Molecular Identification of Six *Steinernema* Isolates and Characterization of their Internal Transcribed Spacers Regions

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Abstract

Molecular identification and genotyping of entomopathogenic nematodes (EPNs) are prerequisites for their proper classification, biodiversity studies, and their potential use in biological control programs. In Palestine, although several isolates of EPNs have been collected, phenotypically characterized, and assessed for their tolerance to cold and heat stresses, the molecular identification and genotyping of most of these isolates have not been accomplished yet. In this study, genomic DNA was isolated from all nematode stages of six *Steinernema* isolates collected in several areas of historical Palestine. The Internal Transcribed Spacers 1 and 2 (ITS1 and ITS2) as well as the 5.8S regions of the 26S rDNA were amplified and sequenced. The obtained sequences were aligned to identify, to explore sequence variations and construct phylogenetic relationships among the isolates. Three of the isolates were identified as *S. feltiae* while the other three as *S. carpocapsae*. At the interspecies level, the 5.8S sequences were more conserved than the ITS sequences which varied in structure and length. Moreover, the ITS2 sequences contained more variable informative sites than those of ITS1. The aligned sequences of the six isolates were analyzed using the neighbor-joining method for two sets of sequences; ITS1-ITS2, and ITS1-5.8S - ITS2. In both cases, similar clustering profiles were produced with slight differences in the branch length of each cluster. These data showed that the association of the different rDNA regions of these *Steinernema* isolates with different evolutionary rates is solely at the species level but not among different isolates of the same species.

Keywords: Entomopathogenic Nematodes, Internal Transcribed Spacers, Phylogenetic Analysis, Ribosomal DNA.

1. Introduction

The use of Entomopathogenic nematodes (EPNs), belonging to the families *Steinernematidae* and *Heterorhabditae* as biocontrol agents of soil-borne pests, necessitates the availability of a collection of EPN strains with various adaptive traits that enhance their efficacy and persistence as biocontrol agents when introduced to different environmental conditions (Grewal *et al*., 2001). In historical Palestine, which is divided into five different environmental and climatic zones that support diverse sets of life forms (Salem, 2008), several isolates of EPNs have been collected, phenotypically characterized, and assessed for their tolerance to cold and heat stresses (Iraki *et al*., 2000; Salame *et al*., 2010). However, molecular identification of the most of these isolates was not conducted yet. Such identification would be a prerequisite for proper classification, biodiversity studies, and pest-management programs.

Because EPNs belonging to the genus *Heterorhabditus* produce hermaphrodites in their life cycle while those of *Steinernema* do not (Muthulakshmi *et al*., 2012), molecular tools are not necessary to differentiate between these two genera. Such tools are, however, very important in order to identify and distinguish between species within each genus, where phenotypic variations become more limited. The Non-coding Internal Transcribed Spacers 1 and 2 (ITS1 and ITS2) of the Ribosomal DNA (rDNA) have provided a powerful tool to identify nematodes since these regions afford species specific polymorphism and in some cases populations specific polymorphism (Powers *et al*., 1997). These two spacers (ITS1 and ITS2) flank a conserved gene coding for the 5.8S of the rRNA. The much more conservative feature of the 5.8S sequences as compared to the two spacers indicates that they vary significantly in their evolutionary rates. Such differences

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can disclose phylogenetic relationships among the different nematode species. The frequently reported intraspecific variation between rDNA sequences of nematodes disagrees with the conception that this gene family has concertedly evolved (Blok *et al*., 1998; Elbadri *et al*., 2002; Hugall *et al*., 1999; Hung *et al*., 1999; Morales-Hojas *et al*., 2001). This study aims at identifying and revealing the evolutionary relationship among six entomopathogenic nematode isolates belonging to the genus *Steinernema* by characterizing the ITS1-5.8S-ITS2 region of their rDNA.

2. Material and Methods

The EPN isolates were provided by Prof. Glazer from the Volcani Center, Bet Dagan, who recovered them from soil samples by baiting with last instar larvae of the wax moth, Galleria mellonella according to Bedding and Akhurst (1975).

2.1. DNA Extraction, Amplification and Sequencing

For each isolate, Infective Juveniles (IJs) were collected from the white trap while the other stages (adults) were gathered from the *G. mellonella* cadaver laying on the white trap. The collected nematodes were put in a 15 ml falcon tube and centrifuged at 10000 rpm for 5 min. The precipitated nematodes were transferred to an Eppendorff tube and ground using a drill. Genomic DNA was then extracted as described in the protocol of Dnasy ™ System (Qiagen GmbH, Leusden, Netherlands). The ITS region from each isolate was amplified in a sterile 0.5 ml tube using the primers described by Vrain *et al*. (1992). PCR amplifications were performed according to Hominick *et al*. (1997) in a PTC-100 thermocycler (MJ research, USA). The amplified ITS regions were electrophoresed in 1% agarose (w/v) gel using 1X TBE buffer at 120 V for 1 hr. Then, they were excised from the gel and purified using QIAquick Gel Extraction Kit (Qiagen GmbH, Leusden Netherlands). Both strands of the purified PCR products were sequenced using sequence specific primers with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystem, USA) carried out on ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, USA). The identity of the sequences was confirmed by a BLAST (Basic Local Alignment Search Tool) search at NCBI (National Center for Biotechnology Information). The obtained sequences were deposited at the NCBI database with the accession numbers listed in Table 1.

Table 1. Gene bank accession numbers for ITS1-5.8S-ITS2 sequences and the result of their BLAST against the Nucleotide collection (nt) database of the NCBI website using the BLASTN 2.2.27+ software (Zhang *et al*., 2000; Morgulis *et al*., 2008).

Isolate	Accession number	Species	Query cover	E value	Max identity
SiS11	KC571262	*S. carpocapsae*	99%	0.0	99%
SiS7	KC571260	*S. carpocapsae*	99%	0.0	99%
IS34	KC571265	*S. carpocapsae*	98%	0.0	99%
SiS15	KC571263	*S. feltiae*	99%	0.0	99%
F17	KC571264	*S. feltiae*	99%	0.0	99%
SiS8	KC571261	*S. feltiae*	99%	0.0	99%

2.2. Phylogenetic Analysis

The sequence sets ITS1+ITS2 and ITS1+5.8S+ITS2 were multialigned with the default parameters of the ClustalX program (Larkin *et al*., 2007). The corresponding sequences of the EPN *Heterorabditis bacteriophora* (NCBI accession number AY321477) were used as outgroup. Phylogenetic relationships between the isolates were established using the MEGA4 program (Tamura *et al*., 2007) following the neighbor-joining (NJ) method (Saitou and Nei, 1987). The evolutionary distances were computed using the LogDet method according to Tamura and Kumar (2002) and are expressed as the units of the number of base substitutions per site. This method permits phylogenetic analysis of sequences that have different base composition such as the ITS regions. The differences in the composition bias among sequences were considered in evolutionary comparisons. Trees were constructed by clustering of associated taxa based on 1000 replicates in the bootstrap test (Felsenstein, 1985).

3. Results and Discussion

For the purpose of identifying the six isolates of EPNs at the species level, their ITS1-5.8S-ITS2 sequences were BLASTed against the nucleotide collection (nr/nt) database. Based on sequence homology, the isolates SiS11 (Sha'alabim), SiS7 (Bet Nir), and IS34 were identified as *S. carpocapsae* while the isolates SiS15 (Alonim), F17 (Karmel), SiS8 (Zichron) were identified as *S. feltiae* (Table 1). Although the length and GC content of both spacers was almost the same within species, they varied at the interspecies level (Table 2).

Such variations are indeed the basis of the frequent use of these spacers to identify nematodes at the species level (Hominick *et al*., 1997). On the other hand, the length and GC content of the 5.8S gene was constant among all isolates (Table 2).

Table 2. Lengths (in bp) and G+C contents (in %) of sequenced rDNA regions of the six *Steinernema* isolates

Isolate	ITS1		5.8S		ITS2	
	(bp)	G+C	(bp)	G+C	(bp)	G+C
		(%)		(%)		(%)
SiS11	278	35	157	41	302	39
SiS7	278	35	157	41	302	39
IS34	279	35	157	41	302	39
SiS15	264	38	157	41	306	36
F17	264	38	157	41	306	37
SiS8	264	38	157	41	306	36

Therefore, the length variations in the rDNA sequences among different *Steinernema* species is mostly due to variations in the ITS region which is in accordance with other reports in the literature (Nguyen *et al*., 2001). Moreover, when the different regions of the rDNA sequences were multiple aligned, the number of variable and informative sites within the non-coding spacers' sequences was 175 sites, which is significantly higher than those of the coding 5.8S sequences which offered only 10 such sites along its entire length (Table 3).

Table 3. Number of variable and constant sites in the six *Steinernema* isolates: total number of sites used for coding (5.8S), noncoding (ITS1, ITS2 or both), and combined sequences (5.8S+ITS1-ITS2).

	ITS1	ITS2	ITS1+ ITS2	5.8S	ITS1+ ITS2+ 5.8S
Length (bp)	299	324	623	157	780
Constant sites	237 (79%)	209 (64%)	447 72%)	147 (94%)	592 (76%)
Variable but uninformative	0	1	1	0	1
Variable and informative	62 (21%)	114 (35%)	175 28%)	10 (6%)	187 (24%)

The results showed also that these informative sites are more available in the ITS2 (35%) than in the ITS1 (21%). Such variations in the rDNA sequence were reported to exist even at the intra-individual level in nematodes (Subbotin *et al*., 2000; Blok *et al*., 1998). The amount and nature of variable and informative sites in multiple-aligned rDNA sequences could be used for revealing the phylogenetic relationship among entomopathogenic nematodes.

Phylogenetic analyses with NJ method were performed using the non-coding ITS sequences alone or by combining the whole rDNA sequences (ITS1-5.8S-ITS2). In both cases trees with similar topologies were produced (Figure 1A, B). The six isolates were separated into two clusters: the first included the *S.*

carpocapsae isolates SiS11 (Sha'alabim), SiS7 (Bet Nir), and IS34, while the second included the *S. feltiae* isolates SiS15 (Alonim), F17 (Karmel), and SiS8 (Zichron). Using *Heterorhabditis bacteriophora* as an outgroup, the clustering of the *S. feltiae* isolates was supported by stronger bootstrap value when the tree was produced based on alignments of the non-coding sequences alone than when the whole rDNA sequences were aligned (99 compared to 89, Figure 1A, B). This might be due to the fact that the percentage of variable and informative sites in the ITS region alone is higher (~30% by dividing the number of variable and informative site over the length of the ITS region) than in the whole rDNA sequence (~25%) which would provide a more reliable phylogram.
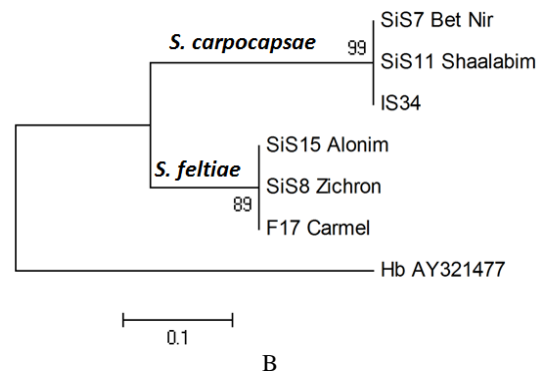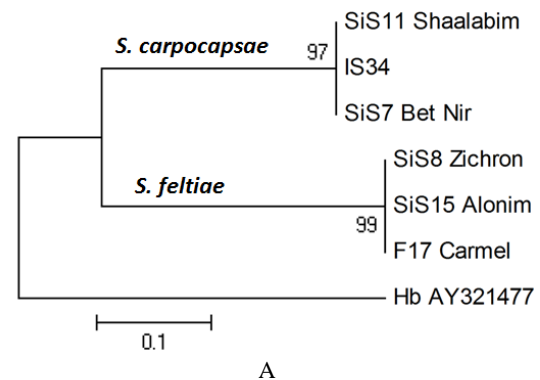

Figure 1. Evolutionary relationships among the six *Steinernema* isolates. The evolutionary history was revealed using the Neighbor-Joining method. The percentage of replicate trees in which the associated isolates clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The tree is drawn to scale with branch lengths calculated using the LogDet method and expressed as the units of the number of base substitutions per site. Two different data sets have been considered for the analysis: (A), ITS1-ITS2 and (B), ITS1-5.8S-ITS2. *Heterorhabditis bacteriophora* isolate (NCBI accession no. AY321477) is included as outgroup. Phylogenetic analyses were conducted in MEGA4.

In conclusion, the data presented in this study showed that the different regions of the rDNA of the six *Steinernema* isolates encompass diverse percentage of variations as follows; ITS2 > ITS1 > 5.8S. Although, these variations were associated with different evolutionary rates at the species level, such variable

rates were not revealed within isolates of the same species.

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