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Diversity of entomopathogenic nematodes (Nematoda: Steinernematidae, Heterorhabditidae) from Arasbaran forests and rangelands in north-west Iran

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Summary – A survey for entomopathogenic nematodes (EPN) was carried out in the Arasbaran forests and rangelands, East Azarbaijan province, north-west Iran, during 2006 to 2008. A total of 691 soil samples were collected from 62 localities across the region of which 21 samples (3%) were positive for EPN, including nine samples (1.3%) with heterorhabditids and 12 (1.7%) with steinernematids. Seven isolates (four *Steinernema* and three *Heterorhabditis*) were recovered from rangelands and 14 (eight *Steinernema* and six *Heterorhabditis*) from forest soil samples. Based on morphology and molecular studies, the *Heterorhabditis* isolates were identified as *H. bacteriophora* and the *Steinernema* isolates as *S. carpocapsae*, *S. bicornutum*, *S. feltiae*, *S. glaseri*, *S. kraussei* and three undescribed species referred to here as *Steinernema* sp. IRAZ7, *Steinernema* sp. IRAZ13 and *Steinernema* sp. IRAZ21. *Heterorhabditis bacteriophora*, the most common species, was present in nine soil samples collected across the forests and rangelands, and of the *Steinernema* species, *S. bicornutum* was obtained from three samples, the other species being found from only one or two samples.

Keywords – *Heterorhabditis bacteriophora*, *Steinernema bicornutum*, *Steinernema carpocapsae*, *Steinernema feltiae*, *Steinernema glaseri*, *Steinernema kraussei*, survey.

Entomopathogenic nematodes (EPN) of the genera *Heterorhabditis* Poinar, 1976 and *Steinernema* Travassos, 1927 are used globally as safe biocontrol agents against soil-borne insect pests (Ehlers, 2005). In a previous survey in Iran, three species of *Steinernema*, including *S. bicornutum* Tallosi, Peters & Ehlers, 1995, *S. carpocapsae* (Weiser, 1955), Wouts, Mráček, Gerdin & Bedding, 1982 and *S. feltiae* (Filipjev, 1934), Wouts, Mráček, Gerdin & Bedding, 1982 and one *Heterorhabditis* species (*H. bacteriophora* Poinar, 1976), were reported from the north-west region of the country (Eivazian Kary *et al.*, 2009). We conducted a second survey in this extensive region but mainly focused on the Arasbaran natural forests and rangelands which are not easily accessible for humans and are likely to be free of introduced nematodes.

Arasbaran is in the north-west region of the Islamic Republic of Iran near the borders of Armenia and Azerbaijan, and is a vast mountainous region with an area of 900 000 ha. The forests are mostly located in the northern section and extend over an area of 120 000 ha between the Qaradagh mountains in the north and the Aras River in the south. Many of the plant communities are not known elsewhere in Iran (Jalili *et al.*, 2003). The area varies in altitude from 200 m (where it borders the Aras river) to 3347 m a.s.l. (Mount Kiamaki). At the higher altitudes the average annual temperature is 5°C; the average of the warmest month is 12°C and that of the coldest −2°C. At lower altitudes the average temperature is 14°C. Dominant species in the Arasbaran forests are hornbeam and oak with 51 and 37% coverage, respectively, but there are three main types including pure oak (altitudes between 500-1000 m a.s.l. are occupied by *Quercus petraea* Krassiin, 1968, 1500-2000 m a.s.l. by *Q. macranthera* Fish & Meyer, 1878 and between 1000-1500 m a.s.l. a mixture of the two species), pure hornbeam, and mixed oak/hornbeam forests (Nikdel & Sadaghian, 2002). Some

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coniferous species occur in a few mixed forests (Sajedi, 2004). The rangelands area of Arasbaran is estimated to occupy 460 000 to 550 000 ha and is located across high mountains, deep valleys and steep slopes. The rangelands occur at *ca* 50 to 2800 m a.s.l.

Given the ecological importance of the Arasbaran region, a survey to study the diversity of EPN was done, focusing solely on the forests and rangelands not covered in the first survey.

Materials and methods

Sampling

A total of 691 soil samples, including 415 samples from forests and 276 from rangelands, were taken from 62 sites during 2006-2008. Ten to 12 soil samples were randomly collected from each site. Each sample was a composite of 15-20 sub-samples taken at a depth of 20 cm. Site location, sampling date, elevation, GPS coordination and associated vegetation were also recorded. The samples were separately placed in a polyethylene bag to prevent water loss during transit and half of each sample (*ca* 1 kg) was used for extraction of EPN (Eivazian Kary *et al*., 2009).

Nematode extraction

EPN were recovered from the samples by baiting with 10-15 last instar larvae of *Galleria mellonella* (L.) (Bedding & Akhurst, 1975) in a polystyrene jar kept at room temperature (25 ± 2°C) for 2 weeks. Soil samples were checked every 2 days from day 5. Dead insects from each sample were collected, rinsed in distilled water and placed on White (1929) traps to collect emerging IJ. Fresh insect larvae were added if needed. To improve recovery rate, a second baiting was done by placing fresh *G. mellonella* larvae into the containers with the same soil.

Morphological characterisation

Nematodes were examined live or heat-killed in Ringer's solution at 60°C. All nematodes studied were reared in *G. mellonella* larvae. Twenty *G. mellonella* larvae were exposed to *ca* 1000 infective juveniles in a Petri dish lined with two moistened filter papers at room temperature (25 ± 3°C). Mature females and males of the first and second generations were isolated by dissecting infected larvae in Ringer's solution 3 and 5 days after infection, respectively (Eivazian Kary *et al*., 2009). First generation males and females were collected from 4-5 day post-inoculated *Galleria* cadavers (dissected in distilled water). Infective juveniles and second generation adults were obtained during the week after their first emergence from *Galleria* cadavers. Nematodes were killed using hot (50-60°C) Ringer's solution (Nguyen & Smart, 1995), fixed in triethanolamine formalin (TAF), processed to anhydrous glycerin by a slow evaporation method (Woodring & Kaya, 1988), mounted on glass slides and studied using an Olympus BX41 microscope equipped with differential interference contrast optics. Specimens were measured using UTHSCSA Image Tool software (Vilcox *et al*., 2002).

Morphological identification was done using criteria suggested by Stock and Kaya (1996) and Hominick *et al*. (1997). Additionally, males and IJ of representative isolates of each species group were examined using scanning electron microscopy. For this purpose specimens were processed following the protocol described by Nguyen and Smart (1995).

Molecular characterisation

Total genomic extraction and ITS-rDNA amplification were done as described by Eivazian Kary *et al*. (2009). Amplified products were purified using a Qiagen Purification kit (Qiagen, Leusden, The Netherlands). Purified DNA was sequenced in IBMP-CNRS, France. The DNA sequences were edited with Chromas 2.01. The edited sequences (ITS1-5.8S-ITS2 for *Heterorhabditis* spp. and ITS1-5.8S-ITS2 and 28S for *Steinernema* spp.) were aligned using ClustalX 1.64 (Thompson *et al*., 1997) with the sequences of other *Heterorhabditis* and *Steinernema* species obtained from GenBank. The sequences of Iranian isolates are deposited in GenBank (Table 1).

Cross-breeding test

To confirm the identity of the *Steinernema* isolates, cross-breeding tests were performed between the isolate under study and known species using the haemolymph hanging drop technique (Poinar, 1967). The following species were used: *S. feltiae* (4CFMO strain), *S. carpocapsae* (All strain), *S. bicornutum* (strain IRA7) and *S. glaseri* (Steiner, 1929), Wouts, Mráček, Gerdin & Bedding, 1982 (NC1), all obtained from the Department of Biology, National University of Ireland, Maynooth, Ireland.

Table 1. *Location, vegetation and accession numbers (ITS and 28s rDNA) of* Heterorhabditis *and* Steinernema *isolates collected from Arasbaran forests and rangelands in north-west Iran.*

Species	Isolate	Locality	Altitude (m a.s.l.)	Latitude/longitude	Vegetation	ITS rDNA (Accession no.)	28s rDNA (Accession no.)
H. bacteriophora	IRAZ1	Ainalou (1)*	1382	N38 53 27/E46 46.50	Forest	FJ860041	–
H. bacteriophora	IRAZ2	Galaeh Darasi (1)*	1490	N38 54 35/E46 5.50	Forest	FJ860042	–
H. bacteriophora	IRAZ4	Vaian	1330	N38 56 35/E46 45 23	Forest	FJ860043	–
H. bacteriophora	IRAZ5	Vaian fork	1329	N38 54 55/E46 48 15	Forest	FJ860044	–
H. bacteriophora	IRAZ6	Nursery	1350	N38 53 41/E46 47 19	Forest	FJ860045	–
H. bacteriophora	IRAZ8	Shirin Boolag	1410	N38 62 88/E47 29.61	Rangeland	FJ860046	–
H. bacteriophora	IRAZ10	Kalaleh Olia	1183	N38 40 57/E46 32 05	Forest	FJ860047	–
H. bacteriophora	IRAZ16	Varzegan	1140	N38 51 56/E47 01.23	Rangeland	–	–
H. bacteriophora	IRAZ17	Bidzar	1263	N38 51 55/E47 00 46	Rangeland	FJ860048	–
S. bicornutum	IRAZ11	Aras Boarder	372	N38 44 47/E46 30.37	Rangeland	FJ860034	FJ860021
S. bicornutum	IRAZ14	Makidi	1630	N39 00 44/E46 41 33	Forest	–	FJ860023
S. bicornutum	IRAZ15	Khan Baghi	485	N38 47 24/E46 30.51	Rangeland	FJ860036	FJ860024
S. carpocapsae	IRAZ9	Galaeh Darasi (2)*	1510	N38 50 34/E46 54.51	Forest	FJ860033	FJ860020
S. feltiae	IRAZ3	Shoja Abad	1745	N38 55 53/E46 46 21	Forest	–	FJ860019
S. feltiae	IRAZ18	Ainalou (2)*	1335	N38 56 13/E46 44.57	Forest	–	FJ860025
S. feltiae	IRAZ22	Ilankesh	2213	N38 52 01/E46 49 52	Rangeland	FJ860040	FJ860028
S. glaseri	IRAZ19	Kolashlou	1424	N38 51 47/E47 01 14	Forest	FJ860037	FJ860026
S. kraussei	IRAZ20	Chichaklu	1820	N38 59 06/E46 53.30	Rangeland	FJ860038	FJ860027
Steinernema sp.	IRAZ7	Forest Station (1)*	1200	N38 53 27/E46 46 50	Forest	FJ860032	–
Steinernema sp.	IRAZ13	Forest Station (2)*	1208	N38 41 34/E46 34 07	Forest	FJ860035	FJ860022
Steinernema sp.	IRAZ21	Karangan	1790	N38 36 44/E46 35 09	Forest	FJ860039	–

* In each of these sites two positive samples were obtained from two different points with a different altitude.

Results

From a total of 691 collected soil samples (415 samples from forests and 276 from rangelands), EPN were recovered from 21 samples (3%), nine of these (42.8%) being positive for heterorhabditids and 12 (57.2%) for steinernematids (Fig. 1; Table 1). Seven of these isolates were recovered from rangelands and the remaining 14 from forest soils. Based on morphological and molecular characters, one species of *Heterorhabditis* and five described species of *Steinernema* were identified. All *Heterorhabditis* isolates were identified as *Heterorhabditis bacteriophora* and *Steinernema* isolates as *Steinernema carpocapsae*, *S. bicornutum*, *S. feltiae, S. glaseri* and *S. kraussei* (Steiner, 1923) Travassos, 1927. There were three undescribed species, *viz*., *Steinernema* sp. IRAZ7, *Steinernema* sp. IRAZ13 and *Steinernema* sp. IRAZ21. Males and females of the undescribed isolates did not hybridise with those of *S. feltiae* 4CFMO, *S. kraussei* IRAZ20, *S. feltiae* IRAZ18 or *S. feltiae* IRAZ3, whilst males and females of each species mated and normal offspring were produced in all self-cross controls.

Forest soils displayed the greatest diversity of EPN species with eight species, namely *H. bacteriophora*, *S. bicornutum*, *S. carpocapsae*, *S. feltiae*, *S. glaseri*, *Steinernema* sp. IRAZ13, *Steinernema* sp. IRAZ21 and *Steinernema* sp. IRAZ7, being obtained from the 14 positive samples. By contrast, four species (*H. bacteriophora*, *S. feltiae*, *S. bicornutum* and *S. kraussei*) were identified amongst the seven rangeland isolates. *Heterorhabditis bacteriophora* was the most common species, being isolated from nine soil samples across both forests and rangelands and, of the *Steinernema* species, *S. bicornutum* was the most common with three isolates, all the other species being recorded from one or two samples. Among the identified species, *S. kraussei* is a first record for Iran. No sample yielded more than one species of EPN. Natural infection of Arasbaran insects with Heterorhabditidae and Steinernematidae nematodes was not detected, but the parasitic nematode, *Hexamermis* sp., was observed at high frequency in immature stages of *Euproctis chrysorrhoea* (L.), *Malacosoma neustria* L. and *Lymantria dispar* L.

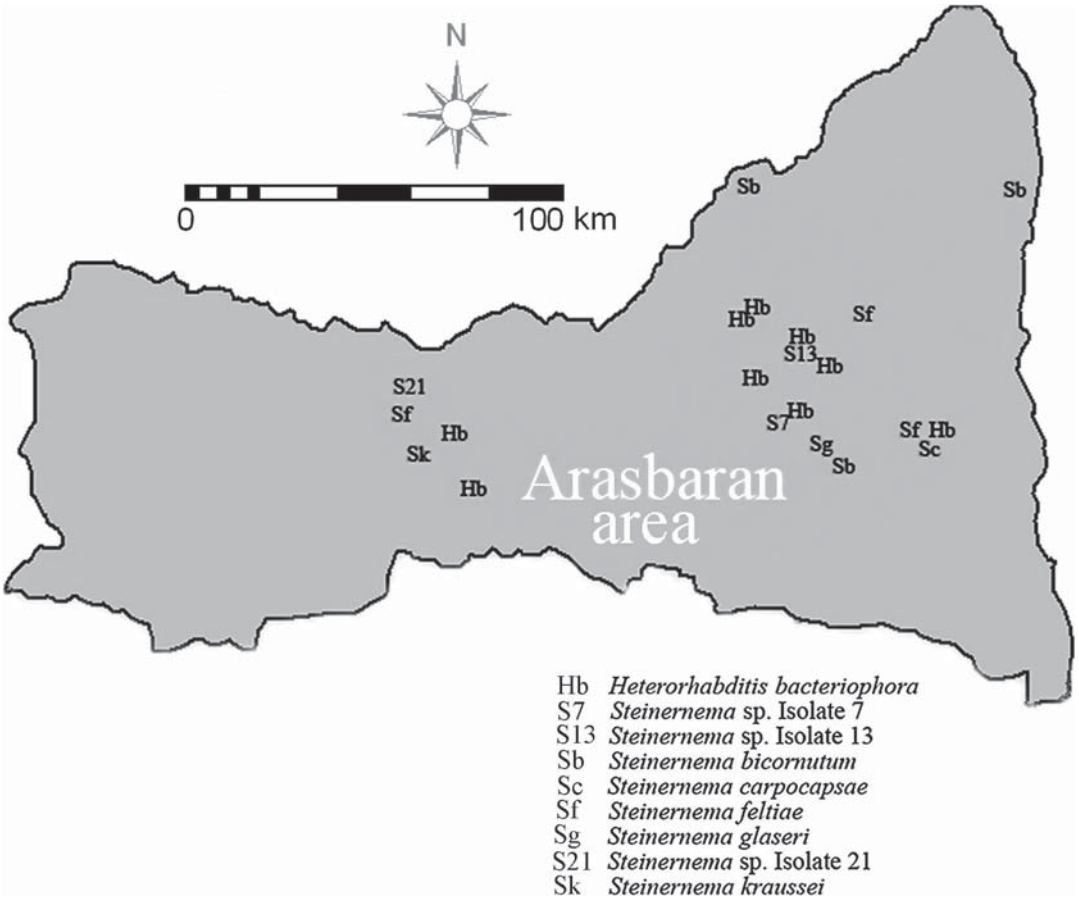

Fig. 1. *Distribution of entomopathogenic nematodes in the Arasbaran forests and rangelands of north-west Iran.*

Discussion

The north-west of Iran is a mountainous region with a cool continental climate. In this region, Arasbaran's forests and grasslands, with more than 1033 plant species, have the highest plant diversity in East Azarbaijan province. From 1976, this region has been registered as one of Iran's nine biosphere reserves under the UNESCO MAB programme. The region shares a border with Turkey where four EPN species have been isolated in three surveys, *viz*., *H. bacteriophora*, *S. affine* (Bovien, 1937) Wouts, Mráček, Gerdin & Bedding, 1982, *S. feltiae* and *S. anatoliense* Hazir *et al.*, 2003 (Özer *et al*., 1995; Susurluk *et al*., 2001; Hazir *et al*., 2003). In the first survey from north-west Iran, four species (*H. bacteriophora*, *S. bicornutum*, *S. carpocapsae* and *S. feltiae*) were reported (Eivazian Kary *et al*., 2009). The present survey adds a further five species to Iran's EPN fauna, including three undescribed *Steinernema* species. The newly recorded species are *S. kraussei*, *S. glaseri*, *Steinernema* sp. IRAZ7, *Steinernema* sp. IRAZ13 and *Steinernema* sp. IRAZ21. Isolates *Steinernema* sp. IRAZ7 and *Steinernema* sp. IRAZ13 are placed in a sister clade with *S. feltiae* and belong to the *feltiae-kraussei* group, but *Steinernema* sp. IRAZ21 is a sister clade to *S. affine* and clusters with the *affine-intermedium* group (Fig. 2). All isolates of *H. bacteriophora* clustered with *H. bacteriophora* isolates reported from other countries (Fig. 3).

Of importance is the fact that nine EPN species have been recorded from natural forests and rangelands, the number of soil samples examined being very low when compared to the area of the region. More intensive survey work will likely recover even more species. Of the 21 positive samples, seven isolates (four *Steinernema* and three *Heterorhabditis*) were detected from rangelands and

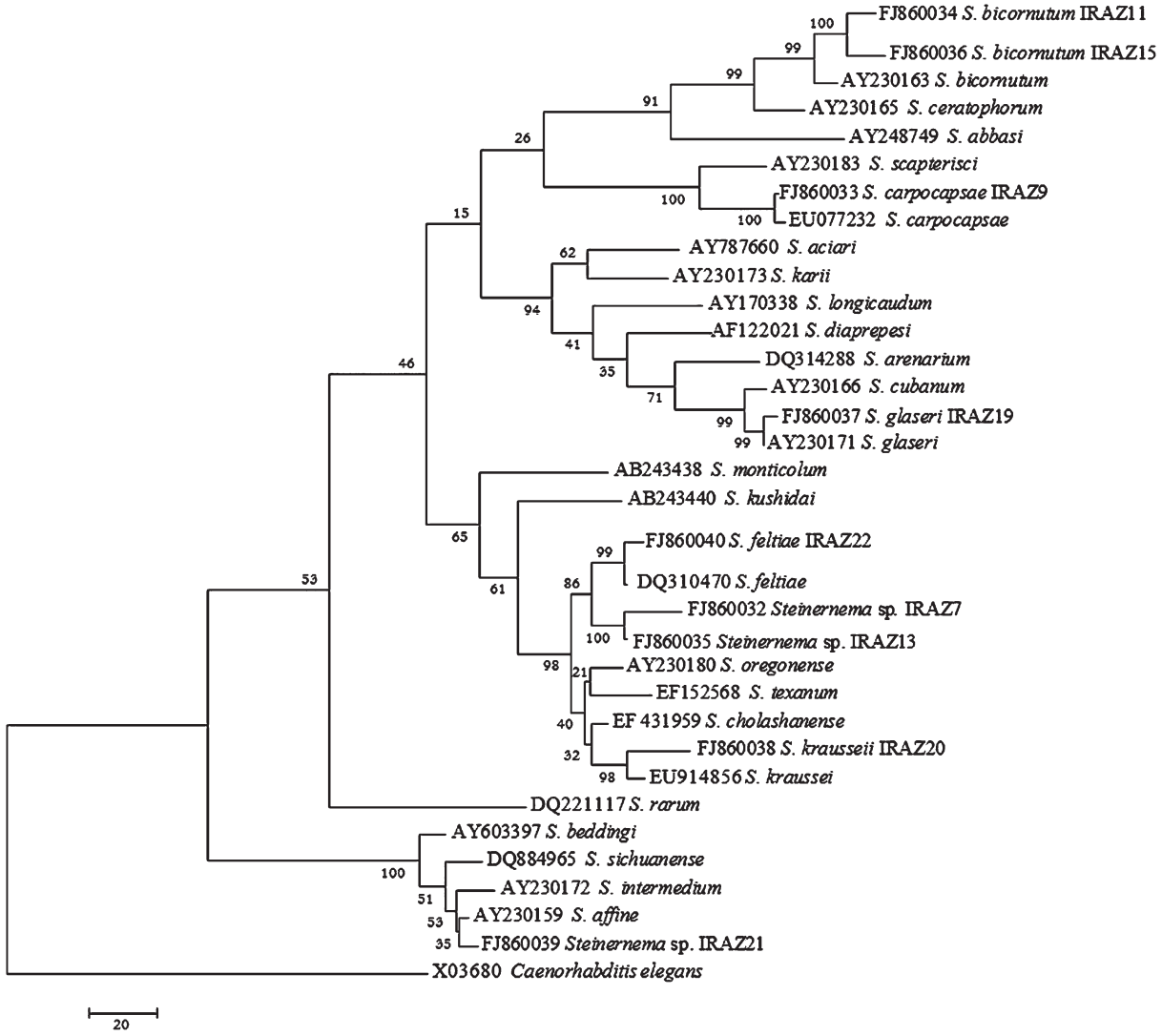

Fig. 2. *Phylogenetic relationships of Iranian steinernematids (five described species and three undescribed isolates) from Arasbaran forest and rangelands with 24 species of* Steinernema *based on analysis of ITS rDNA regions. Two undescribed Iranian isolates form a monophyletic group with four species of the* feltiae-kraussei *group whereas* Steinernema *sp. IRAZ21 clustered with the* affine-intermedium *group and each of the five nominal species forms a monophyletic group with other conspecific isolates with high bootstrap support (99-100%).*

14 (eight *Steinernema* and six *Heterorhabditis*) from forest soil samples. The results indicate that *Steinernema* spp. and/or strains appear to be more dominant and diverse than *Heterorhabditis* spp. in rangelands, but especially so in forests. These findings are in agreement with reports of other surveys (Stock *et al.*, 1999, 2008; Sturhan & Lišková, 1999; Stock & Gress, 2005) where the greatest diversity of EPN was found in woodland and forest habitats.

The identification of native species of EPN and their distribution in the Arasbaran region were the primary goals of the present work. Although EPN were recovered at a low rate (3% of sampled sites) in our study, a similar recovery rate (3.2%) was reported in the first survey from Iran (Eivazian Kary *et al.*, 2009) and also from other regions of the world (Choo *et al.*, 1995; Rosa *et al.*, 2000; Hazir *et al.*, 2003; Canhilal *et al.*, 2006).

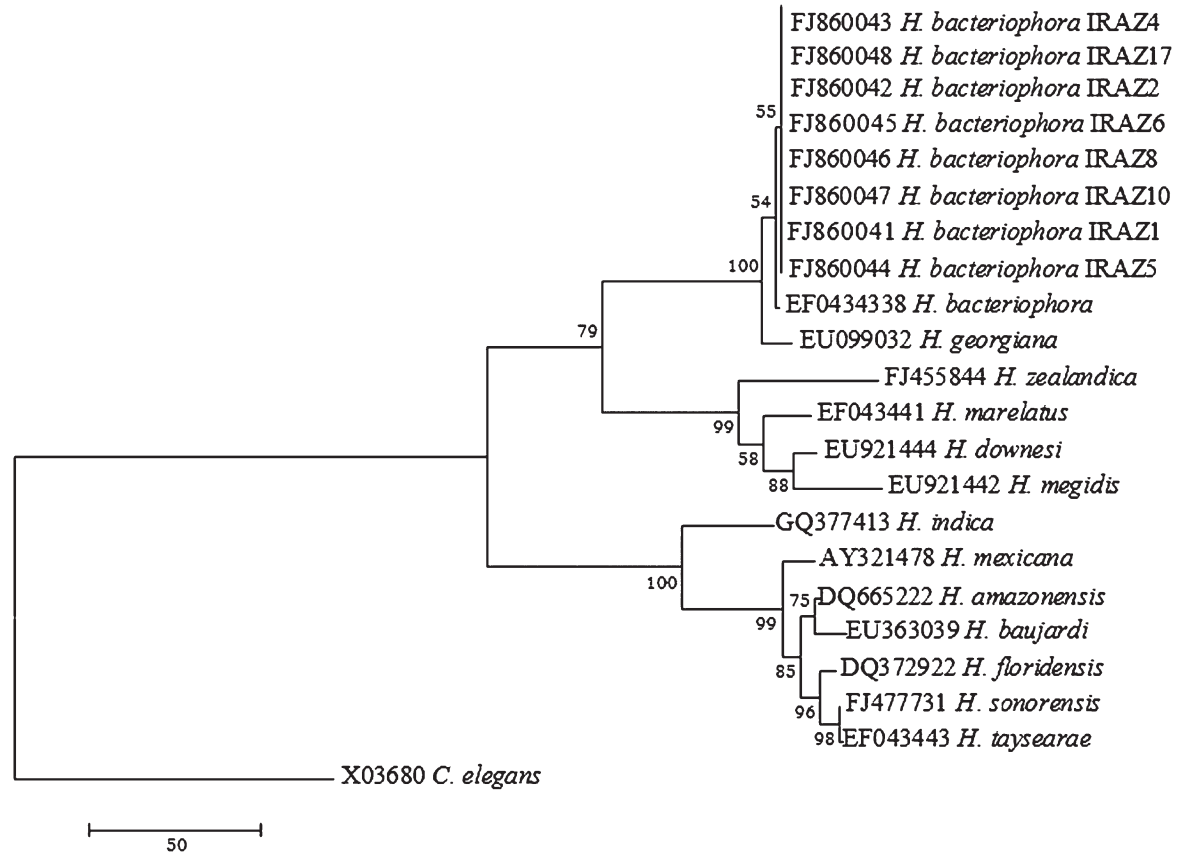

Fig. 3. *Phylogenetic relationships of Iranian isolates of* Heterorhabditis bacteriophora *from Arasbaran forests and rangelands with one other isolate of* H. bacteriophora *and 12 other species of* Heterorhabditis *based on analysis of ITS rDNA regions.*

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