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Phylogenetic relationship of Pakistani entomopathogenic nematode strains on the basis of ITS-1, 5.8S and ITS-2, D2-D3/28S (LSU) rDNA region and 12S rDNA mitochondrial gene

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Abstract

Entomopathogenic nematodes (EPNs) belong to families Steinernematidae and Heterorhabditidae have proved as extremely useful biological control agents and infected a wide range of insect pests. Phylogenetic relationships among Pakistani entomopathogenic nematode strains; steinernematids and heterorhabditid were estimated by nucleotide sequences using three molecular makers viz; ITS-1, 5.8S and ITS-2 rDNA (seventy nine); D2-D3 and 28S (LSU) sequences of rDNA region (fifteen) and 12S rDNA mitochondrial gene (twenty nine) to investigate the genetic diversity. Phylogenetic trees were constructed using two different methods maximum parsimony (MP) and Bayesian inference (BI) in which most of them form highly to moderately supported clades.

Keywords: Phylogenetic relationship, Pakistani entomopathogenic nematode strains, ITS-1, 5.8S, ITS-2, D2-D3/28S, rDNA region, 12S rDNA mitochondrial gene

The entomopathogenic nematodes (EPNs) belong to the Phylum Nematoda (Groombridge, 1992; Wilson, 2000). EPNs of the families Steinernematidae and Heterorhabditidae have proved as tremendously useful biological control agents and infected a wide range of insect pests (Gaugler, 2002). These nematodes were mutualistically associated with bacteria, i.e., genus *Xenorhabdus* spp. (Thomas & Poinar, 1979) for steinernematids and *Photorhabdus* spp. (Boemare *et al*., 1997) for heterorhabditids. Infective juveniles (IJs) release their symbiotic bacteria after entering the host insect's haemocoel that kill the host within 24-48 hrs producing fatal disease. Following 1-3 generations, IJs enter into the soil from insect cadavers and search for new hosts (Dowds & Peters, 2002).

A genomic assessment of EPN from multiple genera has the advantage of decades of ecological research and easy to understand adaptation and convergent evolution in addition to revealing just how similar or different this niche exploitation. There were more than 60 described species in the world within *Steinernema* (Nguyen & Hunt, 2007a; Nguyen *et al*., 2007b; Nguyen *et al*., 2008; Tarasco, 1997; Edgington *et al*., 2009; Spiridonov *et al*., 2010; Khatri-Chhetri *et al*., 2011; Nguyen & Buss, 2011; Stokwe *et al*., 2011). A few from these strains were tested about the host-range and specificity of insect pests but their infection varied and diverse. *Steinernema carpocapsae* was the most profoundly studied steinernematid; extremely broads host range and proficient of infecting more than 250 species of insects across 10 orders (Poinar, 1979).

Different molecular markers and techniques have been used not only for diagnostic purposes, sorting out cryptic species and strains, but also to assess evolutionary relationships along with these nematodes. According to Stock (2002) polymerase chain reaction (PCR) and DNA sequencing has

revolutionized nematode taxonomy and genetics. Distinguishing methods were based on the PCR including random fragment length polymorphic PCR-RFLP and random amplified polymorphic DNA (RAPD) assisted species identification by non-experts (Grenier *et al*., 1996; Joyce *et al*., 1994; Liu & Berry, 1995; Reid & Hominick, 1993; Stock *et al*., 1998). Internal nucleotide data was used to restrict nematode species and conclude their evolutionary history (Adams *et al*., 1998; Blaxter, 1998; Kampfer *et al*., 1998; Nadler, 1992; Nadler & Hudspeth, 1998, 2000; Nadler *et al*., 2000).

Internal Transcribed Spacer (ITS) region of rDNA composed of three gene regions, ITS-1, 5.8S and ITS-2 which was used to distinguish species of EPNs and also to assess their evolutionary history (Nguyen *et al*., 2001; Perlman *et al*., 2003; Stock & Reid, 2003; Spiridonov *et al*., 2004). Evolutionary relationship of EPNs in *Steinernema*, mitochondrial DNA genes have been considered (Nadler *et al*., 2006) and phylogenetic relationship of mtDNA sequence (12S rDNA) and cytochrome oxidase subunit 1 (cox1) gene also considered (Stock *et al*., 2009).

In the present study phylogenetic relationships among Pakistani entomopathogenic nematode strains steinernematids and heterorhabditid were analyzed using three molecular makers; seventy nine (ITS-1, 5.8S and ITS-2 rDNA); fifteen (D2-D3 and 28S (LSU) sequences of rDNA) and twenty nine (12S rDNA mitochondrial gene) to investigate the genetic diversity. Maximum Parsimony (MP) and Bayesian Inference (BI) methods were adopted to construct a phylogenetic tree.

Materials and Methods

Nematode sources: EPNs were extracted from samples collected from five zones of Pakistan viz., Khyber Pakhtunkhwa (The North and North western mountainous areas), Punjab (Upper Indus plain), Balochistan (the plateau of Balochistan and its desert), Sindh (the coastal areas, lower Indus plain) and Kashmir on the basis of climatic regions. Strains used in this study were reared only on last instar larvae of *Galleria mellonella* L., which exposed about 3000-4000 infective juveniles (IJs) in a 9 cm diam. Petri dish, contained with moist filter paper (Whatman No.1) and sealed with Para film. The Petri dishes were incubated at 30 ± 2 °C. Larvae died after 24 hrs of exposure. The insect cadavers were transferred to White trap (White, 1927) and incubated at 30 ± 2 °C for 4-5 days for the emergence of IJs. The nematodes were collected in 1.5 ml Eppendorf tube in 70% ethanol and stored at -20 °C before use.

DNA extraction: Individual isolates were crushed in 30 µl of worm lysis buffer in micro centrifuge tube (1.5 ml) on ice box. The tube was kept at -80 ºC for 10 min, incubated at 65 ºC for 1 h, following by 95 ºC incubation for 10 min to lysed the cells, digest the proteins, inactive Proteinase K, respectively. Later, the tube was ice-cold, then centrifuged at 12,000 rpm for 2 min and 5 µl of supernatant contain the DNA further used in PCR reaction (Mehreen & Shahina, 2013).

PCR amplification: The PCR mix was prepared by combining the components with primer sets and reactions carried out in a volume of 50 μl. The reactions were set up on ice and the following were added: 5 μl 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 8.4, 15 mM $MgCl_2$, 1% Triton X-100), 1 μl of both reverse and forward primer (10 μM), 2 μl of dNTP mixture (5 mM for each), 1 μl Tag DNA polymerase (Promega), 30 μl distilled water and 10 μl of DNA extract.

The reactions were conducted in thermocycler (Eppendorf, Germany). A numeral of primer sets had been considered

for amplification of nuclear, mitochondrial genes and successfully applied to *Steinernema* and *Heterorhabditis* for identification. Primers used for PCR amplification were 18S: 5'-TTGATTACGTCCCTGCCCTTT-3' and 26S: 5'-TTTCACTCGCCGTTACTAAGG-3' (Vrain *et al*., 1992); AB28 5'-ATATGCTTAAGTTCAGCGGGT-3' TWB1 5'-GTTTCCGTAGGTGAACCTGC-3' (Joyce *et al*., 1994) ITS rDNA; D2F: 5'-CCTTAGTAACGGCGAGTGAAA-3' and 536: 5'-CAGCTATCCTGAGGAAAC-3' (Nguyen *et al*., 2006) 391: 5'-AGCGGAGGAAAAGAAACTAA-3' and 501: 5'-TCGGAAGGAACCGCTACTA-3'(Stock *et al*., 2001) 28S rDNA.

The cyclic conditions were as following: 4 min at 94 °C, 35 cycle of 30 sec at 94 °C, reannealing 30 sec at 48 °C and extension at 72 °C for 2 min, followed by 10 min incubation at 72 °C. While (505) 5'-GTTCCAGAATAATCGGCTAGAC-3' and (506) 5'-TCTACTTTACTACAACTTACTCCCC-3'.

PCR program setting used for 12S rDNA amplification, included denaturation at 3 min at 94 °C followed by 37cycles of 94 °C for 30 sec, reannealing 50 °C for 30 sec and extension at 72 °C for 45 sec followed by 7 min incubation at 72 °C, respectively (Nadler *et al*., 2006). PCR product was separated by 1.0 % agarose gel electrophoresis and images were obtained in UV-transilluminator. PCR products were purified using the QIA quick Gel Extraction Kit (Qiagen, Valencia, CA) and cloned into pGemT easy vector System I (Promega, Madison, WI).

The plasmids were transformed into *Escherichia coli* strain JM109 competent cells (Promega, Madison, WI) according to the manufacturer's protocols. Clones containing putative rRNA inserts were identified through blue/white color selection. The plasmid preparations were extracted using the QIAquick Spin Miniprep Kit (Qiagen, Valencia, CA). From the positive results plates, two clones for each EPN individuals were sequenced using an ABI Prism 377 sequencer (PE Applied Biosystem, Foster City, CA, USA) and dye terminator sequencing reagents at the University of Karachi, Karachi, Pakistan.

Phylogeny: These sequences were compared with already registered or published sequences available in NCBI Genbank database using Blast (http://www.blast.ncbi.nlm.nih.gov), multiple alignments of the new sequence data together with others sequences selected from NCBI databases were performed using Clustal W2 software (Larkin *et al*., 2007). On the basis of ITS, D2D3 regions and 12S rDNA gene, phylogenetic analysis was performed by MP with the help of software PAUP* 4.0b8 (Swofford, 2003) with a suitable outgroup in each case. Using MrBayes 2.1.2 (Huelsenbeck *et al*., 2001) the sequence dataset were analyzed as Bayesian inference (BI).

According to Nylander (2002), the best fit model of DNA evolution for BI was obtained using the program MrModeltest 2.2 with the Akaike Information Criterion in union with PAUP. BI analysis under the GTR + I + G model was initiated with a random starting tree and run with the four Metropolis-coupled Markov chain Monte Carlo (MCMC) for 106 generations. The MCMC were sampled at intervals of 100 generations. After approximately 103 generations, the log-likelihood values of the sample points were stabilized. The topologies were used to generate a 50% majority rule consensus tree bootstrap analysis. By using MrEnt 2.0 (Zuccon & Zuccon, 2010), the resulting trees were envisioned. On the basis of ITS rDNA sequences, *Caenorhabditis elegans* (EU131007) was applied as an out group during calculation of the trees; *Cervidella alutus* (AF331911) together with *Panagrellus redivivus* (AF331910) used as outgroup for calculation of

the trees based on D2-D3 sequences. *Caenorhabditis elegans* (X03680) placed as outgroup taxa during calculation of the trees based on 12S rDNA gene.

Results and Discussion

Sequences with positive result and high peak were selected, initially aligned using the Bioedit built-in ClustalW2 program (gap opening penalty = 10, gap extension penalty = 5, delay divergent sequences = 50%) as shown in Fig. 1, Fig. 6 and Fig. 11. The use of multiple methods increases the reliability of the inferred phylogeny if they produce the same results.

Phylogenetic analysis of ITS rDNA region: Specimens information and Genbank accessions number of seventy nine *Steinernema* and *Heterorhabditis* isolates using ITS-1, 5.8S and ITS-2 for the phylogenetic analysis was presented in Table 1. Alignment report of ITS rDNA sequences of the *Steinernema* and *Heterorhabditis* species were shown in Fig. 1.

Steinernema and *Heterorhabditis* tree was constructed using the ITS region sequences performed using heuristic methods with Tree-Bisection Reconnection (TBR) branch swapping and minimum of 100,000 replicates of random stepwise addition. The ITS region sequences of *Caenorhabditis elegans* EU131007 was used as an out group. For *Steinernema* ITS regions, MP analysis showed that the alignment resulted in 802 characters of which 148 are constant, 100 variable characters are parsimony uninformative and 554 characters are parsimony informative. For MP tree length = 3424, CI = 0.3881, RI = 0.6658, RC = 0.2735, HI = 0.6119 (Fig. 2). For *Heterorhabditis* ITS regions MP analysis showed that the alignment resulted in 802 characters of which 148 are constant, 100 variable characters are parsimony uninformative and 554 characters are parsimony-informative. For MP tree length = 2024, CI = 0.6881, RI = 0.6658, RC = 0.4735, HI = 0.3119 (Fig. 3). Bayesian tree inferred analysis was performed for *Steinernema* and *Heterorhabditis* ITS region sequences (Fig. 4 and 5). Model test 3.06 (Posada & Crandall, 1998) was performed for nucleotide substitution model. Both the likelihood ratio test, Akaike information criterion selected the general time reversible (GTR) model with invariable sites (I) and a γ-shaped distribution of substitution rates (Γ) as the best fitting substitution model.

Phylogenetic analysis of D2-D3/28S rDNA region: Fifteen (D2-D3/28S rDNA gene) indigenous samples from *Steinernema* and one from *Heterorhabditis* isolates along with registered species have been included in the analysis (Table 2). For *Steinernema* D2-D3/28S rDNA regions MP analysis showed that the alignment resulted in 682 characters of which 128 are constant, 100 variable characters are parsimony uninformative and 454 characters are parsimony-informative. For MP tree length = 2424, CI = 0.3681, RI = 0.6558, RC = 0.2835, HI = 0.6219 (Fig. 7). While for *Heterorhabditis* D2-D3/28S rDNA regions MP analysis showed that the alignment resulted in 722 characters of which 128 were constant, 90 variable characters parsimony uninformative and 554 characters parsimony informative. For MP tree length = 1024, CI = 0.6581, RI = 0.6358, RC = 0.4745, HI = 0.3219 (Fig. 8).

Bayesian analysis was used to reconstruct phylogenetic relationships among the fourteen isolates of *Steinernema* and one from *Heterorhabditis* with other worldwide species (Fig. 9-10). *Steinernema litorale* PAK.P.*S*.7 (JQ795723) formed clade with '*feltiae*-group' with 99% similarity. *S. bifurcatum* n. sp., (JQ838179), *S. maqbooli* (JQ838176, JQ838177) *Steinernema* n. sp., A (JQ838178) and *Steinernema* n. sp., B (JX068817) formed clade with '*bicornutum* group', *Steinernema balochiense* n. sp., (JX068821) and *Steinernema* n. sp., C (JX068820) formed monophyletic group with '*carpocapsae*-group'. Whereas, Heterorahabditis isolate PAK.S.*H*.92A (JQ838180) formed sister clade with *Heterorhabditis indica*.

Table 1. Specimens information and Genbank accession number of seventy nine *Steinernema* and *Heterorhabditis* isolates by using ITS-1, 5.8S and ITS-2 used for phylogenetic analysis.

S.NO	*PCR No.	New Code	Accession No.	Similarity
1.	-	PAK.S.*S*.34	GU130183	*S. siamkayai*
2.	-	PAK.S.*S*.38	GU130184	*S. siamkayai*
3.	-	PAK.S.*S*.49	GU130185	*S. siamkayai*
4.	-	PAK.S.*S*.58	GU130186	*S. siamkayai*
5.	-	PAK.S.*S*.59	GU130187	*S. siamkayai*
6.	-	PAK.S.*S*.23	GU130182	*S. carpocapsae*
7.	-	PAK.S.*H*.56	GU130179	*H. indica*
8.	-	PAK.P.*S*.43	GU130180	*S. pakistanense*
9.	-	PAK.P.*S*.44	GU130181	*S. pakistanense*
10.	2	PAKS.*S*.71	JF775374	*S. pakistanense*
11.	4N	PAK.S.*S*.72	JF775375	*S. pakistanense*
12.	16	PAK.S.*S*.10	JF892544	*S. siamkayai*
13.	5	PAK.S.*S*.73	JF892545	*S. siamkayai*
14.	8	PAK.P.*S*.7	JF892546	*S. litorale*
15.	24	PAK.S.*S*.74	JN157768	*S. pakistanense*
16.	26	PAK.S.*S* 75	JN157769	*S. maqbooli*
17.	12	PAK.S.*S*.12	JN157770	*Steinernema* n. sp., A
18.	20	PAK.S.*S*.76	JN157771	*Steinernema* n. sp., A
19.	39	PAK.P.*S*.79	JN157772	*S. siamkayai*
20.	36	PAK.S.*H*.78	JN157773	*Heterorhabditis* n. sp., A
21.	27	PAK.S.*H*.77	JN157774	*H. indica*
22.	48	PAK.P.*S*.80	JN571085	*S. siamkayai*
23.	60	PAK.S.*S*.15	JN571086	*S. abbasi*
24.	21	PAK.S.*H*.82	JN571087	*Heterorhabditis* n. sp.,A
25.	14	PAK.P.*S*.37	JN571088	*S. bifurcatum* n. sp.
26.	15	PAK.S.*S*.63	JN571089	*Steinernema* n. sp., B
27.	6	PAK.S.*H*.81	JN571090	*H. indica*
28.	53	PAK.S.*S*.50	JN571091	*S. pakistanense*
29.	41	PAK.S.*S*.84	JN571092	*S. siamkayai*
30.	49	PAK.P.*H*.67	JN571093	*Heterorhabditis* n. sp., A
31.	42	PAK.P.*H*.70	JN571094	*Heterorhabditis* n. sp., A
32.	46	PAK.S.*S*.86	JN571095	*Steinernema* n. sp. C
33.	71	PAK.S.*S*.16	JN571096	*S. abbasi*
34.	73	PAK.S.*S*.97	JN571097	*S. maqbooli*
35.	32	PAK.S.*S*.104	**AP	*Steinernema* sp.
36.	51	PAK.P.*S*.64	JQ894821	*S. siamkayai*

37.	61	PAK.S.*S*.89	JQ894822	*S. siamkayai*
38.	63	PAK.P.*S*.90	JQ894823	*S. siamkayai*
39.	73	PAK.S.*S*.101	JQ894824	*S. maqbooli*
40.	1(61)	PAK.S.*S*.89	JQ894828	*S. siamkayai*
41.	6(69)	PAK.S.*H*.92A	JQ894826	*H. indica*
42.	7(50)	PAK.P.*S*.112	JQ894827	*S. siamkayai*
43.	72	PAK.B.*S*.200	JX110444	*S. siamkayai*
44.	348	PAK.P.*S*.150	JX110445	*S. siamkayai*
45.	A	PAK.P.*S*.99	JX110446	*S. siamkayai*
46.	37	PAK.S.*S*.118	JX135549	*S. pakistanense*
47.	75	PAK.S.*S*.142	JX135545	*S. pakistanense*
48.	76	PAK.S.*S*.120	AP	*S. pakistanense*
49.	77	PAK.S.*S*.125	JX135546	*S. pakistanense*
50.	45	PAK.B.*S*.85	JX135547	*S. balochiense* n. sp.
51.	78	PAK.S.*S*.130	JX135550	*S. pakistanense*
52.	91	PAK.K.*S*.251	JX068812	*S. abbasi*
53.	94	PAK.K.*S*.254	JX068813	*S. abbasi*
54.	81	PAK.S.*H*.123	JX068814	*H. indica*
55.	82	PAK.S.*S*.127	JX068815	*S. siamkayai*
56.	85	PAK.S.*S*.133	JX068816	*S. siamkayai*
57.	88	PAK.S.*S*.137	JX135555	*S. siamkayai*
58.	86	PAK.S.*S*.139	JX135556	*S. siamkayai*
59.	84	PAK.S.*S*.136	JX135557	*S. siamkayai*
60.	78	PAK.S.*S*.135	JX135551	*S. pakistanense*
61.	66 (J)	PAK.S.*S*.91	JX135554	*S. siamkayai*
62.	135	PAK.S.*S*.153	JX135558	*S. pakistanense*
63.	109	PAK.P.*H*.163	JX144733	*H. indica*
64.	-	PAK.K.*S*.261	JX144735	*S. abbasi*
65.	139	PAK.K.*S*.419	AP	*S. pakistanense*
66.	13f	PAK.S.*S*.145	JX135553	*S. abbasi*
67.	124	PAK.P.*S*.157	JX144734	*S. pakistanense*
68.	128	PAK.S.*S*.109	JX135552	*S. maqbooli*
69.	125	PAK.N.*S*.301	JX144736	*S. siamkayai*
70.	70	PAK.S.*S*.96	AP	*Steinernema* n. sp. C
71.	113	PAK.S.*H*.302	AP	*Heterorhabditis* n. sp., A
72.	42	PAK.P.*S*.94	JX144742	*S. abbasi*
73.	134	PAK.S.*S*.305	JX232282	*S. pakistanense*
74.	96	PAK.K.*S*.262	JX232283	*S. abbasi*
75.	69	PAK.S.*H*.92	JX144743	*H. indica*
76.	-	PAK.P.*S*.37(1)	JX144741	*S. bifurcatum* n. sp.
77.	136	PAK.S.*S*.307	JX232285	*S. maqbooli*
78.	79	PAK.S.*S*.303	JX144744	*S. pakistanense*
79.	45	PAK.B.*S*.304	JX232284	*S. abbasi*

*PCR No. the samples stored at NNRC's molecular lab;**AP= Applied for accession No.

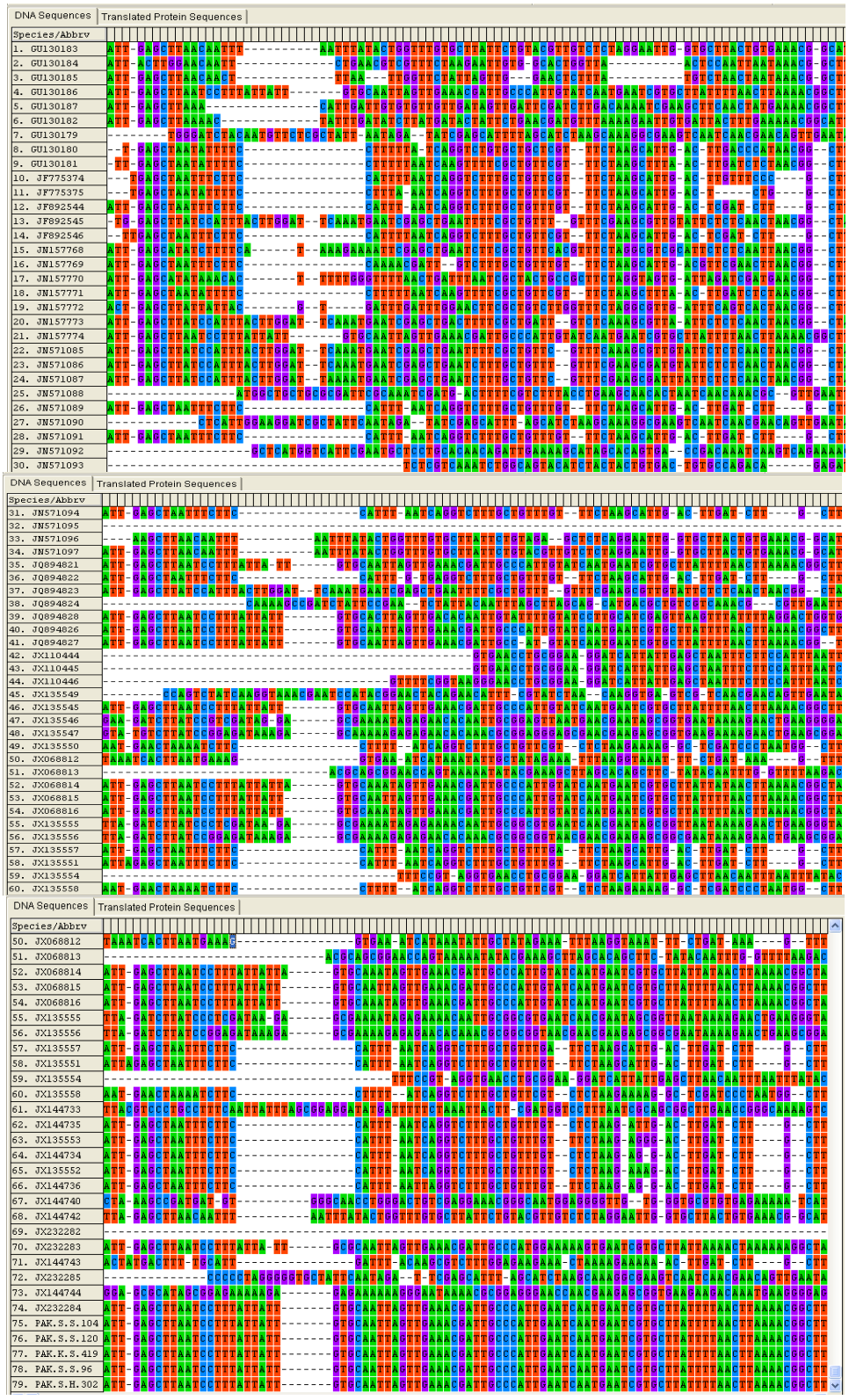

Fig.1. Alignment report of ITS rDNA sequences of the *Steinernema* and *Heterorhabditis* species.

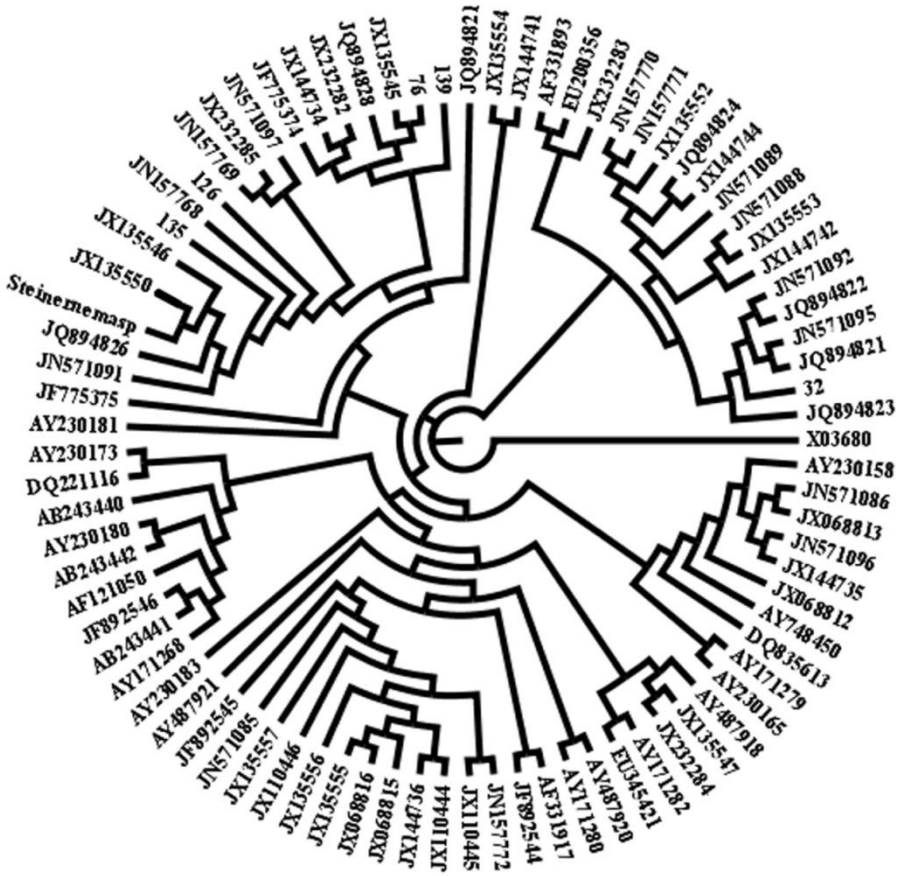

Fig. 2. Maximum parsimony (MP) tree of *Steinernema* sp., based on the alignment of ITS region sequences.

Fig. 3. Maximum parsimony (MP) tree of *Heterorhabditis* sp., based on the alignment of ITS region sequences.

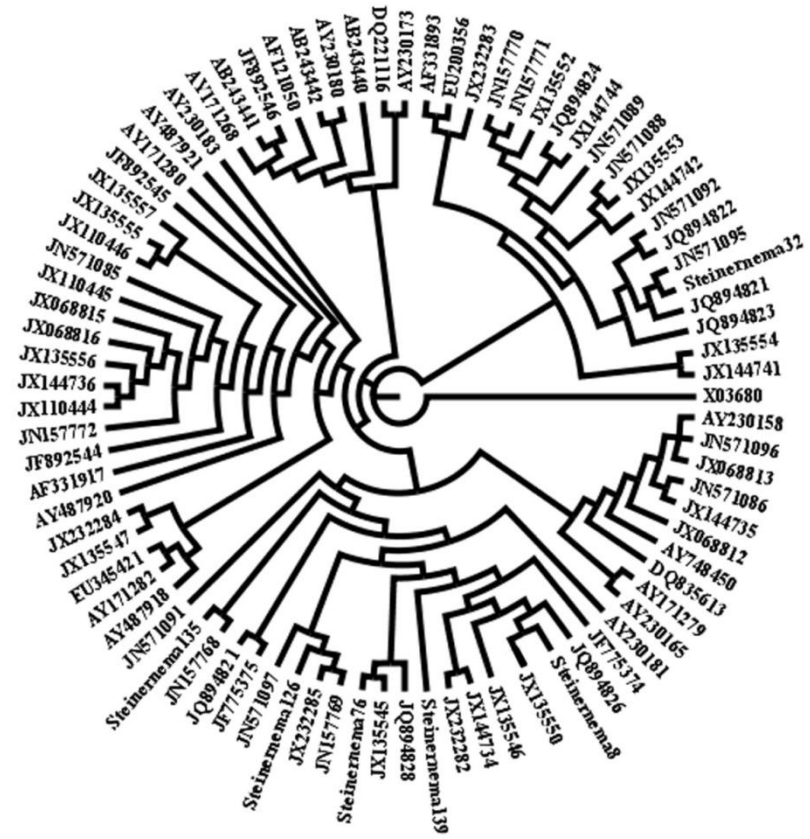

Fig. 4. Bayesian inferred (BI) tree of *Steinernema* sp., based on the alignment of ITS region sequences.

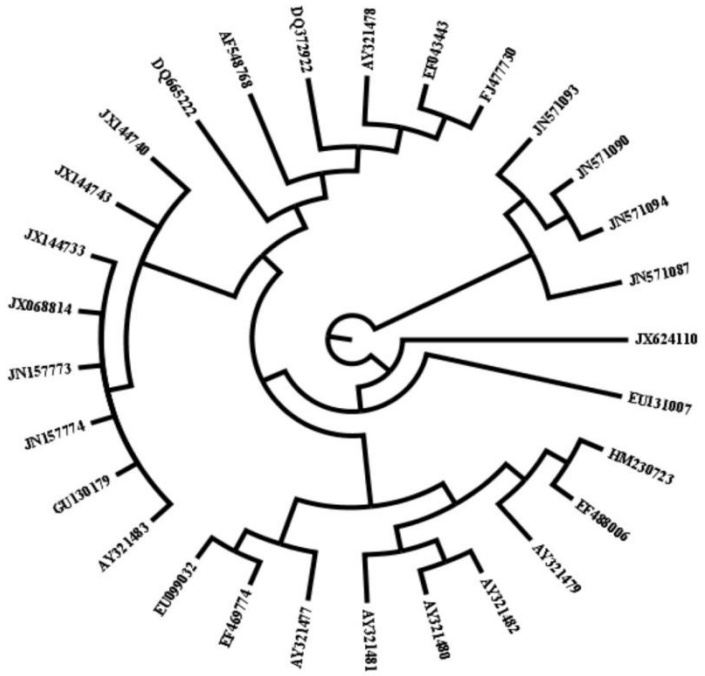

Fig. 5. Bayesian inferred (BI) tree of *Heterorhabditis* sp., based on the alignment of ITS region sequences.

Table 2. Specimens information and Genbank accession numbers of *Steinernema* and *Heterorhabditis* isolates by using D2-D3/28S ribosomal RNA gene for phylogenetic analysis.

S. No.	PCR No.	New code	Accession No.	Similarity index/species
1	8	PAK.P.S.7	JQ795723	*Steinernema litorale***
2	26	PAK.S.S.75	JQ838176	*S. maqbooli*
3	136	PAK.S.S.97	JQ838177	*S. maqbooli*
4	20	PAK.S.S.76	JQ838178	*Steinernema* n. sp. A
5	14	PAK.P.S.37	JQ838179	*S. bifurcatum* n. sp.
6	6 (69)	PAK.S.*H*.92A	JQ838180	*Heterorhabditis indica*
7	15	PAK.S.S.63	JX068817	*Steinernema* n. sp. B
8	77	PAK.S.S.125	AP*	*S. pakistanense*
9	-	PAK.P.S.37	JX068825	*S. bifurcatum* n. sp.
10	46	PAK.S.S.86	AP*	*Steinernema* n. sp. C
11	45	PAK.B.S.85	JX068821	*S. balochiense* n. sp.
12	-	PAK.S.S.44	JX068824	*S. pakistanense***
13	53	PAK.S.S.50	JX068822	*S. pakistanense***
14	-	PAK.S.S.43	AP*	*S. pakistanense***
15	70	PAK.S.S.96	JX068820	*Steinernema* n. sp. C

AP= *Applied;** First time sequenced D2-D3.

Fig. 6. Alignment report of D2-D3/ 28S rDNA sequences of the *Steinernema* and *Heterorhabditis* species.

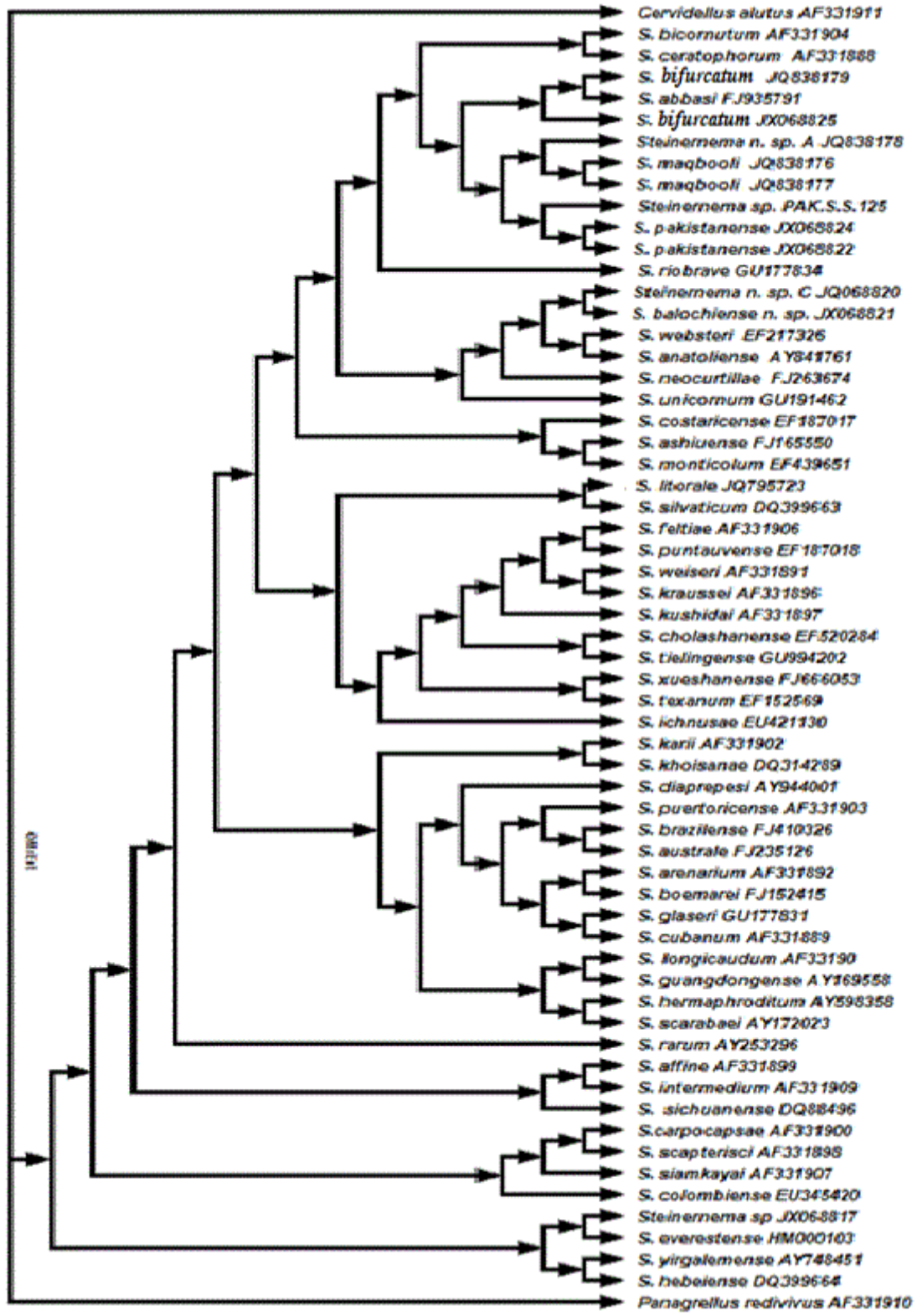

Fig. 7. Maximum parsimony (MP) tree of *Steinernema* sp. based on the alignment of D2-D3/28SrDNA sequences.

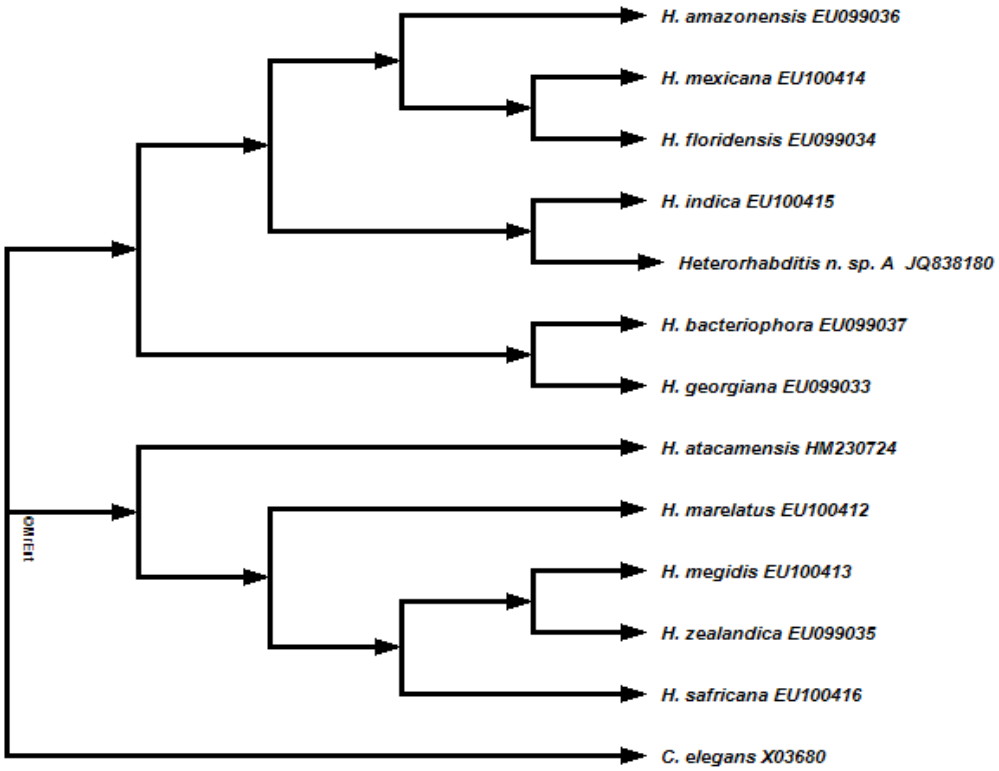

Fig. 8. Maximum parsimony (MP) tree of *Heterorhabditis* sp. based on the alignment of D2-D3/28S region sequences.

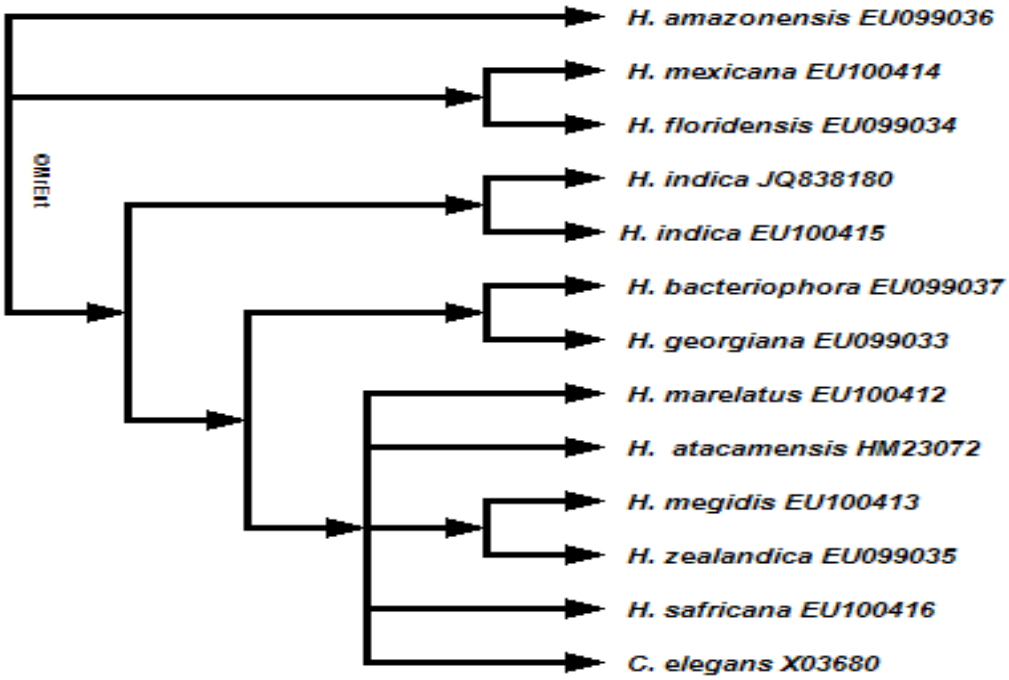

Fig. 9. Bayesian inferred (BI) tree of *Heterorhabditis* sp. based on the alignment of D2-D3/28S region sequences.

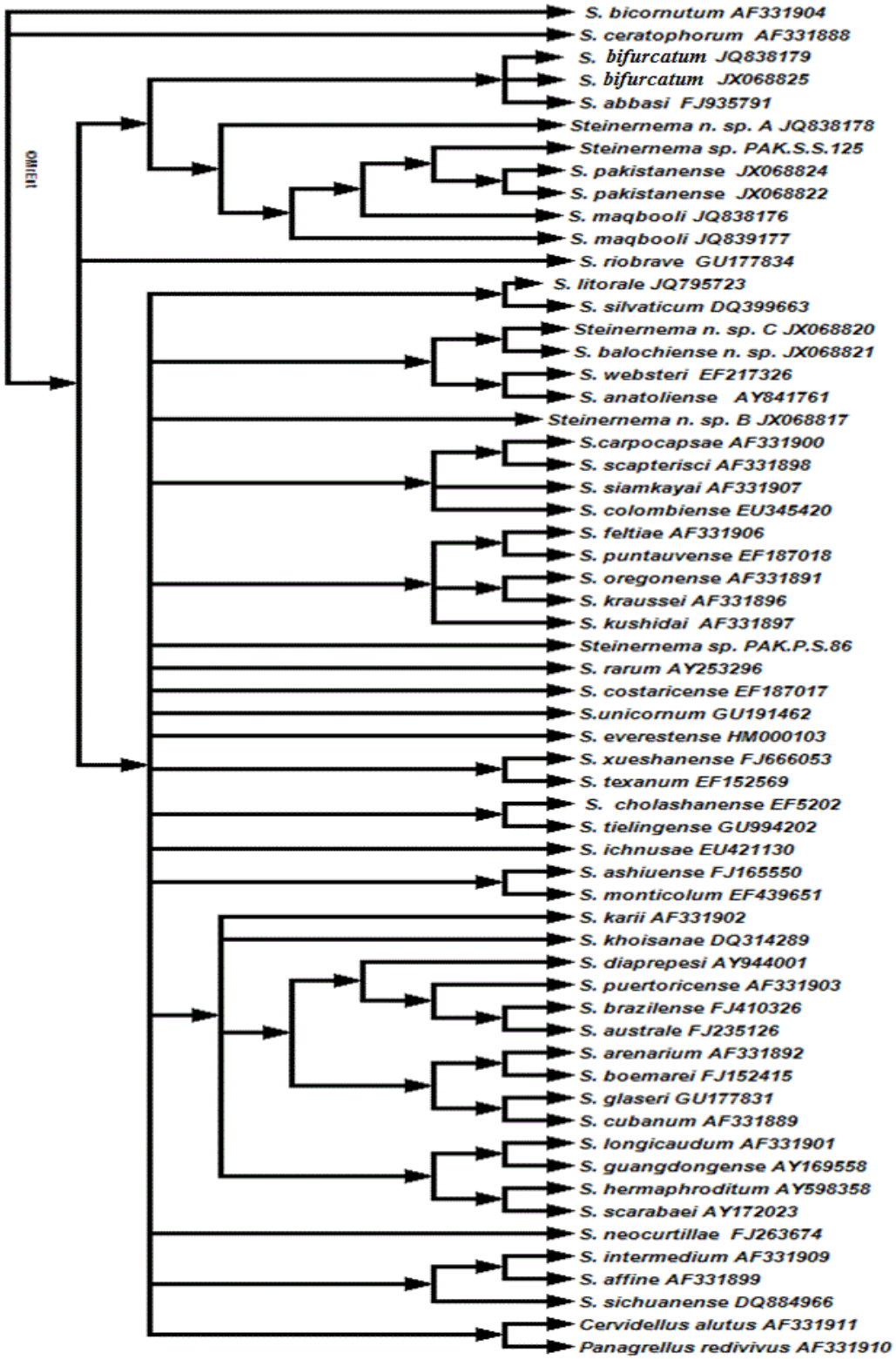

Fig. 10. Bayesian inferred (BI) tree of *Steinernema* sp. based on the alignment of D2-D3/28S region sequences.

Fig. 11. Alignment report of 12S ribosomal DNA gene sequences of the *Steinernema* and *Heterorhabditis* species.

Phylogenetic analysis of the 12S rDNA gene: Twenty four isolates from *Steinernema* and five from *Heterorhabditis* isolates were included in the analysis 12S rDNA gene (Table 3). For *Steinernema*12S rDNA gene MP analysis showed that the alignment resulted in 504 characters of which 47 are constant, 199 variable characters are parsimony uninformative and 258 characters are parsimony-informative. For MP tree length = 1455, CI = 0.4481, RI = 0.6952, RC = 0.2783, HI = 0.5519 (Fig. 12) while for *Heterorhabditis* 12S rDNA gene MP analysis showed that the alignment resulted in 522 characters of which 185 constant, 303 variable characters parsimony uninformative and 34 characters parsimony-informative. For MP tree length = 445, CI = 0.7581, RI = 0.6458, RC = 0.6145, HI = 0.2419 (Fig. 13).

Bayesian analysis was used to reconstruct phylogenetic relationships among the isolates of *Steinernema* and *Heterorhabditis* with other worldwide species (Fig. 14-15). *Steinernema litorale* PAK.P.S.7 (JQ795723) formed clade with '*feltiae*-group', *S. bifurcatum* n. sp., (JQ838179), *S. maqbooli* (JQ838176, JQ838177), *Steinernema* n. sp., A (JQ838178) and *Steinernema* n. sp., B (JX068817) formed clade with '*bicornutum* group' and *S. balochiense* n. sp., (JX068821) and *Steinernema* n. sp., C (JX068820) formed monophyletic group with '*carpocapsae*-group'. *Heterorhabditis* sp. was first time successfully sequenced on the basis of 12S rDNA.

Table 3. Specimens information and Genbank accession numbers of twenty nine *Steinernema* and *Heterorhabditis* isolates by 12S rDNA gene used for phylogenetic analysis.

S. No.	PCR No.	New code	Accession No.	Similarity index
1	41	PAK.S.*S*.84	JN571098	*S. siamkayai*
2	42	PAK.P.*H*.70	JN571099	*Heterorhabditis* n. sp., A
3	43	PAK.P.*S*.94	AP*	*Steinernema* sp.
4	45	PAK.B.*S*.85	JN571100	*S. balochiense* n. sp.
5	46	PAK.S.*S*.86	JN571101	*Steinernema* n. sp., C
6	48	PAK.P.*S*.80	JN571102	*S. siamkayai*
7	49	PAK.P.*H*.67	JN571103	*Heterorhabditis* n. sp., A
8	51	PAK.P.*S*.64	JN571104	*S. siamkayai*
9	55	PAK.B.*S*.93	JN571105	*S. siamkayai*
10	56	PAK.S.*H*.17	JN571106	*H. bacteriophora*
11	57	PAK.S.*S*.87	JN571107	*S. siamkayai*
12	58	PAK.S.*S*.88	JN599138	*S. siamkayai*
13	59	PAK.P.*S*.24	JN599139	*S. siamkayai*
14	60	PAK.S.*S*.15	JN599140	*S. abbasi*
15	61	PAK.S.*S*.89	JN599141	*S .siamkayai*
16	63	PAK.P.*S*.90	JN599142	*S. siamkayai*
17	66	PAK.S.*S*.91	JN599143	*S. siamkayai*
18	69	PAK.S.*H*.92	JN599144	*H. indica*
19	40	PAK.P.*S*.99	JN599145	*S. siamkayai*
20	70	PAK.S.*S*.96	JN599146	*Steinernema* n. sp., C
21	8	PAK.P.*S*.7	JN599147	*S. litorale*
22	-	PAK.S.*S*.12	JQ838181	*Steinernema* n. sp., A
23	-	PAK.S.*S*.76	JQ838182	*Steinernema* n. sp., A
24	-	PAK.S.*S*.75	JQ838183	*S. maqbooli*
25	-	PAK.S.*H*.149	JX144737	*Heterorhabditis* sp.
26	-	PAK.S.*S*.63	JX068819	*Steinernema* n. sp., B
27	-	PAK.S.*S*.43	JX144739	*S. pakistanense*
28	-	PAK.S.*S*.37(1)	JX068826	*S. bifurcatum* n. sp.
29		PAK.S.*S*.50	JX144738	S. *pakistanense*

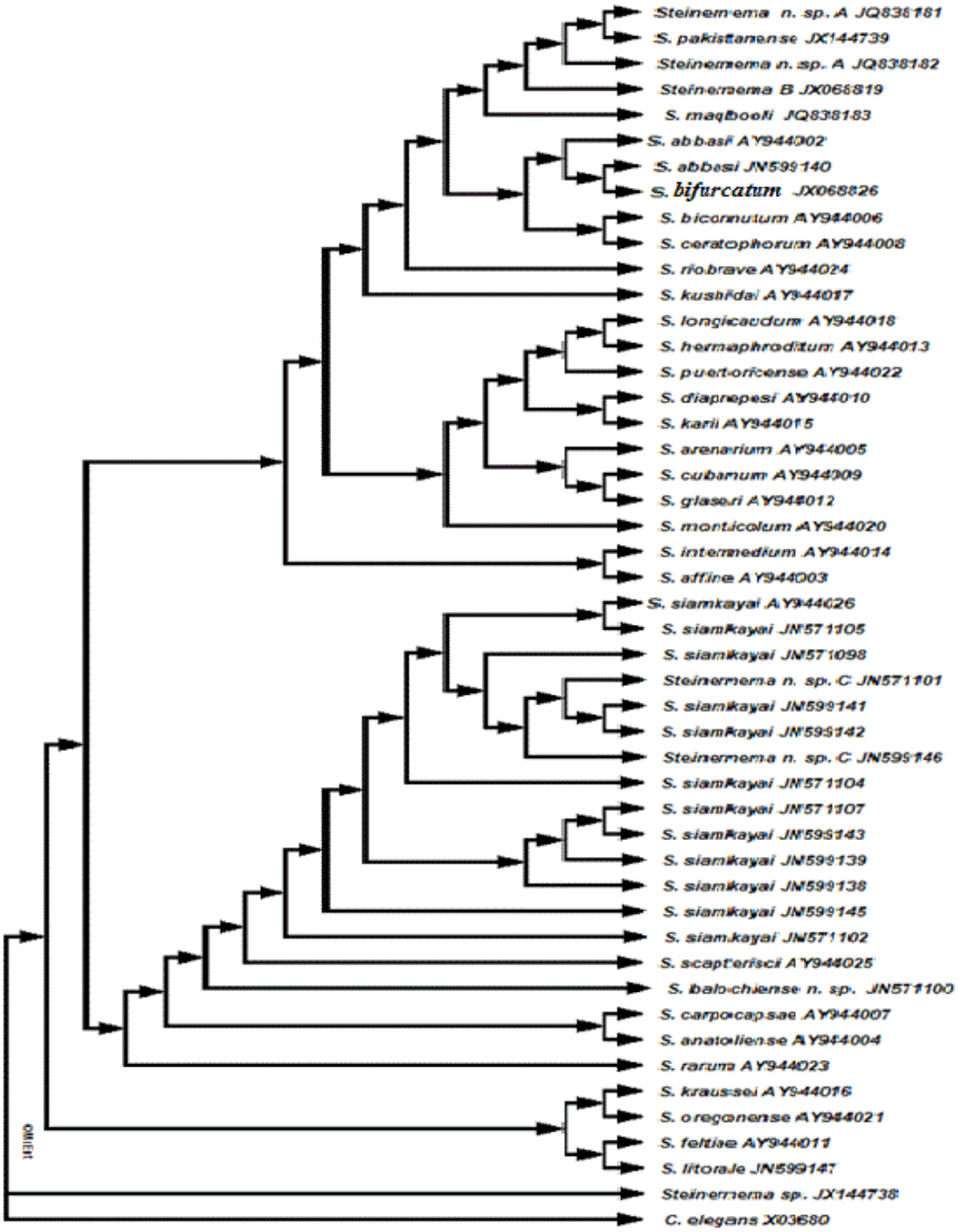

Fig. 12. Maximum parsimony (MP) tree of *Steinernema* sp. based on the alignment of 12S rDNA gene sequences.

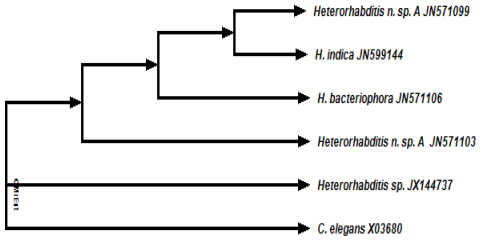

Fig. 13. Maximum parsimony (MP) tree of *Heterorhabditis* sp., based on the alignment of 12S rDNA gene sequences.

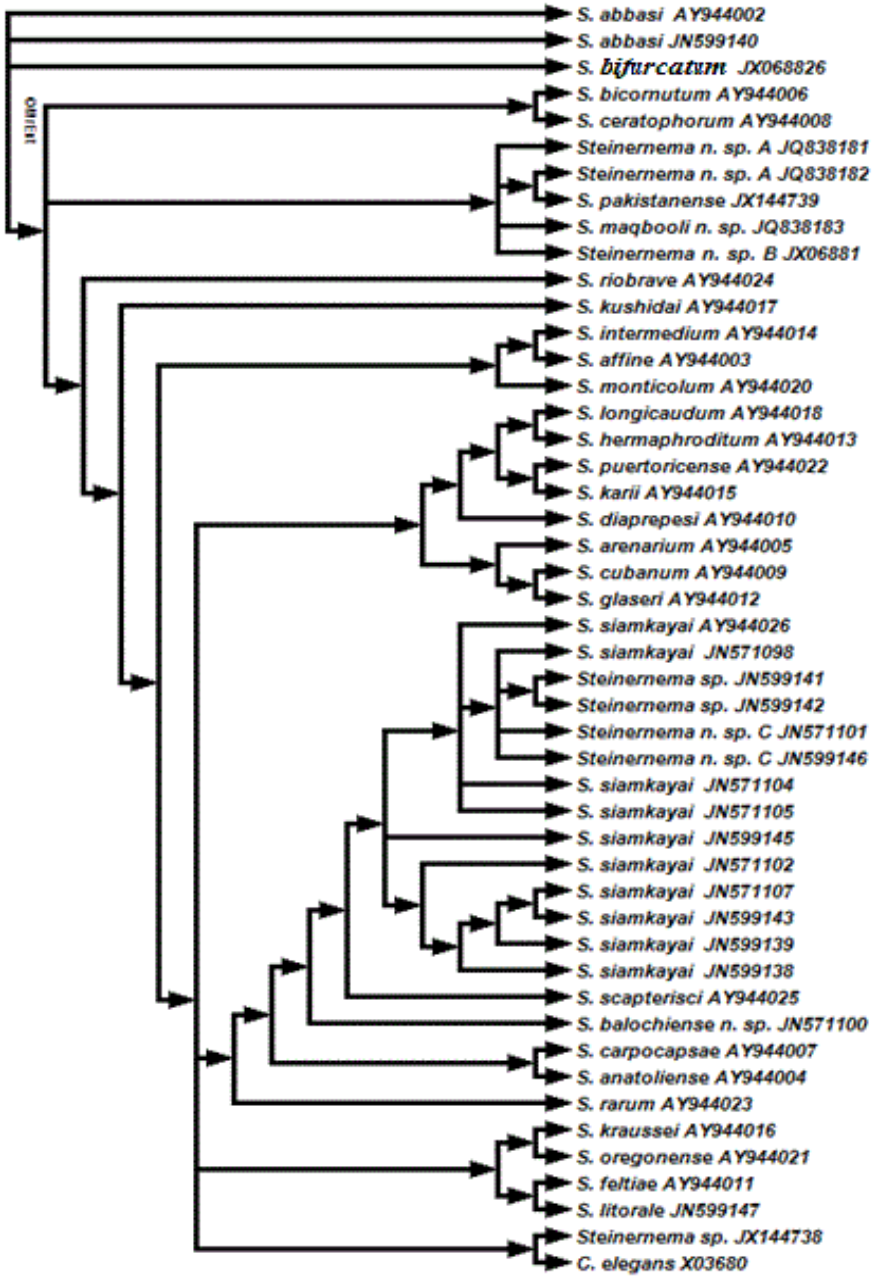

Fig. 14. Bayesian inferred (BI) tree of *Steinernema* sp. based on the alignment of 12S rDNA gene sequences.

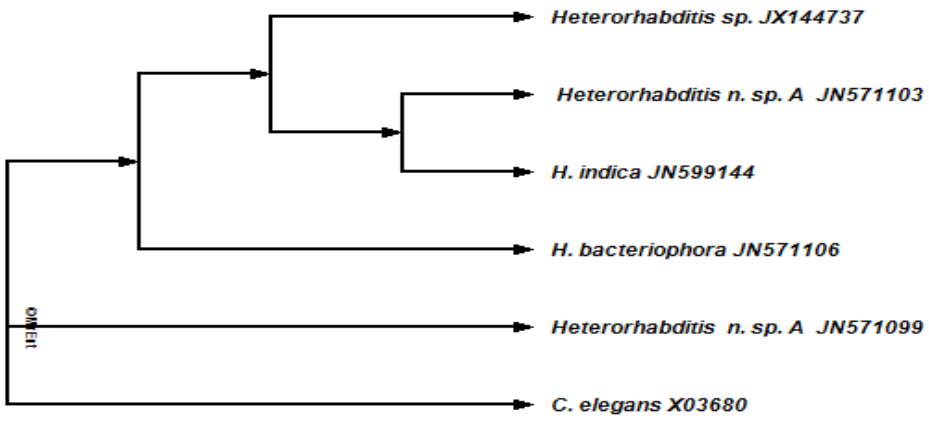

Fig. 15. Bayesian inferred (BI) tree of *Heterorhabditis* sp. based on the alignment of 12S rDNA sequences.

DNA markers considered for phylogenetic and population genetic studies (Nuclear ribosomal DNA) had established extremely useful and employed extensively to study nematode systematics at the molecular level. Numerous of these approaches have provided motivating and significant insights into biodiversity and evolution, chiefly for EPNs (Akhurst, 1987; Reid & Hominick, 1992; Gardner *et al*., 1994; Liu & Berry, 1995; Reid *et al*., 1997; Adams *et al*., 1998; Stock *et al*., 2001). This study indicated that Pakistan has a rich and diverse entomopathogenic nematode fauna, from which novel strains/species can provide a new and improved potential for the successful application of EPNs and their symbiotic bacteria as biological control agents. Phylogenetic analysis at the species level, small subunit (SSU) rRNA had been considered insignificant when attempting to distinguish closely related species (Liu *et al*., 1997), while the less conserved regions of the large ribosomal subunit (LSU) and the internal transcribed spacer (ITS) that separates rDNA coding regions were proved more informative (Nguyen *et al*., 2001; Stock *et al*., 2001; Adams *et al*., 2006; Nadler *et al*., 2006).

Advances made in molecular biology and software technology have revitalized the discipline of taxonomy. Molecular taxonomy herein considered as an additional step for species descriptions several sequences of steinernematid and heterorhabditid nematode isolates from different regions of Pakistan were identified by using different molecular markers. Phylogenetic relationships among Pakistani EPN strains steinernematids and heterorhabditid, ITS-1, 5.8S, ITS-2 (seventy nine); D2-D3 and 28S (LSU) sequences of rDNA (fifteen) and 12S rDNA mitochondrial gene (twenty nine) were estimated by nucleotide sequences using three molecular makers to investigate the genetic diversity. Molecular identification resulted in the nine identified species viz., *S. abbasi* Elawad *et al*., 1997; *S. asiaticum* Anis *et al*., 2002; *S. carpocapsae* (Weiser, 1955) Wouts *et al*., 1982; *S. feltiae* (Filipjev, 1934) Wouts *et al*., 1982; *S. litorale* Yoshida, 2004; *S. pakistanense* Shahina *et al*., 2001; *S. siamkayai* Stock *et al*., 1998; *Heterorhabditis indica* Poinar *et al*., 1992; *H. bacteriophora* Poinar, 1976 and seven unidentified species viz., *S. bifurcatum* n. sp., (Shahina *et al*., 2014); *S. maqbooli* (Shahina *et al*., 2013); *S. balochiense* n. sp., (Shahina *et al*., 2013a); *Steinernema* n. sp. A; *Steinernema* n. sp. B; *Steinernema* n. sp., C and *Heterorhabditis* n. sp. A.

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