PHYLOGENETIC RELATIONSHIPS OF INSECT-ASSOCIATED FREE-LIVING RHABDITID NEMATODES FROM EASTERN MEDITERRANEAN REGION OF TURKEY

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Abstract. Free-living nematodes play an important role in nutrient cycling. The study was designed to characterize insect-associated free-living soil nematodes in Rhabditida order from Eastern Mediterranean region of Turkey using their 28S rDNA sequences and phylogenetic analysis. A total of 105 soil samples were taken from different habitats. *Galleria* baiting technique was used to obtain free-living nematodes. The nematodes were harvested from cadavers of last instar *Galleria mellonella* larvaeafter trapping process. Free-living soil nematodes were detected in 14 soil samples (13.3%). PCR products obtained from 28S rDNA of isolates were sequenced and phylogenetic trees created. BLASTN homology searches and phylogenetic tree results constructed by Neighbour Joining and Bootstrap Tree methods. Results showed that these insect-associated isolates were determined as species belonging to Rhabditidae (*Rhabditis terricola* and *Mesorhabditis* sp.), Chambersiellidae (*Macrolaimus* sp.) and Cephalobidae (*Cephalobus* sp., *Pseudacrobeles* sp. and *Acrobeloides* sp.) families in Rhabditida. Free-living nematodes interact with many arthropods and other invertebrate species. These interactions are also important for their use in biological control program. In this study, these interactions between free-living nematode-insect,–arthropod and–invertebrate are discussed.
Keywords: *DNA sequence analysis, phylogeny, nematode-insect association, free-living soil nematode, Rhabditida*

Introduction

Nematodes (roundworms) are the most common, abundant, and genetically diverse metazoan organisms found in many habitats particularly soils and sediments, even in the most extreme environments (Baldwin et al., 1999; Derycke et al., 2008). Rhabditida order (rhabditids) is an interesting nematode group found in abundance in all kinds of soil. The free-living forms of this organisms display different feeding habits such as saprophagous or bacteriophagous, and as animal parasites (enthomopathogenic forms) (Yeates et al., 1993; Abolafia and Pena-Santiago, 2007). Because of their abundance, rapid life cycle, and strong interactions with other soil microbes and predators, free-living nematodes play an important role in nutrient cycling (Yeates and Wardle, 1996; Ferris et al., 1997; Carrascosa et al., 2014). Their functional guilds respond to food web enrichment, environmental perturbation, and recovery (Wang et al., 2004). They

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indirectly contribute to nitrogen mineralization by grazing on decomposer microbes, excreting ammonium, and immobilizing nitrogen in live biomass (Ferris et al., 1998; Neher, 2001). Due to their high occurrence, diversity, different tropic levels, and sensitivity to abiotic alterations free-living nematodes also contribute as bioindicators to environmental pollution and soil quality studies (Levi et al., 2012). Their high phenotypic plasticity among populations reduces the number of diagnostic characters in rhabditid nematode groups. Thus their classification has been a matter of long and strong discussions and diversity is far from being well known (Nadler, 2002; Abolafia and Pena-Santiago, 2007; Derycke et al., 2008). Molecular techniques and phylogenetic analyses can potentially overcome this problem and are promising tools to assess biodiversity (Derycke et al., 2008). In recent years, DNA sequence data have brought a revival in the field of systematic (Blaxter et al., 1998; De Ley and Blaxter, 2002; Subbotin et al., 2006). Rhabditids have been identified on the basis of large subunit (LSU or 28S) ribosomal DNA (rDNA) sequences. Trees inferred from LSU or 28S rDNA sequences have been used in deducing relationships among certain closely related species, primarily congeners (De Ley et al., 1999; Stock et al., 2001; Nadler et al., 2003).

The study was planned to characterize insect-associated free-living soil nematodes in Rhanditida order from East-Mediterranean Turkey using their 28S rDNA sequences and phylogenetic analysis.

Materials and methods

Nematode isolation

A total of 105 soil samples were taken from different locations and habitats in Adana, Osmaniye and Kahramanmaraş provinces at October 2010 (Table 1) (Karabörklü, 2012). Soil samples were baited with last instar *Galleria mellonella* L. (Lep.: Pyralidae) larvae (Bedding and Akhurst, 1975) for screening entomopathogenic nematodes (EPN) in our previous study (Karabörklü, 2012; Karabörklü et al., 2015a). However, many rhabditid nematodes also detected during these procedures (Duncan et al., 2003, Campos-Herrera et al., 2012). These rhabditid nematodes harvested from dead *G. mellonella* larvae were placed individually into modified White traps (Kaya and Stock 1997) at room temperature (~25 ºC). Harvested nematodes were washed in dH_2O and stored at 10ºC, 60%±5 relative humidity and full darkness until DNA extraction (Karabörklü, 2012; Karabörklü et al., 2015a).

DNA extraction and PCR amplification

DNA extraction and PCR amplification were done as described in our previous study with some modifications in method suggested by Williams et al. 1994 (Karabörklü, 2012; Karabörklü et al., 2015a,b). DNA was extracted by using 10–20 nematodes. In 20 µl of worm lysis buffer (50 mM KCl, 0.05% gelatin, 10 mM Tris pH 8.2, 10 mM DTT, 0.45% Tween 20, 2.5 mM $MgCl_2$ and 60 µg $µl^{-1}$ proteinase K) nematodes were cut and transferred to sterile microfuge tubes (0.5 ml) on ice. Lysate were frozen at -80ºC for 20 min and then incubated at 65ºC for 1 h, followed by inactivation of proteinase K for 15 min at 95ºC (Zhang et al., 2008). The lysates were centrifuged at 6000 rpm for 30 sec after cooling. Then, 2.5 µl DNA suspension were added to a PCR reaction mixture containing 2.5 µl of 10X PCR buffer, 1.5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP

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mixture, 1 µl of each primer pair (100 µM), 0.25 µl *Taq* polymerase (5 U μl^{-1}) and completed to 25 µl with distilled water. 28S rDNA primer pairs 5'-F'AGCGGAGGAAAAGAAACTAA-3' and 5'-R'ATCCGTGTTTCAAGACGGG-3' were used in amplification reaction (Nadler and Hudspeth, 1998). PCR was adjusted at 94 ºC for 3 min, followed by 33 cycles of 94 ºC for 30 sec, 52 ºC for 30 sec, and 72 ºC for 1 min, followed by final extension at 72 ºC for 7 min (Stock et al., 2001). Electrophoresis was performed on 1% agarose gel.

Phylogenetic analysis

PCR products were purified using of PCR purification kit (Fermentas) and sequenced by RefGen Biotechnology Laboratory (METU, Turkey). Alignments of sequences were performed using the National Centre for Biological Information's (NCBI) Basic Local Alignment Search Tool (BLAST) to compare the sequence data with known sequences submitted on the NCBI. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura et al., 2011; Yilmaz et al., 2012). Phylogenetic trees were constructed using the Neighbor Joining (NJ) and Bootstrap Tree (BT) methods (based on 1000 bootstrap replications) of the MEGA version 5 program. Alignment gaps and missing data were eliminated in pairwise sequence comparisons (Karabörklü et al., 2015a).

Results

Isolates

Free-living soil nematodes were detected in 14 out of 105 soil samples (13.3%) (*Table 1*). Nematodes were harvested from cadavers of last instar *G. mellonella* larvae after trapping process. Nematode densities on cadavers varied depending on isolates. SK-3, SK-16, SK-18 and SK-75 were the highest, SK-20 and SK-51 were medium, and SK-13, SK-29, SK-30, SK-32, SK-34, SK-76, SK-94 and SK-101 isolates were the lowest in density. Nematodes were isolated from forests, grasslands, and agricultural fields. Percentage of the nematodes isolated from forest, grassland, agronomical and horticultural areas were found to be 13.3, 13.3, 46.7 and 26.7%, respectively (*Table 1*).

Table 1. *Nematode isolation regions and their habitation properties*

Isolate	Isolation region	Habitat
SK-3	Pozantı/Adana	Forest (Pinery)
SK-13	Kozan/Adana	Horticultural area (Citrus orchard)
SK-16	Sülemişli-Feke/Adana	Grassland
SK-18	Saimbeyli/Adana	Grassland
SK-20	Pınarlar-Tufanbeyli/Adana	Agronomical area (Wheat field)
SK-29	Selimiye/Osmaniye	Agronomical area (Cornfield)
SK-30	Akyar/Osmaniye	Agronomical area (Cornfield)
SK-32	Toprakkale/Osmaniye	Agronomical area (Cornfield)
SK-34	Ceyhan/Adana	Horticultural area (Olive orchard)
SK-51	Çona/Osmaniye	Agronomical area (Peanut field)
SK-75	Süleymanlı Yolu/Kahramanmaraş	Forest (Pinery)
SK-76	Süleymanlı/Kahramanmaraş	Horticultural area (Grape orchard)
SK-94	Yeniköy/Osmaniye	Agronomical area (Cornfield)
SK-101	Buruk-Sarıçam/Adana	Horticultural area (Citrus orchard)

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Molecular identification

28S rDNA PCR products of the isolates were individually purified and sequenced. Isolates were clustered on the basis of genus. Isolates compared with 20 nematode species showing highest similarity. Detailed phylogenetic analysis was given previously for each isolate. SK-3 and SK-75 isolates were indicated 99 and 98% identity with *Rhabditis terricola* (EF417152) after BLASTN homology search, respectively. Phylogenetic tree was constructed using the Neighbor Joining (NJ) and Bootstrap Tree (BT) methods for two nematode species were given in *Figure 1*. Similarly, SK-13 displayed 95% identity with *Pseudacrobeles variabilis* (AF143368) (*Fig. 2*). Similarity rates and Bootstrap Trees (BT) of other isolates were given in *Table 2* and *Figures (3−6)*.

Table 2. *Similarity rates of free-living nematode isolates with accession numbers*

Isolates (AN)	Species (AN)	Similarity (%)
SK-3 (KP027186)	*Rhabditis terricola* (EF417152)	99
SK-13 (KP027183)	*Pseudacrobeles variabilis* (AF143368)	95
SK-16 (KP027182)	*Acrobeloides maximus* strain JB-89 (DQ903097)	95
SK-18 (KP027184)	*Acrobeloides* sp. JB-68 (DQ903091)	99
SK-20 (KP027191)	*Cephalobus* sp. PS-1143(DQ903100)	99
SK-29 (KP027193)	*Macrolaimus* sp. SAN-2005 (DQ145639)	94
SK-30 (KP027181)	*Acrobeloides nanus* (EF417139)	88
SK-32 (KP027189)	*Acrobeloides* sp. JB-68 (DQ903091)	97
SK-34 (KP027190)	*Acrobeloides* sp. JB-68 (DQ903091)	99
SK-51 (KP027192)	*Cephalobus* sp. PS-1143(DQ903100)	99
SK-75 (KP027185)	*Rhabditis terricola* (EF417152)	98
SK-76 (KP027194)	*Mesorhabditis longespicula* strain DF5017 (EU195980)	92
SK-94 (KP027187)	*Acrobeloides* sp. DWF-1106 (DQ903080)	80
SK-101 (KP027188)	*Acrobeloides* sp. DWF-1106 (DQ903080)	89

AN: Accession Number

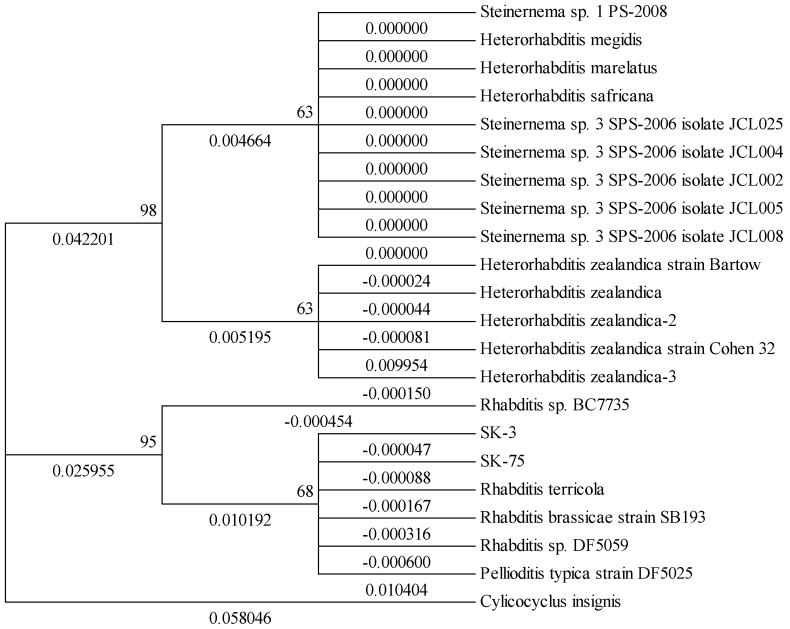

Figure 1. *Phylogenetic relationships of different nematode species with SK-3 and SK-75 isolates. Phylogeny inferred from the alignment of the 719 and 583bp of 28S rDNA region, respectively. The horizontal bar represents 0.005% differences in nucleotide identities*

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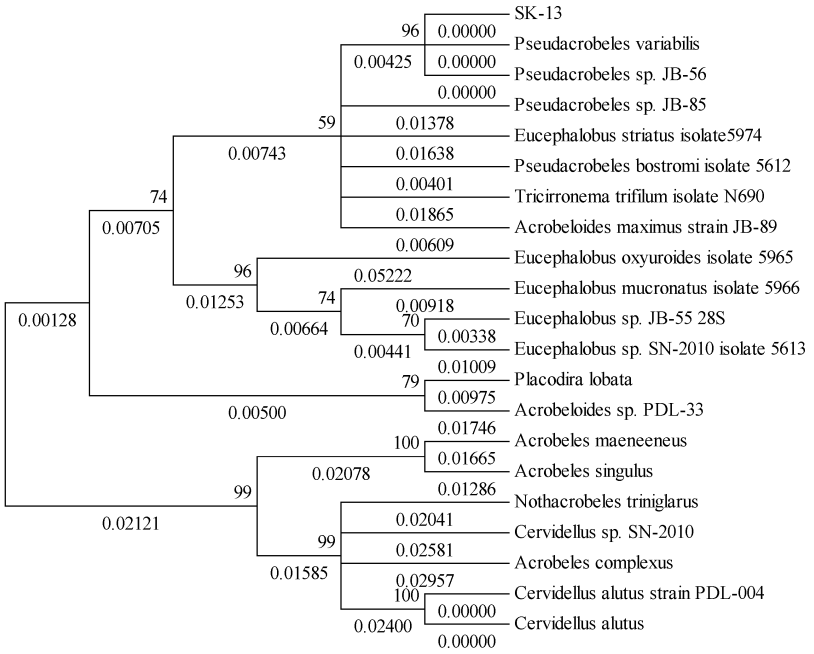

Figure 2. *Phylogenetic relationships of different nematode species with SK-13 isolate. Phylogeny inferred from the alignment of the 620bp of 28S rDNA. The horizontal bar represents 0.005% differences in nucleotide identities*

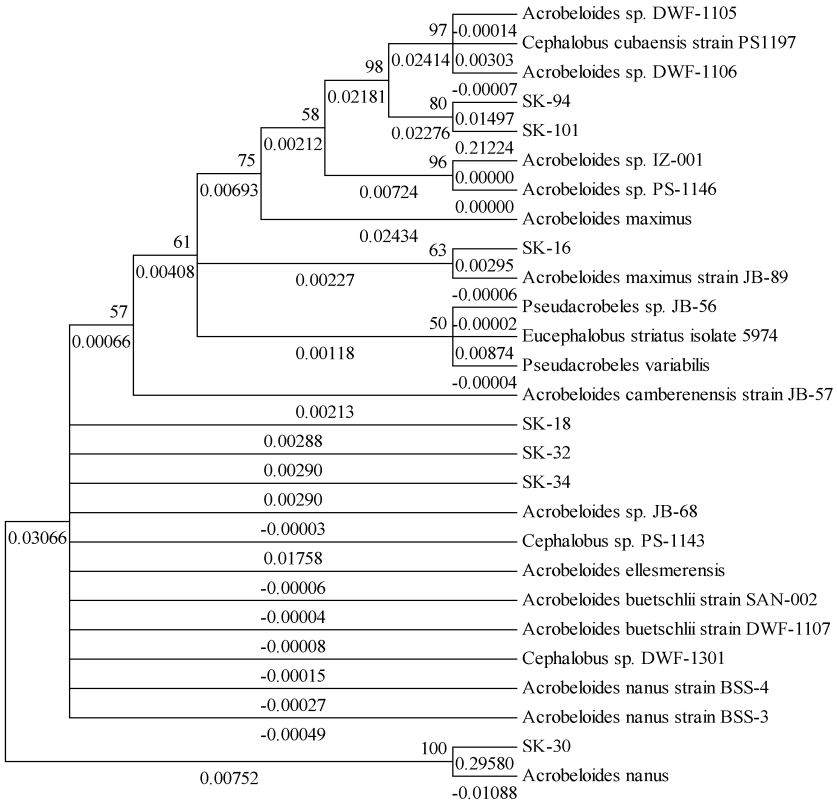

Figure 3. *Phylogenetic relationships of different nematode species with SK-16, SK-18, SK- 30, SK-32, SK-34, SK-94 and SK-101 isolates. Phylogeny inferred from the alignment of the 727, 919, 763, 752, 934, 679 and 684bp of 28S rDNA, respectively. The horizontal bar represents 0.005% differences in nucleotide identities*

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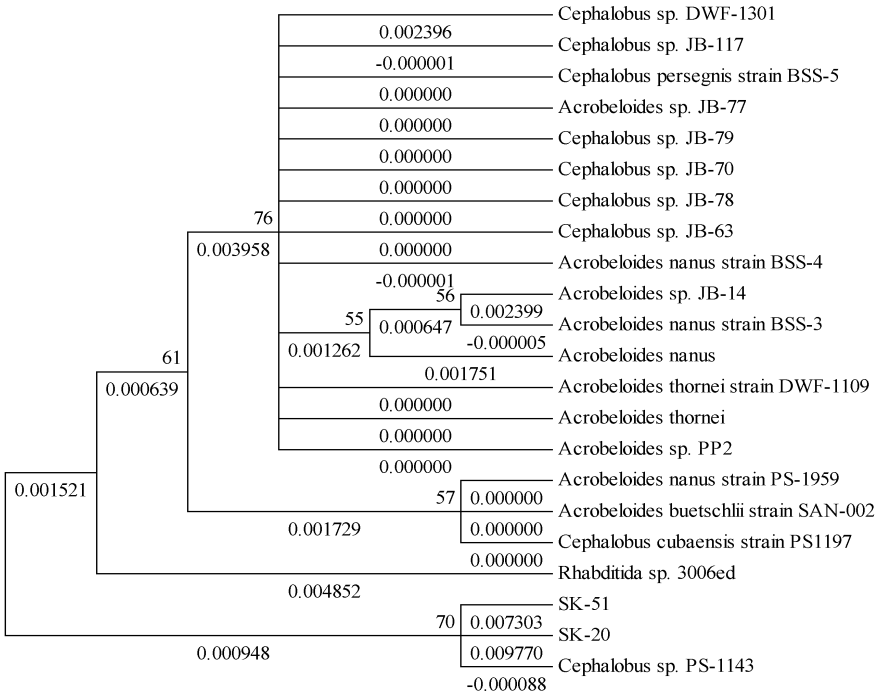

Figure 4. *Phylogenetic relationships of different nematode species with SK-20 and SK-51 isolates. Phylogeny inferred from the alignment of the 746 and 927bp of 28S rDNA, respectively. The horizontal bar represents 0.005% differences in nucleotide identities*

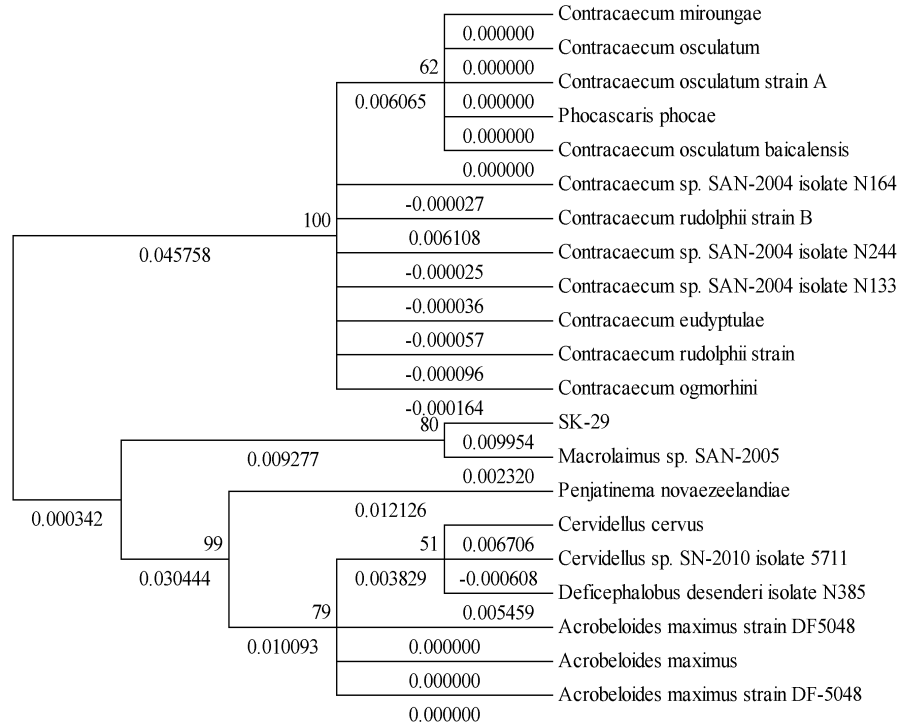

Figure 5. *Phylogenetic relationships of different nematode species with SK-29 isolate. Phylogeny inferred from the alignment of the 632bp of 28S rDNA. The horizontal bar represents 0.005% differences in nucleotide identities*

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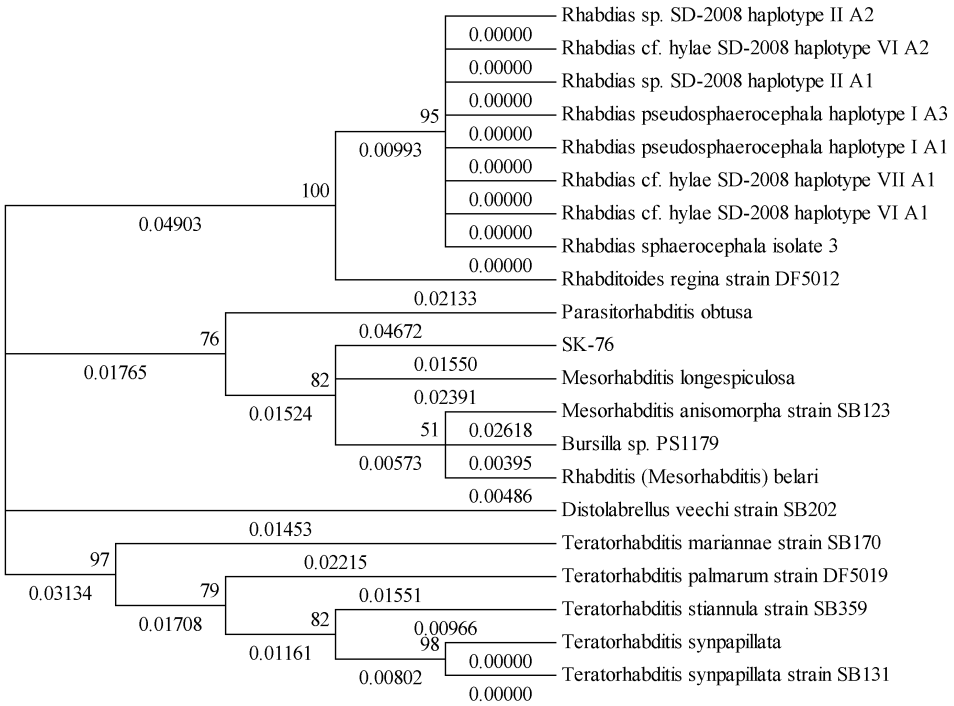

Figure 6. *Phylogenetic relationships of different nematode species with SK-76 isolate. Phylogeny inferred from the alignment of the 688bp of 28S rDNA. The horizontal bar represents 0.005% differences in nucleotide identities*

Discussion

The free-living nematodes undertake an important role in nutrient cycling. Besides, contributions of these organisms to nutrient cycling, nitrogen mineralization and distribution have been well documented (Ferris et al., 1997, 1998; Neher, 2001). Entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) are also included in free-living rhabditid nematode groups and used as excellent biocontrol agents for many insect pests (Grewal et al., 2005).

In this study, 14 free-living nematode isolates were determined. Free-living nematodes, isolated from different habitats of East-Mediterranean region of Turkey, were characterized after BLASTN homology and phylogenetic analysis based on Neighbor Joining and Bootstrap Tree methods. The free-living nematode isolates were identified as *Rhabditis terricola* (SK-3 and SK-75), *Mesorhabditis* sp. (SK-76) (Rhabditidae), *Macrolaimus* sp. (SK-29) (Chambersiellidae), *Cephalobus* sp. (SK-20 and SK-51), *Pseudacrobeles* sp. (SK-13) and *Acrobeloides* sp. (SK-16, SK-18, SK-30, SK-32, SK-34, SK-94 and SK-101) (Cephalobidae) from order Rhabditida. Isolates were mainly obtained from agricultural fields with a rate of 73.3%. *Rhabditis terricola* strains were obtained from forest area, but *Pseudacrobeles* sp. and *Mesorhabditis* sp. strains were isolated from horticultural fields of different regions. On the other hand *Cephalobus* sp. and *Macrolaimus* sp. strains were also isolated from various agronomical fields. *Acrobeloides* sp. strains were collected from grassland, agronomical and horticultural fields. Our results indicated that the free-living nematodes can be obtained from habitats as forest, grassland and agricultural fields. Abolafia and Pena-

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Santiago (2007) supports our finding in that rhabditid nematodes are very abundant in all types of soil and sediments.

In the current study, the free-living nematode strains (*Rhabditis terricola*, *Pseudacrobeles* sp., *Mesorhabditis* sp., *Cephalobus* sp., *Macrolaimus* sp. and *Acrobeloides* sp.) were isolated from last instar *G. mellonella* larvae cadavers. Large numbers of *Rhabditis terricola* determined in earthworm cocoons (*Lumbricus rubellus*, Hoffmeister and *Eisenia foetida*, Savigny) and earthworm cultures suffered extensive productivity loss (Taboga, 1981). The relationship of the *Rhabditis* species with arthropods could be described as phoretic, moderately pathogenic, and facultatively parasitic (Carta and Osbrink, 2005). Poinar (1971) reported that *Rhabditis adenobia* Poinar lived and reproduced within the colleterial glands and endophallic passages of the male dynastid beetle *Oryctes monoceros* L. (Col.: Scarabaeidae) without apparent harm to the host. *R. brevispina* (Claus) was found on the body surface of mole crickets, *Gryllotalpa unispina* (Ort.: Gryllotalpidae) and has phoretic relationship (Gulyamova, 1990).

Rhabditis sp. nematodes are also reported as opportunistic invader of cadavers (Garcia et al., 2011). However, *Rhabditis blumi* Sudhaus showed high pathogenicity (>78%) against major cruciferous insect pests, *Artogeia rapae* L. (Lep.: Pieridae), *Mamestra brassicae* L. (Lep.: Noctuidae) and *Plutella xylostella* L. (Lep.: Plutellidae) in laboratory experiments (Park et al., 2012). Dauer larvae of *Rhabditis rainai* Carta and Osbrink (2005) experimentally infested two subterranean termite species *Reticulitermes flavipes* Kollar and *R. hesperus* Banks (Massey, 1971). Schulte (1989) reported that *Rhabditis (Oscheius) necromena* Sudhaus and Schulte infected three millipede species, Australian *Akamptogonus novarae* (Humbert and Saussure) (Dipl.: Paradoxosomatidae), Australian *Oncocladostoma castaneum* (Attems) (Dipl.: Paradoxosomatidae) and Portuguese pest *Ommatoiulus moreletii* (Lucas) (Dipl.: Julidae) at different levels. It is also reported that dauer juveniles of *R. necromena* remain inside the haemocoel of this host until it dies and resume their development after feeding on bacteria present in the decaying carcass ('necromeny'). Moreover, soil bacteria that are attached to the surface of the invading nematode juveniles regularly cause a lethal contamination in the non-adapted *O. moreletii* (Massey, 1971). It was also reported that *Rhabditis* species use many mollusk species as host (Grewal et al., 2003).

Mesorhabditis irregularis (Körner) was found on body surface of mole crickets, *G. unispina*. *M. irregularis* was associated only with host phoretically (Poinar, 1971). *M. irregularis* and *M. quercophila* (Rühm) were observed in *Melolontha afflicta* Ball. (Col.: Scarabaeidae). *M. oschei* Körner has relationship with *Ips sexdentatus* (Börner) (Col.: Curculionidae) (Grewal et al., 2003). On the other hand *M. spiculigera* species were ectoparasite of *Scarabaeus sacer* (Col.: Scarabaeidae) (El-Kifl et al., 1971). *Macrolaimus* species are commonly recovered from bark infested with beetles and they may be carried by other insects (Massey, 1974). *M. canadensis* isolated from the frass of the bark beetle *Phloeosinus canadensis* Swaine (Col.: Curculionidae) (Sanwal, 1960). *M. crucis* was isolated from *Pissodes piniphilus* Herbst. (Col.: Curculionidae) (Poinar, 1975). The other species belong the genus *Cephalobus* was indicated to have interaction with some mollusks, annelids and insect species. *Cephalobus (Acrobeloides) buetschlii* de Man and *C. persegnis* use mollusk species *Polygyra albolabrus* Say and *Helix hortensis* Muller as host (Grewal et al., 2003). *C. persegnis* species was determined to have infestation on earthworm cocoons and act as ectoparasite on *Scarabaeus sacer* L. (Col.: Scarabaeidae) (El-Kifl et al., 1971). No study has been conducted previously

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concerning the relationship of *Pseudacrobeles* and *Acrobeloides* with insect and arthropod species. However, *Acrobeloides nanus* (De Man) infestation was reported in earthworm cocoons (Kraglund and Ekelund, 2002). Contribution of free-living nematodes to soil nutrient cycle and soil mineralization are evident. During these processes, free-living nematodes interact with many arthropods and other invertebrate species. Interactions of free living nematodes with arthropods and other invertebrates vary from phoretic to pathogenic relations. These interactions are also important for their use in biological control program. However, more detailed studies need to be carried out to clarify these relationships.

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