Molecular Characterization of Several Isolates of Entomopathogenic Nematode *Heterorhabditisbacteriophora* Poinar (Rhabditida, Heterorhabditidae) from Soil of Citrus Orchards in Latakia, Syria

Ghadah Zeini[#1], Nabil AboKaf*[2], Mazen Al- Body[#3], Majda Mofleh[#3]
#Postgraduate Student (Doctorate), Department of Plant Protection, Faculty of Agriculture, Tishreen University, Latakia, Syria
**Professor, Plant Protection Department, Faculty of Agriculture, Tishreen University,Latakia, Syria*
#Researcher, Plant ProtectionDepartment, General Commission for Scientific Agriculture Research, Latakia. Syria

Abstract

Heterorhabditisbacteriophora was isolated from the soil of citrus orchards in different localities of Latakia Governorate, Syria. Collected material was identified based on the morphometric and morphological characters. Internal transcribed spacer 1 (ITS1) of five isolates was amplified using the PCR. Sequences of collected material have compared with available genetic data in the GeneBank. The identity of the collected material with the available sequence reached up to 99%. Phylogeny tree of the isolates inferred based on the ITS1 sequences of five Syrian isolates and some international sequences of the same and other related species using Steinernemafeltiae as an outgroup. Syrian isolates of H. bacteriophora grouped in the same clad with other related isolates of neighboring countries.

Keywords: *Entomopathogenic, Heterorhabditis, ITS1, Isolate, Syria.*

I. INTRODUCTION

Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae are lethal pathogens of insects. Their success in this role can be attributed to the unique symbiotic relationship between these nematodes and a lethal insect-pathogenic bacterium. Because of their biocontrol potential, considerable attention has been directed over the past few decades to *Heterorhabditis* and *Steinernema* and their respective bacterial endosymbionts, *Photorhabdus* and *Xenorhabdus* [4].
Morphological and morphometric characteristics can be used to identify species of *Steinernema*, but they are less reliable for species differentiation in *Heterorhabditis*. Molecular characterization is necessary for final confirmationof identity [14]. Noncoding internal transcribed spacers 1 and 2 (ITS1 and ITS2) of the ribosomal DNA (rDNA) were indicated as a powerful tool to identify species because it can supply with species-specific polymorphism[22].

The first report of this entomopathogenic nematode, *H. bacteriophora* was from Australia, where it was recovered from the larvae of *Helicoverpapunctigera*[9].
To date, only 16 species under the genus*Heterorhabditis* have been described in addition to 95 species belong to the genus *Steinernema* [14]. Species of the genus *Heterorhabditis*have beendivided into three groups, *bacteriophora* group with three species, *megidis* group which includes six species while, *indica* group includes seven species [13], [21]. Recently, a few species of genus *Heterorhabditis* have been described based on the molecular as well as morphological characters, these species namely, *H. amazonensis*[23], *H. floridensis* [11], and *H. mexicana* [10]. However, four species of *Heterorhabditis* were synonymized based on the molecular data [5].

Two entomopathogenic nematodes species *H. bacteriophora* and *S. feltiae* were reported for first time from Lebanon [6]. These two species of nematodes are common of Mediterranean region. Five isolates of the species *H. bacteriophora* and two isolates of *S. feltiae*were reported from different localities of Eastern Black Sea region, Turkey [24].
Recently, several survey studies were conducted in differentagro-ecosystems of different climate zones of Syria revealed a good number of new records of EPNs.The first report of the species *H. bacteriophora* was from Deer Al-Zoor, and Al-RakaGovernorates of Eastern part of Syria [19].So far, one isolate of *S. cubanum*and12 isolates of *H. bacteriophora* were recovered from stone fruit

orchards in Latakia, costal area of Syria [25]. However, *H. zealandica*was reported for the first time from Damascus, Southern of Syria [1], Later several species namely, *H. bacteriophora*, *H. indica*and *H. zealandica*were reported from soils of mainly stone fruit and apple in addition to samples of vineyards, citrus and walnut orchards of Damascus countryside governorate, southern part of Syria [2]. Furthermore, eight isolates were recovered from soil samples of citrus orchards and identified as *H. bacteriophora* [17].

This study aimed at confirming the identity and revealing the phylogenetic relationship of five-entomopathogenic nematode isolates belonging to the species*H. bacteriophora*by characterizing the *ITS1* region of rDNA.

II. MATERIALS AND METHODS

A. *Soil sample and isolation of nematodes*:

EPNs were recovered from soil samples by the insect baiting technique [18], using seventh instar larvae of the greater wax moth *Galleriamellonella*. Infective juveniles (IJs) were harvested from *G. mellonella* cadavers using White traps [7]. Emerging juveniles were inoculated to live *G.mellonella* larvae to confirm Koch's postulates of pathogenicity. Nematode suspensions were kept in shallow layer of water in 25 cm^2 tissue culture flasks for storage and further studies. Only five positive samples are selected for further studies. For each sample, several details were recorded and summarized (Table, 1).

B. *Morphological and morphometric:*

Thirty IJs of each isolate were observed. Measurements were made and the followingcharacters of IJs were analyzed [17]: Body length (L), maximum body width (MBW), distance from anterior end to excretory pore (EP), distance from anterior end to nerve ring (NR), distance from anterior end to end of pharynx (ES), tail length (T), D%= EP/ESx100, E%= EP/Tx100. Preliminary identification of the EPN was based on general morphology[3], [12].

C. *DNA extraction*

For extraction of the DNA of the nematodes, Twenty ofinfective juveniles for each isolate were suspendedin 400 µL of lysis buffer (10 mMTris-HCl, pH 8.0; 100 mM EDTA; 100 mMNaCl; 1% sodiumdodecyl sulfate [SDS]) Proteinase K(500)µg/ml was added and digested for 2 h at 55° C and keep shaking the tubes every 15 min. For the extraction, three steps were followed: in the first, an equal volume of phenol was used; in the second, 200 µl of phenol and 200 µl of chloroform/isoamyl alcohol (24/l) were used; and in the third, 400 µl of chloroform/isoamyl alcohol (24/l) was used. After each extraction step, phases were separated by spinning at 12,000 rpm for 3 min.The aqueous phase finally obtained was precipitated with l/10 volume of 3 M sodium acetate and 2.5 volumes of -20° C cooled absolute ethanol and refrigerated at -20° C for one hour to overnight. The spooled DNA or pellet obtained was washed in 70% ethanol, centrifuged at 12,000- 13,000 rpm for 5-10 min at 4° C, and briefly air dried (inverting the tube on a paper towel).The precipitated DNA was eluted in a small volume (20-50 µl) of TE Buffer (10 mMTris-HCl, pH 7.6; 1 mM EDTA) [15], [16].

Table 1. Collection details of entomopathogenic nematodeH. bacteriophorafrom some citrus orchards in Latakiagovernorate

Isolate codes	Location	Date of collection	Coordinate
GA1	Gio	Oct. 2016	N 35˚28.3' E 035˚56.9'
MG2	Al-Magreet	May 2016	N 35˚36.2' E 35˚48.9'
BA1	Al-Bassa	Oct. 2016	N 35˚29.9' E 035˚50.5'
SR1	Al-Sorsokia	July 2016	N 35˚42.4' E 035˚53.9'
B1	Borj Al-Qasab	May 2016	N 35˚35.7' E 035˚47.6'

D. *DNA amplification*

ITS1 region of the extracted DNA was amplified by PCR in a 50 µL reaction mixture which was containing: 5 µL of 10X PCR buffer (200 mMTris-Hcl pH8 500 mMKcl), 2 µL of MgCl2 (25mM), 1 µL of dNTP mixture (10 mM of each dNTP), 1.5µL (1 unit) of *Taq* DNA polymerase and 33.5 µL of double distilled water, 1 µL of the forward primer TW81: 5'GTTTCCGTAGGTGAACCTGC–3', and 1 µL of the reverse primer AB28: 5'–ATATGCTTAAGTTCAGCGGGT–3', 5µL of the extracted DNA. Program of thermocycler for the PCR reaction was as: one cycle at 94°C for 2 min. (Initial denaturation), 35 cycles at 94°C for 1min. (Denaturation), follow 55°C for 45 sec. (Annealing) and 72°C for 2 min. (Extension) then 72°C for 10 min. (Final extension) [20], [24].

E. *Purification and quantification of PCR products*

10µL of the product was loaded on a 1.2% agarose gel stained with 0.5 µg/ml ethidium bromide,

with 1X TBE buffer running solation and under voltage 80 v for two hours. 100-bp DNA ladder was used for approximant quantification of the DNA product. The gel was visualized under ultraviolet light and photographed using a gel documentation system (Bio.Doc. Analyze).

The target bands were purified using a gel purification kit NucleoSpin®Extract II Kit (Doreen, Germany).PCR cycle sequencing was performed (SEQ-8000–Germany) using the same primers (TW81 and AB28) used in PCR amplification reactions. DNA extraction, DNA amplification, Purification and quantification of PCR products were conducted in Technical University of Dresden, Institute of Botany, Germany.

F. Statistical analysis of sequences

ITS1 sequences of the Syrian isolates were compared with NCBI GeneBankdatabase of nucleotides through BLAST Tool. DNA sequences of the five Syrian isolates were submitted to GeneBank (NCBI) and accession for each sequence is provided and summarized in the (Table, 2). Sequences of selected isolates were aligned and analyzed usingMEGA-X® with sequences of some isolates of the same species and some other related species of the same genus available in the GeneBank. Accessions and localities of selected isolates are included in the Table 2. The phylogeneticrelationship of the Syrian and some related isolates was evaluated using the MEGA-X® software. Phylogeny tree of the selected sequences inferred using neighbor joining method with 1000 bootstrap replicates.

Table 2. Details of local and international isolates of entomopathogenic nematode *H.bacteriophora*and some related species used in the phylogeny study of genetic diversity.*Steinernemafeltiae*is used as an outgroup

No.	Taxon	NCBI GenBank Accession	Country
1	*Heterorhabditisbacteriophora* GA1	MK474645.1	Syria
2	*Heterorhabditisbacteriophora* MG2	MK474643.1	Syria
3	*Heterorhabditisbacteriophora* BA1	MK474644.1	Syria
4	*Heterorhabditisbacteriophora* SR1	MK474617.1	Syria
5	*Heterorhabditisbacteriophora* B1	MK474619.1	Syria
6	*Heterorhabditisbacteriophora*	MK211207.1	Iraq
7	*Heterorhabditisbacteriophora*	JX544064.1	Turkey
8	*Heterorhabditisbacteriophora*	HM140690.1	Lebanon
9	*Heterorhabditisfloridensis*	DQ372922.1	USA
10	*Heterorhabditisgeorgiana*	EU099032.1	USA
11	*Heterorhabditisindica*	FJ935792.1	India
12	*Heterorhabditismarelatus*	EF043441.1	USA
13	*Heterorhabditismegidis*	EF043439.1	Ireland
14	*Heterorhabditis_mexicana*	AY321478.1	Ireland
15	*Heterorhabditissafricana*	EF488006.1	USA
16	*Heterorhabditiszealandica*	AY321481.1	USA
17	*Steinernemafeltiae*	MK294325.1	Poland

III. RESULTS AND DISCUSSION

A. Molecular phylogenetic

ITS1 sequences were supporting the morphometric and morphological study of these isolates. The identity *percent* of the Syrian isolates reached up to 99% when compared with GeneBank database through the BLASTTool.

Based on *ITS1* sequences phylogenetic tree obtained using neighbor joining method with 1000 bootstrap. Result showed that the Syrian populations of *H. bacteriophora* are monophyletic group with high support value of bootstrap (100%) and *S. feltiae*is used as an outgroup (Fig. 1). Isolates of neighboring countries are grouped with local isolates of Syria in which support the identity at morphometric and morphological level [17].

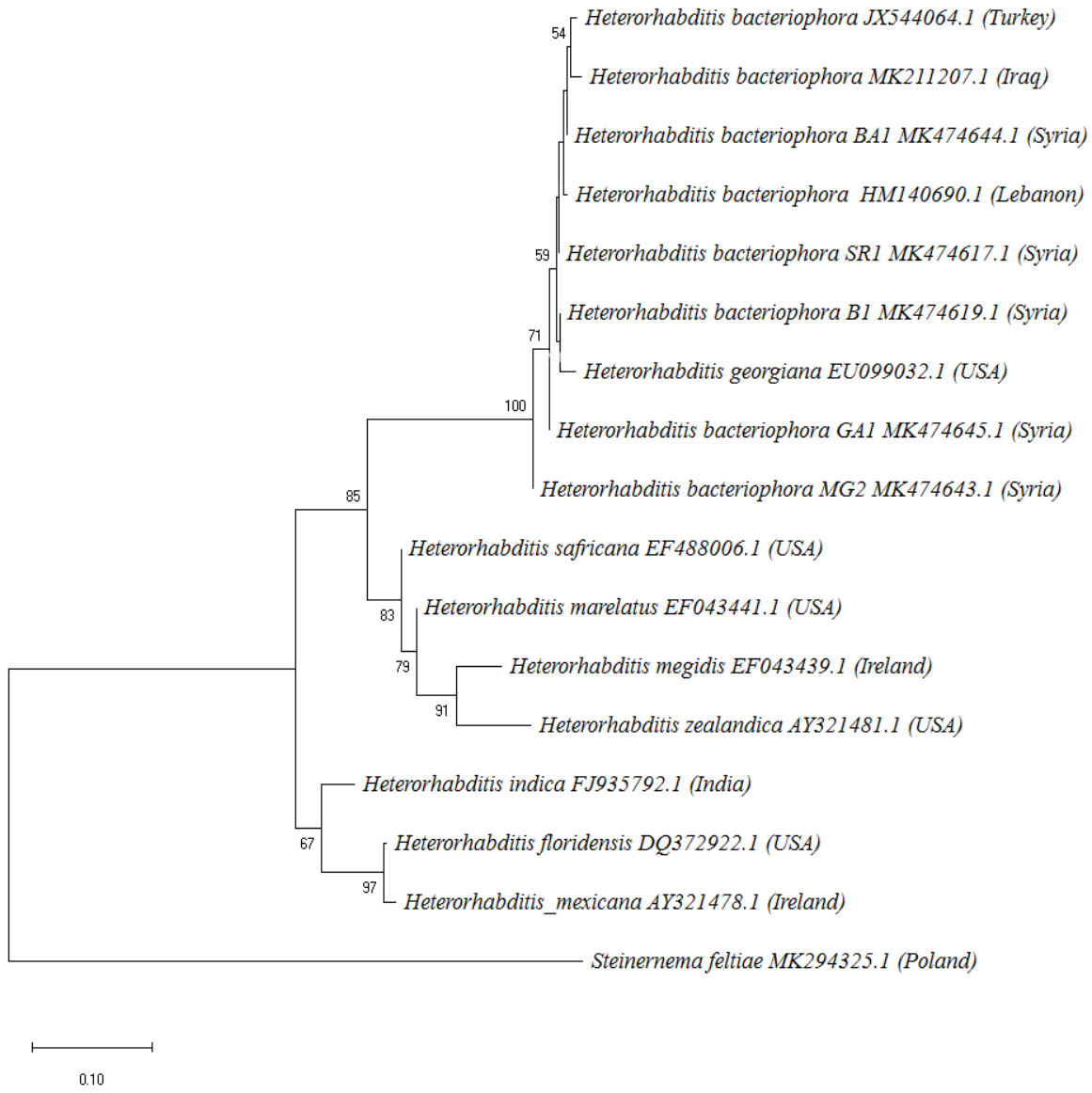

Figure (1) Phylogenetic relationships of the Syrian and neighboring countries isolates of *H.bacteriophora* with other species under same genus based on analysis of *ITS1*regions using the neighbor joining method. Numbers higher than 50% on branches indicate the percentage of 1000 bootstrap replicates.*Steinernemafeltiae*used as outgroup.

Al-Magreet isolate is a sister group of the other isolates of *H. bacteriophora* (Fig. 1). Species *H. georgiana*, placed in the same clad of *bacteriophora*, the close relationship of *H. bacteriophora* strain with *H. georgiana* was reported [13]. It is very difficult to distinguish these two species;also, *H. georgiana* is an American species with no more information about its occurrence from Europe [8].While other related species under the genus *Heterorhabditis*are separated in two clads supporting the division of the genus into three clads [13], [21].

IV. CONCLUSION

May it is not the first study dealing with molecular identification of ENPs from Syria. However, sequences of Syrian isolates are shared internationally through the GeneBank database during this study. This is a preliminary step in the way of integrative taxonomy of entomopathogenic nematodes from Syria using the molecular tools as well as morphological features. This technique can be used at the commercial production of this entomopathogenic nematode as bio-control agent in future. Due to the difficulties in discrimination of the species belong to genus *Heterorhabditis*,it is necessary to maintain the DNA profile of each species under the genus globally.

ACKNOWLEDGMENT

Authors would like to thank Marko Höffman and Ralf Olbrischt, University Kassel andHafez Mahfoud, General commission for Scientific Agricultural Research in Latakiafor technical assistance and helpful discussions, andthanks are given to MahranZeity for his assistance in software analysis and improvement the quality of the paper. Authors are grateful to General commission for Scientific Agricultural Research for providing the material and facilities of this investigation.

REFERENCES

[1] A.Jawish. Occurrence of entomopathogenic nematodes at Damascus Governorate and evaluating its pathogenicity on some insect hosts. M.SC. Thesis, Faculty of Agriculture. Damascus University, Syria, pp. 99, 2010.

[2] A.Jawish, K. Al-Assasand A. Basheer. A survey of entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) in Damascus countryside of Syria. Bulletin OEPP/EPPO Bulletin 45(1): 81–89, 2015.

[3] A.Lucskai. Identification Key to Entomopathogenic Nematode Species. ActaPhytopathologica et EntomologicaHungarica. 34(4): 317–325, 1999.

[4] T. Griffin, N. E. Boemare and E. E. Lewis. Biology and behaviour. In: Grewal PS, Ehlers RU, Shapiro-Ilan DI (eds) Nematodes as biocontrol agents. CABI Publishing, Oxon, pp 47–64, 2005.

[5] J. Hunt and S. A. Subbotin. Taxonomy and systematics. In: Nguyen KB (ed) Hunt DJ, vol 12. Advances in entomopathogenic nematode taxonomy and phylogeny. Nematology monographs and perspectives. Brill Publishing, Leiden, pp 13–58, 2016.

[6] Noujeim, C. Khater, S. Pages, J. Ogier, P. Tailliez, M. Hamze, and O. Thaler. The first record of entomopathogenic nematodes (Rhabiditiae: Steinernematidae and Heterorhabditidae) in natural ecosystems in Lebanon: A biogeographic approach in the Mediterranean region. Journal of Invertebrate Pathology Elsevier Inc. (107): 82–85, 2011.

[7] F. White. A method for obtaining infective nematode larvae from cultures. Science Journal, 66, 302-303, 1927.

[8] I.Majić, A. Sarajlić, T. Lakatos, T. Tóth, E. Raspudić, Z. Puškadija, G. KanižaiŠarić and Ž. Laznik. Virulence of new strain of Heterorhabditisbacteriophorafrom Croatia against Lasiopterarubi. Plant Protect. Sci., 55: 134–141, 2019.

[9] J.R. G. O. Poinar. Description and biology of a new insect parasitic rhabditoid, Heterorhabditisbacteriophora n. gen. n. sp. (Rhabditida; Heterorhabditidaen. fam.). Nematologica, 21, 463-470, 1976.

[10] K.B. Nguyen, D. I. Shapiro-Ilan, R. J. Stuart, C. W. Mccoy, R. R. James and B.J. Adams. Heterorhabditismexicana n. sp. (Rhabditida: Heterorhabditidae) from Tamaulipas, Mexico, and morphological studies of the bursa of Heterorhabditis spp. Nematology 6, 231-244, 2004.

[11] K.B. Nguyen, U. Gozel, H. S. Koppenhöfer and B. J. Adams. Heterorhabditisfloridensis n. sp. (Rhabditida: Heterorhabditidae) from Florida. Zootaxa 1177, 1-19, 2006.

[12] K.B. Nguyen and D. J. Hunt. Entomopathogenic nematodes: systematics, phylogeny and bacterial symbionts. Nematology monographs and perspectives. Leiden, E.J. Brill Eds. Vol. 5, 816 pp, 2007.

[13] K.B.Nguyen, D. I. Shapiro-Ilan and G. N. Mbata. Heterorhabditisgeorgiana n. sp. (Rhabditida: Heterorhabditidae) from Georgia, USA. Nematology 10, 433-448. 2008.

[14] K.B.Nguyen. Tabular keys to species of Steinernema and Heterorhabditis. In: Nguyen KB (ed) Hunt DJ, vol 12. Advances in entomopathogenic nematode taxonomy and phylogeny. Nematology monographs and perspectives. Brill Publishing, Leiden, pp 59–109, 2016.

[15] M.D. Bargues, F. G. Marquez and S. Mas-Coma. Rapid nonradioactive and non-fluorescent rDNA sequencing: Application to parasites. Research and Reviews in Parasitology 55: 199–205, 1995.

[16] M.D. Bargues and S. Mas-Coma. Phylogenetic analysis of lymnaeid snails based on 18S rDNA sequences. Molecular Biology and Evolution 14: 569–577, 1997.

[17] N.Abo Kaf, M. Al- Body,M. Mofleh andG. Zeini. Natural Occurrence of Entomopathogenic Nematodes (EPNs) in Citrus Orchards in Lattakia Governorate, Syria. Faculty of Agriculture.Tishreen University Journal for Research and Scientific Studies 40(5):31-47, 2018.

[18] R.A. Bedding and R. J Akhurst. A simple technique for the detection of insect parasitic rhabditid nematode in soil. Nematologica, 221, 109-110, 1975.

[19] R.Canhilal, W. Reid, H. Kutuk and M. El-Bouhssini. Natural Occurrence of entomopathogenic nematodes (Rhabditida: SteinernematidaeandHeterorhabditidae) in Syrian soils. Research Journal of Agriculture and Biological Sciences, 2(6): 493-497, 2006.

[20] S.A. Joyce, A. Reid, F. Driver and J. Curran. Application of polymerase chain reaction (PCR) methods to the identification of entomopathogenic nematodes. In:Burnell AM, Ehlers RU, Masson JP, (Eds). Genetics of Entomopathogenic Nematode-Bacterium Complexes. European Commission Public. EUR 15681 EN: 178-187, 1994.

[21] S.E. Spiridonov, S. A. Subbotin, Phylogeny and phylogeography of Heterorhabditis and Steinernema. In: Nguyen KB (ed) Hunt DJ, vol 12. Advances in entomopathogenic nematode taxonomy and phylogeny. Nematology monographs and perspectives. Brill Publishing, Leiden, pp 413–427, 2016.

[22] T.O. Powers, T. C. Todd, A. M. Burnell, P. C. Murray, C. C. Fleming, A. L. Szalanski, B. A. Adams and T.S. Harris. The rDNA Internal transcribed spacer region a taxonomic marker for nematodes. J Nematol., 29:441-450,1997.

[23] V.Andaló, K. B. Nguyen and A. Moino. Heterorhabditisamazonensis n. sp. (Rhabditida: Heterorhabditidae) from Amazonas, Brazil. Nematology 8(2006), 853-867,2007.

[24] Z.Erbas,C. Gokce, S. Hazir, Z. Demirbag and IDemir. Isolation and identification of entomopathogenic nematodes (Nematoda: Rhabditida) from the Eastern Black Sea region and their biocontrol potential against Melolonthamelolontha (Coleoptera: Scarabaeidae) larvae. Turk J Agric For, 38: 187-197,2014.

[25] Z.Mosalam. Efficacy of entomopathogenic nematodes against Capnodis spp. in stone-fruit orchards. PHD. Thesis, Faculty of Agriculture. Tishreen University, Syria, pp. 117, 2009.