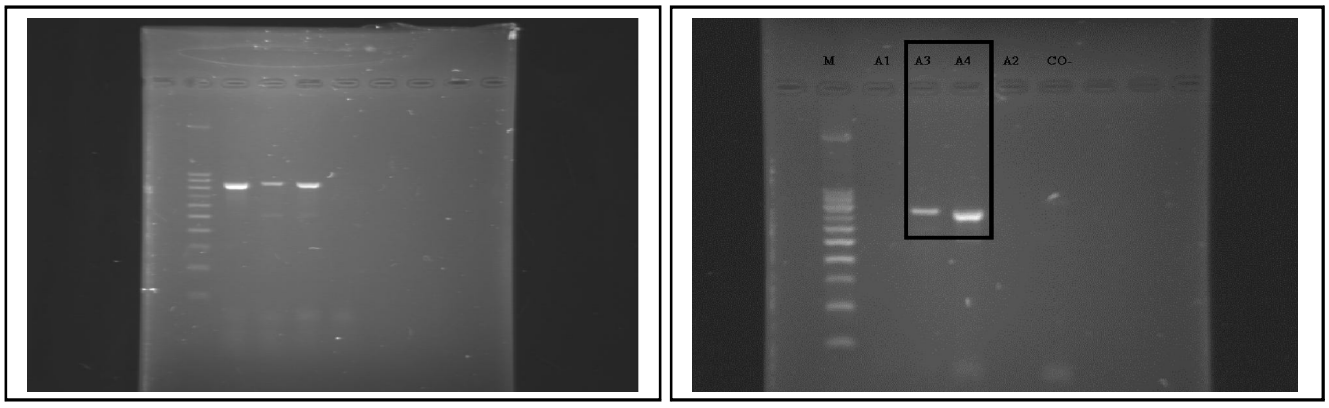


Fig. 1: PCR amplifiedene product for ITs gene.

Amplification of internal transcribed spacer (ITS)

For ITS region amplification, the primers TW81 and AB28 (Joyce *et al*., 1994) were used. Then, PCR reactions were conducted in a thermo-cycle (A300 Fast Thermal Cycler, Hangzhou Longgene Scientific Instruments Co. Ltd., China), with cycling profile according to Joyce *et al*., (1994). Initial denaturation at 94°C for four minutes; followed by 35 cycles of 94°C for one minute, 55°C for one minute and 72°C for two minute; and a final extension at 72°C for 20 minutes the PCR product was electrophoresed for one hour on 1% agarose gels with 10X TBE buffer 5% and green-viewer (SYBR) was used to stain the gel by loading 3µl from the PCR product. Basically, the final volume of PCR reaction was always 25µl. The size of amplified products was identified by comparing with 100-bp molecular DNA ladder (Parstous, Iran) (www.parstous.com).

Sequencing, sequence analysis and sequence alignment

The products were sequenced by Macrogen Co. (Korea). First, the DNA sequence was blasted to check the sequence quality. Then, Bio Edit software (Hall, 1999) was used to check the chromatograms of sequences. Also, DNA Baser was utilized to assemble the consensus sequences. Thirty one valid and verified sequences of ITS gene were retrieved from the Genbank and aligned together with the sequence from this study using Clustal X (Larkin *et. al*., 2007).

Results and Discussion

The isolated entomopathogen nematodes are characterize by the narrowing at the pre-end in one side at the body larvae. While the posturer of the female is more width than larvae male specificity in pharynx area.

The studied isolates were identified and characterized based on ITS of the DNA. The target base pair was presented on electrophoresis between 700-800bp (Fig. 1). For assessment and study the identification of indigenous parasitic nematodes that were collected from long horn date palm stem borer larvae which infested date palms trees in Iraq, two primers were used: TW81 and AB28 to identify the species based on ITS region.

Analysis and sequence of ITS region

The length of ITS gene for IRQ isolate was

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

Rhabditis_blumi_IRQ.																															
Rhabditis_blumi	0.176																														
DQ121436_Rhabditis_blumi	0.176	0.000																													
AJ291722_Oscheius_sp.	0.817	0.890	0.890																												
AJ297891_Oscheius_sp.	0.817	0.890	0.890	0.000																											
AJ297897_Oscheius_tipulae	0.766	0.824	0.824	0.118	0.118																										
AJ297898_Oscheius_tipulae	0.766	0.824	0.824	0.118	0.118	0.000																									
AJ297899_Oscheius_tipulae	0.767	0.824	0.824	0.121	0.121	0.003	0.003																								
AM398816_Pellioditis_marina	0.591	0.708	0.708	0.640	0.640	0.702	0.702	0.694																							
AM398818_Pellioditis_marina	0.591	0.692	0.692	0.646	0.646	0.692	0.692	0.685	0.036																						
AM398825_Pellioditis_mediterranea	0.614	0.696	0.696	0.679	0.679	0.690	0.690	0.683	0.170	0.183																					
AY293284_Heterorhabditis_megidis	0.633	0.683	0.683	0.620	0.620	0.591	0.591	0.591	0.387	0.399	0.387																				
AY321481_Heterorhabditis_zealandica	0.610	0.648	0.648	0.610	0.610	0.598	0.598	0.598	0.391	0.395	0.390	0.117																			
DQ121441_Rhabditella_axe	0.422	0.430	0.430	0.969	0.969	0.886	0.886	0.886	0.668	0.709	0.686	0.674	0.638																		
EF469774_Heterorhabditis_bacteriophora	0.583	0.658	0.658	0.683	0.683	0.677	0.677	0.677	0.372	0.384	0.437	0.229	0.241	0.601																	
EF503690_Heterorhabditidoides_chongmingens	0.614	0.651	0.651	0.637	0.637	0.639	0.639	0.633	0.489	0.510	0.550	0.520	0.498	0.619	0.559																
EF530041_Heterorhabditis_zealandica	0.610	0.648	0.648	0.610	0.610	0.598	0.598	0.598	0.391	0.395	0.390	0.117	0.000	0.638	0.241	0.498															
EU131007_Caenorhabditis_elegans	0.938	0.941	0.941	1.185	1.185	1.190	1.190	1.207	0.847	0.895	0.845	1.005	0.939	0.856	0.926	0.956	0.939														
EU273598_Oscheius_chongmingensis	0.614	0.651	0.651	0.637	0.637	0.639	0.639	0.633	0.489	0.510	0.550	0.520	0.498	0.619	0.559	0.000	0.498	0.956													
FJ473361_Heterorhabditis_safricana	0.621	0.648	0.648	0.589	0.589	0.592	0.592	0.592	0.352	0.368	0.370	0.065	0.092	0.679	0.190	0.503	0.092	0.946	0.503												
FJ547241_Oscheius_carolinensis	0.598	0.653	0.653	0.572	0.572	0.616	0.616	0.623	0.457	0.472	0.499	0.449	0.481	0.676	0.525	0.296	0.481	0.906	0.296	0.469											
FM992594_Cloacina_caenis	0.673	0.760	0.760	0.631	0.631	0.647	0.647	0.640	0.421	0.420	0.443	0.392	0.402	0.706	0.474	0.562	0.402	0.953	0.562	0.365	0.448										
FM992611_Cloacina_robertsi	0.675	0.777	0.777	0.630	0.630	0.632	0.632	0.639	0.421	0.420	0.444	0.401	0.402	0.692	0.484	0.568	0.402	0.930	0.568	0.374	0.453	0.014									
FR854206_Labiosimplex_irma	0.721	0.788	0.788	0.599	0.599	0.605	0.605	0.612	0.472	0.471	0.489	0.383	0.412	0.674	0.453	0.563	0.412	1.023	0.563	0.383	0.434	0.086	0.092								
FR854211_Labiosimplex_major	0.673	0.758	0.758	0.605	0.605	0.600	0.600	0.606	0.480	0.473	0.480	0.369	0.393	0.695	0.454	0.578	0.393	1.006	0.578	0.361	0.435	0.071	0.080	0.045							
Heterorhabditidoides	0.614	0.651	0.651	0.637	0.637	0.639	0.639	0.633	0.489	0.510	0.550	0.520	0.498	0.619	0.559	0.000	0.498	0.956	0.000	0.503	0.296	0.562	0.568	0.563	0.578						
Heterorhabditidoides_rugolensis	0.626	0.664	0.664	0.656	0.656	0.659	0.659	0.652	0.494	0.510	0.551	0.514	0.492	0.619	0.559	0.011	0.492	0.989	0.011	0.496	0.297	0.561	0.567	0.562	0.577	0.011					
HM230723_Heterorhabditis_atacamensis	0.614	0.640	0.640	0.589	0.589	0.592	0.592	0.592	0.347	0.363	0.366	0.074	0.095	0.686	0.190	0.508	0.095	0.949	0.508	0.008	0.474	0.357	0.365	0.374	0.352	0.508	0.502				
JQ002565_Heterorhabditidoides_sp.	0.626	0.664	0.664	0.656	0.656	0.659	0.659	0.652	0.494	0.510	0.551	0.514	0.492	0.619	0.559	0.011	0.492	0.989	0.011	0.496	0.297	0.561	0.567	0.562	0.577	0.011	0.000	0.502			
JQ359018_Heterorhabditis_indica	0.661	0.705	0.705	0.682	0.682	0.669	0.669	0.669	0.423	0.412	0.468	0.304	0.292	0.666	0.226	0.537	0.292	0.923	0.537	0.264	0.484	0.480	0.479	0.460	0.444	0.537	0.542	0.264	0.542		
JX155307_Strongylida_environmental	0.682	0.785	0.785	0.631	0.631	0.656	0.656	0.649	0.426	0.425	0.449	0.406	0.403	0.714	0.500	0.574	0.403	0.941	0.574	0.379	0.453	0.016	0.019	0.099	0.083	0.574	0.573	0.379	0.573	0.496	
JX155345_Strongylida_environmental	0.697	0.797	0.797	0.618	0.618	0.632	0.632	0.639	0.427	0.426	0.445	0.397	0.398	0.722	0.496	0.575	0.398	0.909	0.575	0.375	0.448	0.030	0.027	0.101	0.086	0.575	0.575	0.375	0.575	0.473	0.019

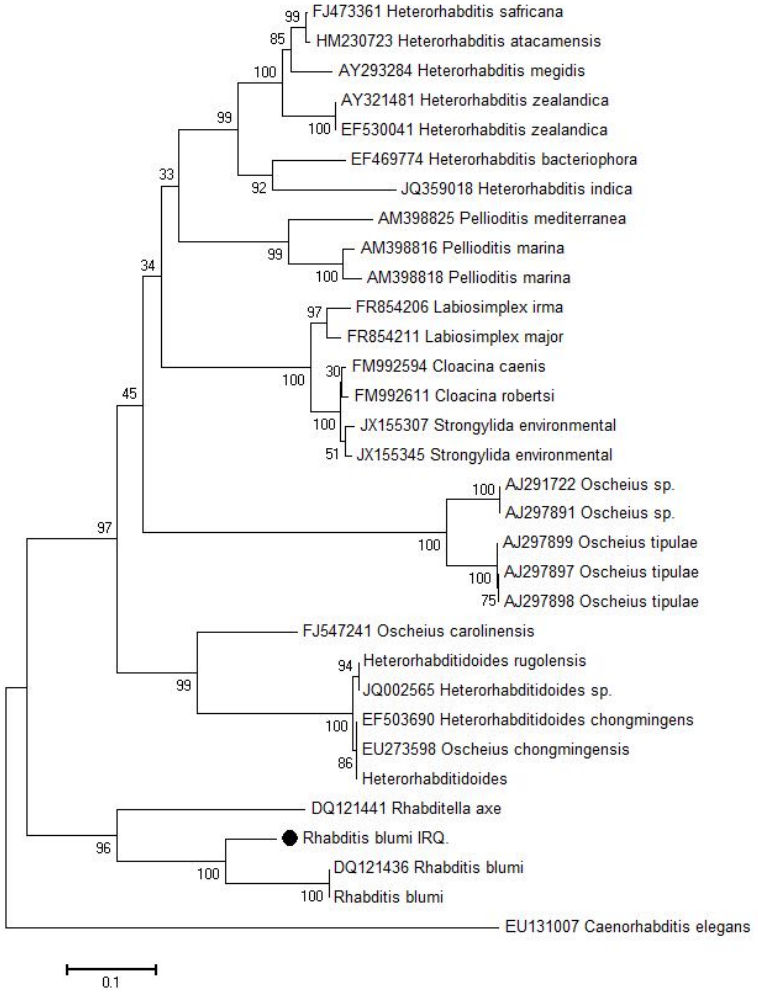


Fig. 2: Phylogenetic relationships of studied *Rhabditis blumi* isolates with 30 *Heterorhabditis* species based on ITS sequences as inferred from Maximum Likelihood (ML) analyses. *Caenorhabditis elegans* (EU131007) was used as the out group. Support values are presented near the nodes in the form: bootstrap in ML.

825 bps and BLAST analysis base on ITS gene for Iraqi population of nematodes attributes 99% similarities and 96% of query coverage with *Rhabditis blumi* (DQ121436) which were the highest match in ITS, the results showed that more than 30 matched with various species of EPN in GenBank belonging to Heterorhabditis genera.

The multiply alignment of a 918 bps segment of ITS gene for 30 (Fig. 2) taxa showed that 101 sites were conserved, 751 sites were variable, 658 sites were singleton and 71 sites were parsimony informative. The phylogenetic tree reconstructed based on ITS sequences, using Neighbor-joining analysis, showed *Rhabditis blumi IRQ.1* forms a monophyletic group with other *Rhabditis blumi* isolates from other regions around the world (DQ121436), *Heterorhabditidoides* isolates and the '*Insectivora*' group of *Rhabditis* were placed in a single clade. (Fig. 2).

Mean interspecific distance of ITS sequences was 0.53% (range 0.00-1.11), which was calculated by the Tamura 3-parameter model. There was 0.17% difference between Iraqi isolates and *Rhabditis blumi* (DQ121436). (Table 2).

Fig. 2 Phylogenetic relationships of studied *Rhabditis blumi* isolates with 30 *Heterorhabditis* species based on ITS sequences as inferred from Maximum Likelihood (ML) analyses. *Caenorhabditis elegans* (EU131007) was used as the out group. Support values are presented near the nodes in the form: bootstrap in ML.

Distribution of EPN species and isolates in different areas could be exhibited significance in behavioral and physiological adaptation. Therefore, it is crucial for the successful use of EPN as a biological control agent to identify and document the locally adapted species or isolates (Stock *et al*., 1999).

Many EPN identifications are based on traditional morphological methods, but recently some scientists have used molecular techniques for the identification of EPNs. The use of molecular methods can greatly reduce the amount of time needed for identification of unknown nematode isolates. PCR offers several advantages compared to more traditional methods of diagnosis: organisms do not need to be cultured prior to processing by PCR, the technique has sensitivity and it is rapid and versatile (Lee *et al*., 1993). Most recently, DNA sequence analysis of internal transcribed spacer region (ITS-1) of rDNA (Nguyen *et al*., 2001).

Molecular characterization of nematodes considered analysis of sequence data from the internal transcribed spacer region (ITS) of rDNA (Hominick *et al*., 1997, Stock *et al*., 2001). We therefore sequenced and aligned partial sequence of ITS rDNA (918 nucleotides). Molecular analysis was performed on adult females because their large size provided an appropriate amount of DNA. Sequencing of the nucleotides of organisms enables use of the sequence differences amongst EPN isolates for identification and affirmation of new species (Szalanski *et al*., 2000). 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).

References

Akhurst, R. and K. Smith (2002). Regulation and safety. In R. Gaugler (ed.), Entomopathogenic Nematology, CAB International, Wallingford, UK., 311-332.

Al-Jboory, I. (2007). Survey and identification of the biotic factors in date palm environment and its application for designing IPM-program of date palm pests in Iraq. *Aden Univ. J. Appl. Nat. Sci*., **11(3):** 1-28.

Al-Zaidawi, J.B. (2019). Isolation and characterization native Iraqi entomopathogenic nematodes and efficacy of virulence on termite *Microcerotermes diversus* (Insecta:Isoptera). Degree of Doctor Philosophy in Agricultural Science. Ferdowsi University of Mashhad, 52.

Bedding, R.A., A.S. Molyneux and R.J. Åkhurst (1983). *Heterorhabditis* spp., *Neoaplectana* spp. And *Steinernema kraussei*: Interspecific and intraspecific differences in infectivity for insects. *Exp. Parasitol*., **55:** 249-257.

Ehlers, R.U. and S.D.I. Ilan Mass production (2005). In: P.S. Grewal, R.U. Ehlers, D.I. Shapiro-Ilan editors. Nematodes as Biocontrol Agents. Wallingford, UK: CABI Publishing, 65-78.

Hall, T.A. (1999). Bio Edit: a user friendly biological sequence alignment editor and analysis program for Windows 95/ 98/*NT. Nucleic Acids Symposium Series*., **41:** 95-98.

Hominick, W.M., B.R. Briscoe, F.G. Del Pino, J.A. Heng, D.J. Hunt, E. Kozodoy, Z. Mracek, K.B. Nguyen, A.P. Reid, S.E. Spiridonov, S.P. Stock, D. Sturhan, C. Waturu and M. Yoshida (1997). Biosystematics of entomopathogenic nematodes: Current status, protocols and *deWnitions. J. Helminthol*., **71:** 271-298.

Joyce, S., A. Reid, F. Driver and J. Curran (1994). Application of polymerase chain reaction (PCR) *methods to identification of entomopathogenic nematodes Cost.*, 178-187.

Kaya, H.K. and S.P. Stock (1997). Techniques in insect nematology. *in* L.A. Lacey, ed. Manual of Techniques in Insect Pathology. San Diego, *CA: Academic Press*., 281-324.

Larkin, M.A., G. Blackshields, N. Brown, R. Chenna, P.A. Mcgettigan, H. Mcwilliam, F. Valentin, I.M. Wallace, A. Wilm and R. Lopez (2007). W. Clustal and Clustal X version 2.0. B*ioinformatics.,* **23:** 2947-2948.

Lee, R.C., R.L. Feinbaum and V.R. Ambros (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell.,* **75:** 843-54.

Nguyen, K.B., J. Maruniak and B.J. Adams (2001). The diagnostic and phylogenetic utility of the rDNA internal transcribed spacer sequences of *Steinernema*. *Journal of Nematology.,* **33:** 73-82.

Spiridonov, S.E., K. Reid, S.A. Podrucka and M. Subbotin (2004). Moenz Phylogenetic relationships within the genus *Steinernema* (Nematoda : Rhabditida) as inferred from analyses of sequences of the ITS 1-5. 8S-ITS2 *region of rDNA and morphological features Nematology.*, **6:** 547-566.

Shapiro-Ilan, D.I., T.E. Cottrell, R.F. Mizell and D.L. Horton (2017). Curative control of the peachtree borer using *entomopathogenic nematodes. J. Nematol*., **48:** 170-176.

Stock, S.P., B.M. Pryor and H.K. Kaya (1999). Distribution of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) in natural habitats in California, USA. Biodivers. *Conserv.*, **8:** 535-549.

Stock, S.P., J.F. Campbell and S.A. Nadler (2001). Phylogeny of Steinernema Travassos, 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. *J. Parasitol.*, **87:** 877-889.

Szalanski, A.L., D.B. Taylor and P.G. Mullin (2000). Assessing nuclear and mitochondrial DNA sequence variation in Steinernema (Rhabditida: Steinernematidae). *Journal of Nematology.,* **32:** 229-233.

Toyce, S.A., A.M. Burnell and T.O. Powers (1994). Characterization of *Heterohabditis* isplates by PCR amplification of segments of mtDNA and rRNA genes. *Journal of Nematology.*, **26:** 260-270.

White, G. F. 1927. A method for obtaining infective nematode larvae from cultures. *Science.,* **66:** 302-303.

Woodring, J.L. and H.K. Kaya (1988). Steinernematid and heterorhabditid nematodes: A handbook of biology and techniques. Southern Cooperative Series Bulletin, Arkansas Agricultural Experiment Station, Fayetteville, AR. 331, 30.