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New data on *Heterorhabditis bacteriophora* Poinar, 1976 from south eastern Iran

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During a survey on the entomopathogenic nematodes from Kerman province of Iran, three new populations of *Heterorhabditis bacteriophora* were recovered from natural areas. Description, measurement and illustration are provided for these isolates. Molecular analysis on ITS sequence of new isolates of *H. bacteriophora* set these populations close to a population from South Africa (EU700310). *H. bacteriophora* and *H. georgiana* formed a monophyletic group. This is the first data of ITS rDNA of *H. bacteriophora* from south eastern Iran (Kerman province). Analysis of Iranian *H. bacteriophora* showed that Kerman isolates place close to Mashhad isolates.

Key words: *Heterorhabditis*, Iran, ITS rDNA, morphology, phylogeny, taxonomy.

INTRODUCTION

The genus *Heterorhabditis* Poinar 1975 belongs to the order Rhabditida and are obligate parasite of insects. The entomopathogenic nematode (EPN) species, *Heterorhabditis bacteriophora* is utilized as biocontrol agents against various insect pests of agricultural significance (Grewal et al. 2005; Kooliyottil et al. 2013). The infective juvenile stage carries a Gram-negative bacterium in anterior part of intestine and transmits to the hemocoel of insect hosts (Poinar et al. 1977). This symbiotic bacterium (*Photorhabdus luminescens*) has been studied in different aspects (Strauch and Ehlers, 1998; Fischer-Le Saux et al. 1999; Duchaud et al. 2003; Ciche et al. 2006). Species of *Heterorhabditis* have been studied previously according to morphology, cross breeding and molecular characteristics (Akhurst, 1987; Curran and Webster, 1989; Dix et al. 1992; Gardner et al. 1994; Griffin et al. 1994; Joyce et al. 1994; Liu and Berry, I996; Naismith et al. 1996; Nguyen and Smart, 1996; Stock and Kaya, 1996; Stock et al. 1996; Adams et al. 1998; Nguyen et al. 2006; Maneesakorn et al. 2011). The advantage of ITS rDNA as a useful marker for phylogenetic study firstly has been revealed by Hillis and Dixon (1991). Joyce et al. (1994), Baldwin et al. (1995) and Adams et al. (1998) also showed that ITS rDNA gene is useful marker for phylogenetic analysis within this genus. At present, 19 species of *Heterorhabditis* has been reported (Edgington et al. 2010; Plichta et al. 2009; Stock et al. 2002; Saleh, 1995; Karimi et al. 2011) and the number of nominal species is increasing over the time. In Iran only *H. bacteriophora* has been reported previously (Karimi et al. 2015). The aims of this study were to addressing diversity of new isolates/species of *Heterorhabditis* genus in south east of Iran. Morever, we attempted to infer the phylogenetic relationship of described and deposited isolates of *Heterorhabditis* in NCBI database as well as three new isolates of *H. bacteriophora* which resulted from the current project. Finally, morphometric and illustration of the studied isolates are given.

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MATERIAL AND METHODS

Nematode collection

During 2012- 2013, we examined 178 soil samples on the occurrence of EPNs in south eastern Iran. The soil samples were collected from beneath trees and orchards located in Kerman (Kerman province, Iran). Last instar larvae of *Galleria mellonella* (Lepidoptera, Galleriidae) placed into 50 ml plastic containers (Bedding & Akhurst, 1975) at room temperature (about 25°C). Soil samples were checked every 2 days from 4th day after baiting with the *Galleria* larvae. Dead insects with reddish, red-violet color from the soil samples were collected, rinsed in distilled water and placed on White (1927) traps to collect emerging Infective Juvenile.

Molecular analysis

For DNA extraction, nematode specimens (10 individuals) were picked into 1.5 ml tube containing 25 µl double distilled water. The tube was frozen in liquid nitrogen and was crushed with a sterile needle, then vortexed. Afterwards, 2 µl Worm Tissue Lysis buffer (WTL) and 2 µl proteinase K (20 mg ml^{-1}) were added. The homogenate was incubated at 56°C for 1 h and then at 95°C for 10 min (Shokoohi et al. 2014). The supernatant was extracted and stored at -20°C. The forward primer 18S (5`-TTGATTACGTCCCTGCCCTTT-3`) and the reverse primer 26S (5`-TTTCACTCGCCGTTACTAAGG -3`) (Vrain et al. 1992) were used in the PCR for amplification of the ITS region. PCR was conducted with 8 µl of the extracted DNA, 2.5 µl of PCR buffer, 0.5 µl of DNTP, 1 µl of $MgCl_2$, 0.3 µl Taq, 1 µl of each primer (10 pmol µl^{-1}) and ddH2O to a final volume of 25 µl. The amplification was carried out using an Eppendorf master cycler gradient (Eppendorf, Hamburg, Germany). The PCR profile was 3 min at 94°C, 37 cycles of 45 s at 94°C, 45 s at 54°C and 1 min at 72°C, and finally one cycle of 6 min at 72°C followed by a holding temperature of 4°C. After DNA amplification, 5 µl of product was electrophoresed on 1% agarose gel for DNA checking. The bands were stained, visualized and photographed under a UV transilluminator. The product was stored at -20°C prior to sequencing. PCR product was purified and sequenced with primers that used for amplification step. Sequencing reactions were performed by the Bioneer Company (South Korea) (http://eng.bioneer.com).

Sequences for the ingroups and outgroups were provided from the GenBank.The ribosomal ITS sequences were analysed and aligned using the program ClustalW implemented in BioEdit (Hall, 1999). Phylogenetic tree was reconstructed using the Bayesian inference method with the Mr Bayes 3.1.2 software (Ronquist and Huelsenbeck, 2003). The analysis was run for 10^6 generations. The ITS rDNA sequence of *Steinernema minutum* Maneesakorn, Grewal, and Chandrapatya, 2010 (GU647156) was used as outgroup. This selection was based on the result of Karimi *et al.* (2011). The tree was visualised with "TreeView" program. ITS sequences of *H. bacteriophora* were deposited in the GenBank under accession numbers KM870815, KM870816 and KM870817, respectively.

RESULTS

Six of the 178 soil samples (3%) were positive for occurrence of EPNs. Among them, five isolates of *Heterorhabditis* genus was isolated. Future study showed that all isolates belonged to species group of "*bacteriophora*".

Heterorhabditis bacteriophora Poinar, 1976
(Fig. 1 A-F)

Measurements. See Table 2.

TABLE 1. Nematode species and GenBank accession number used for phylogenetic study. Iranian sequences are presented in bold.

Species	GenBank accesion number	Fragment length	Reference	Origin
Heterorhabditis amazonensis	DQ665222	1045 bp	Andalo et al. 2006	Brazil
Heterorhabditis atacamensis	HM230723	730 bp	Edgington et al. 2011	Chile
Heterorhabditis bacteriophora	EU598233	819 bp	Eivazian Kary et al. 2009	Iran
Heterorhabditis bacteriophora	EU598236	819 bp	Eivazian Kary et al. 2009	Iran
Heterorhabditis bacteriophora	EU598237	819 bp	Eivazian Kary et al. 2009	Iran
Heterorhabditis bacteriophora	EU598231	819 bp	Eivazian Kary et al. 2009	Iran
Heterorhabditis bacteriophora	EU598227	819 bp	Eivazian Kary et al. 2009	Iran
Heterorhabditis bacteriophora	EU598228	819 bp	Eivazian Kary et al. 2009	Iran
Heterorhabditis bacteriophora	EU598232	819 bp	Eivazian Kary et al. 2009	Iran
Heterorhabditis bacteriophora	KM870815	599 bp	Present study	Iran
Heterorhabditis bacteriophora	KM870816	596 bp	Present study	Iran
Heterorhabditis bacteriophora	KM870817	595 bp	Present study	Iran
Heterorhabditis bacteriophora	EU700310	880 bp	de Waal and Malan., 2008	South Africa
Heterorhabditis bacteriophora	EU598225	819 bp	Eivazian Kary et al. 2009	Iran
Heterorhabditis bacteriophora	EU598224	819 bp	Eivazian Kary et al. 2009	Iran
Heterorhabditis bacteriophora	EU598222	819 bp	Eivazian Kary et al. 2009	Iran
Heterorhabditis bacteriophora	EU598223	818 bp	Eivazian Kary et al. 2009	Iran
Heterorhabditis bacteriophora	EF469774	822 bp	Shahina et al. 2008	Pakistan
Heterorhabditis bacteriophora	FJ653913	1014 bp	Karimi et al. 2009	Iran
Heterorhabditis bacteriophora	AY170328	1024 bp	Qiu et al. 2006	China
Heterorhabditis bacteriophora	EU163272	1041 bp	Karimi et al. 2007	Iran
Heterorhabditis bacteriophora	FJ860043	784 bp	Nikdel et al. 2010	Iran
Heterorhabditis bacteriophora	DQ100262	725 bp	Saeb and Grewal., 2005	USA
Heterorhabditis bacteriophora	JX164230	771 bp	Hassani-Kakhki et al. 2012	Iran
Heterorhabditis bacteriophora	EU715291	900 bp	Malan et al. 2011	South Africa
Heterorhabditis bacteriophora	EU598230	816 bp	Eivazian Kary et al. 2009	Iran
Heterorhabditis bacteriophora	DQ333349	628 bp	Saeb et al. 2006	USA
Heterorhabditis bacteriophora	DQ100255	734 bp	Saeb and Grewal., 2005	USA
Heterorhabditis bacteriophora	DQ100256	471 bp	Saeb and Grewal., 2005	USA
Heterorhabditis bacteriophora	DQ100258	577 bp	Saeb and Grewal., 2005	USA
Heterorhabditis bacteriophora	EU200360	840 bp	Stock et al. 2008	Jordan
Heterorhabditis bacteriophora	EU200357	838 bp	Stock et al. 2008	Jordan
Heterorhabditis bacteriophora	EU200361	837 bp	Stock et al. 2008	Jordan
Heterorhabditis bacteriophora	EU180070	660 bp	Emelianoff et al. 2008	France
Heterorhabditis bacteriophora	EU180069	660 bp	Emelianoff et al. 2008	France
Heterorhabditis bacteriophora	EF043438	1063 bp	Regeai and Burnell., 2006	Ireland
Heterorhabditis bacteriophora	EU435140	809 bp	Valadas et al. 2008	Portugal
Heterorhabditis bacteriophora	HM140691	864 bp	Noujeim et al. 2011	Lebanon
Heterorhabditis bacteriophora	EU716332	695bp	de Waal and Malan, 2008	South Africa
Heterorhabditis bacteriophora	EU598234	816 bp	Eivazian Kary et al. 2009	Iran
Heterorhabditis bacteriophora	EU598235	818 bp	Eivazian Kary et al. 2009	Iran
Heterorhabditis bacteriophora	EU516355	819 bp	Eivazian Kary et al. 2009	Iran
Heterorhabditis bacteriophora	FJ860041	777 bp	Nikdel et al. 2010	Iran
Heterorhabditis bacteriophora	FJ860042	824 bp	Nikdel et al. 2010	Iran
Heterorhabditis bacteriophora	FJ860044	826 bp	Nikdel et al. 2010	Iran
Heterorhabditis bacteriophora	FJ860045	700 bp	Nikdel et al. 2010	Iran
Heterorhabditis bacteriophora	FJ860046	911 bp	Nikdel et al. 2010	Iran
Heterorhabditis bacteriophora	FJ860047	811 bp	Nikdel et al. 2010	Iran
Heterorhabditis bacteriophora	FJ860048	814 bp	Nikdel et al. 2010	Iran
Heterorhabditis bacteriophora	JF920961	850 bp	Karimi et al. 2011	Iran
Heterorhabditis bacteriophora	GU362544	707 bp	Karimi and Mokaram, 2011	Iran
Heterorhabditis bacteriophora	JF358015	786 bp	Karimi et al. 2011	Iran
Heterorhabditis bacteriophora	GQ848095	634 bp	Tanha Maafi et al. 2011	Iran
Heterorhabditis bacteriophora	KC675180	844 bp	Kamali and Karimi., 2013	Iran
Heterorhabditis bacteriophora	EU598226	818 bp	Nikdel et al. 2008	Iran
Heterorhabditis bacteriophora	EU598229	817 bp	Nikdel et al. 2008	Iran
Heterorhabditis baujardi	EU363039	854 bp	Dolinski et al. 2008	Brazil
Heterorhabditis baujardi	AF548768	795 bp	Phan et al. 2003	Vietnam
Heterorhabditis brevicaudis	DQ020278	556 bp	Hsieh et al. 2009	China

Heterorhabditis brevicaudis	DQ177908	556 bp	Hsieh et al. 2008	China
Heterorhabditis downesi	EU921444	791 bp	Lakatos and Toth, 2008	Hungary
Heterorhabditis downesi	AY321482	990 bp	Nguyen et al. 2004	Mexico
Heterorhabditis floridensis	DQ372922	1057 bp	Nguyen et al. 2006	USA
Heterorhabditis georgiana	HQ225901	901 bp	Maneesakorn et al. 2011	USA
Heterorhabditis georgiana	HQ225898	901 bp	Maneesakorn et al. 2011	USA
Heterorhabditis hawaiiensis	AF029707	550 bp	Adams et al. 1998	USA
Heterorhabditis hepialius	AF029709	559 bp	Adams et al. 1998	USA
Heterorhabditis indica	KF247222	948 bp	Gokte-Narkhedkar et al. 2013	India
Heterorhabditis marelatus	AY321479	995 bp	Nguyen et al. 2004	Mexico
Heterorhabditis marelatus	DQ100271	560 bp	Saeb and Grewal, 2005	USA
Heterorhabditis megidis	HQ225905	888 bp	Maneesakorn et al. 2011	USA
Heterorhabditis megidis	EU921442	793 bp	Lakatos and Toth, 2008	Hungary
Heterorhabditis safricana	FJ791249	732 bp	Malan, 2009	South Africa
Heterorhabditis safricana	FJ473361	878 bp	Malan, 2008	South Africa
Heterorhabditis sonorensis	FJ477731	880 bp	Stock et al. 2009	USA
Heterorhabditis sonorensis	FJ477730	880 bp	Stock et al. 2009	Mexico
Heterorhabditis taysearae	KC633186	1021 bp	DarIssa et al. 2013	Gaza Strip
Heterorhabditis taysearae	EF043443	1052 bp	Regeai and Burnell	USA
Heterorhabditis zealandica	EU031650	777 bp	Malan et al. 2011	South Africa
Heterorhabditis zealandica	EU860184	796 bp	Malan et al. 2011	South Africa
Steinernema minutum	GU647156	852 bp	Maneesakorn et al. 2010	Thailand

Material from province of Kerman (five females, four males and four Iinfective Juveniles in a good state of preservation)

Female. Body length slightly curved ventrad after fixation. Cuticle smooth. Lateral field not visible. Lip region offset, having six round lips, bearing small papillae. Stoma rhabditoid, 8-11 μm long, with distinct cheilo-, gymno- and stegostom. Cheilostom finely cuticularized. Gymnostom longer than cheilostom, having well cuticularized lumen. Stegostom not having glottoid apparatus without denticles. Pharyngeal corpus 3-4 times isthmus length, with procorpus longer than metacorpus. Metacorpus distinct, swollen. Isthmus robust, distinctly separated from metacorpus. Basal bulb ovoid, with valvular apparatus. Cardia conoid, surrounded by intestinal tissue. Nerve ring at isthmus level, at 62-65% of neck length. Excretory pore opening at basal bulb level, at 60- 74% of neck length. Deirid not visible. Intestine without distinct specialization. Reproductive system didelphic-amphidelphic. Vulva protruding, located posterior to middle part of body. Oviduct short. Vagina with fine walls, extending inward 0.1 of the body width. Rectum 0.9-1.2 times anal body diameter. Tail conical, with acute end (Fig. 1. C, E, D).

Male. General morphology similar to that of female. Body curved ventrally after fixation. Genital system monorchic, with testis reflexed anteriorly. Tail conical, curved ventrally, with pointed tip. Bursa peloderan, opening anteriorly with nine pairs of papillae, three precloacal and seven postcloacal, arranged in 1+2+3+3 pattern, the GP1th and GP4th shorter. Manubrium rounded; calamus short and offset; lamina curved ventrad and expanded with rounded tip. Gubernaculum curved ventrally, expanded in its anterior part (Fig. 1. A, B).

Juvenile. Body elongate after fixation. Cuticle clearly, smooth. Lateral field not visible. Lip region continuous with neck, having six round lips, bearing small papillae. Stoma rhabditoid, 15-18 μm long, with distinct cheilo-, gymno- and stegostom. Cheilostom finely cuticularized. Gymnostom longer than cheilostom, having well cuticularized lumen. Stegostom without glottoid apparatus. Pharyngeal corpus 3.3-3.8 times isthmus length, with procorpus longer than metacorpus. Isthmus robust, distinctly separated from metacorpus. Basal bulb ovoid, 12-20 μm long, with valvular apparatus. Cardia conoid, 3-4 μm long, surrounded by intestinal tissue. Nerve ring at isthmus level, at 42-62% of neck length. Excretory pore opening at bulb level, at 70-76% of neck length. Deirid not visible. Intestine without distinct specialization. Tail conical, with acute end (Fig. 1. F).

Locality and habitat. The specimens were found in Lalezar (province of Kerman; N: 29°52'17"; E: 56°81'06"), in association with the rizosphere of *Pinus* sp.

Other materials examined. Very similar to females of Kerman population but having shorter body and pharynx.

Remark. The Iranian specimens of *H. bacteriophora* fit well with previously studied materials (Poinar, 1976; Karimi et al. 2011; Nikdel et al. 2012; Erbas et al. 2014). In comparison with the material examined by Poinar (1976), it differs in female body length (1500-3425 mm *vs* 512-671 mm), tail length (49-97 µm *vs* 83-112 µm) and gubernaculum length (12-32 µm *vs* 18-25 µm). Karimi et al. (2011) reported this species with shorter body length (*vs* 702-808 µm) and spicules (*vs* 29-36 µm). Compared with the specimens studied by Nikdel et al. (2012), there are no significant differences. Erbas et al. 2014 studied this species with shorter body length (*vs* 540-643 µm).
The lengths of ITS sequence of *H.bacteriophora* amplified were 595-599 bp (see Table 1). The ITS sequences aligned clearly with previous described isolates of *H. bacteriophora*. The Bayesian analysis implied the strongly supported clades for all studied isolates (Fig. 2).
Pairwise Maximum Composite Likelihood distance among the ITS rDNA region of *H. bacteriophora* molecularly identified sequences showed that *H. bacteriophora* populations from Kerman (KM870815; KM870816; KM870817; Iran) have the highest genetic variation (0.570, 0.590 and 0.593, respectively) with *H. bacteriophora* from Tehran (FJ653914; Iran). (see Table 3)

DISCUSSION

Phylogenetic analysis

The reconstructed phylogenetic tree contained three main clades: I) *H. bacteriophora* and *H. georgiana*; II) *H. megidis, H. downesi, H. zealandica, H. safranica, H. atacamensis, H. marelatus and H.* hepilaius; and III) *H. sonorensis, H. taysearae, H. floridensis, H. amazonesis, H. baujardi, H. brevicaudis* and *H. indica.*
The phylogenetic relationships among the 80 *Heterorhabditis* isolates are presented in Figure 2. The 54 isolates of *H. bacteriophora* comprise a paraphyletic group by analysis of the ITS region. In this clade, the two isolates of *H. georgiana* places within the isolateisolates of *H. bacteriophora*. This result obtained by some scientists (Erbas et al. 2014; Karimi et al. 2014; Malan et al. 2014). Maneesakorn et al. (2011) showed close relationship of *H. bacteriophora* and *H. georgiana*. The later species (*H. georgiana*) was described by Nguyen et al. (2008) from Georgia (USA). According to Nguyen et al. (2008), *H. georgiana* is the closest species to *H. bacteriophora*. Both species similar in having female tail (conical, 22-36 *vs* 29-41 µm; see Nguyen et al. 2008), male tail (conical with peloderan bursa), spicule and gubernaculum shape and bursal papillae (1+3+3+3). However, they differ in spicules length (36-44 *vs* 41-49 µm), gubernaculum length (18-25 *vs* 20-28 µm), gubernaculum/spicules% (50 *vs* 51-64) (see Nguyen et al. 2008). In addition both species having the same ITS region (Nguyen et al. 2008), but they differ in 29 aligned nucleotides position. *H. bacteriophora* and *H. georgiana* form a monophyletic group with highly supported values (posterior probability, 1.00). Both species have similar characters (Nguyen et al. 2008). However, male posterior end (e.g. spicule shape and papillae arrangement) is different. It seems that more study is needed to distinguish between these two species. This result obtained by Nguyen et al. (2008). They classified both species in the "bacteriophora"group. They considered both species as sister group according to ITS rDNA region. The pairwise divergence between taxa ranged from 0.005 to 0.633 (Table 3). The result indicates that *H. bacteriophora* has the lowest divergence with *H. georgiana* (0.031; Table 3).
During the survey on EPN in Kerman province, five isolates of *H. bacteriophora* were recovered from 178 soil samples collected throughout the province. According to the present study, revealed that *H. bacteriophora* is the dominant species of this genus in Kerman province. Sequences of the ITS region and morphological characters, have confirmed validity of the studied species in arid region of Iran.

The occurrence of EPNs in the study area of our survey was relatively low (3%). The main reasons for low recovery rate for EPNs for this region may be due to nearby the studied region with desert. In addition more samples may increase the species diversity and the number of positive samples.

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