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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Determination of Entomopathogenic Nematode Fauna in Şırnak, Türkiye

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Abstract: With increasing concerns over the adverse environmental impacts of chemical pesticides, there has been a growing interest towards sustainable pest management strategies worldwide. In this context, entomopathogenic nematodes (EPNs) have emerged as promising biological control agents against insect pests in agricultural production. This study focused on the isolation, identification, and characterization of entomopathogenic nematodes (EPNs) in Şırnak Province, Türkiye. Over the period from July to September 2023, a comprehensive survey involving the collection of 256 soil samples led to the successful isolation, of 11 EPN isolates, which accounted for 4.3% of the samples. Morphological examinations and molecular diagnostics, including mitochondrial COI and 18S rDNA gene regions, facilitated the precise identification of these isolates. Notably, our findings revealed a diverse population of EPNs, predominantly *Heterorhabditis bacteriophora*, comprising five isolates, alongside four distinct species of *Steinernema* (one *Steinernema carpocapsae*, one *Steinernema affine*, two *Steinernema feltiae* and two *Steinernema* sp.). Phylogenetic analysis, utilizing the mitochondrial cytochrome oxidase subunit I (COXI) and the 18S rDNA gene regions, indicated low genetic variability within *H. bacteriophora*, whereas a higher level of diversity was observed among *Steinernema* species. Notably, the presence of diverse EPN species highlights their capacity as effective biocontrol agents against a range of insect pests. These findings provide valuable insights into the biodiversity of EPNs in Şırnak Province and underscore the significance of utilizing local isolates for sustainable agricultural practices. Further research is warranted to elucidate the efficacy of these isolates in integrated pest management strategies tailored to the region's specific agroecological conditions.

Keywords: 18S rDNA, COXI, entomopathogenic nematodes, *heterorhabditis*, *steinernema*, şırnak.

Şırnak İli Entomopatojen Nematod Faunasının Belirlenmesi

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Öz: Kimyasal pestisitlerin olumsuz çevresel etkilerine ilişkin artan endişelerle birlikte, dünya genelinde tarımsal zararlılarla mücadelede sürdürülebilir mücadele yöntemlerine yönelim artmıştır. Bu bağlamda, entomopatojen nematodlar (EPN'ler), tarımsal üretimde zararlı böceklere karşı umut vaat eden biyolojik mücadele ajanları olarak ortaya çıkmaktadır. Bu çalışma, Şırnak ili entomopatojen nematodlarının (EPN) izolasyonu, tanımlanması ve karakterizasyonuna odaklanmaktadır. 2023 yılı Temmuz ve Eylül ayları arasında toplam 256 toprak örneği toplanmış ve bu örneklerden 11 EPN izolatı başarıyla elde edilmiştir. Bu da örneklerin % 4.3'ünü temsil etmektedir. Morfolojik incelemeler ve mitokondriyal COI (COXI) ile 18S rDNA gen bölgelerini içeren moleküler teşhis yöntemleri kullanılarak bu izolatlar tanımlanmıştır. Çalışma sonucunda beş adet *Heterorhabditis bacteriophora* izolatı ve dört adet *Steinernema* türüne ait izolat (bir adet *Steinernema carpocapsae*, bir adet *Steinernema affine*, iki adet *Steinernema feltiae* ve iki adet *Steinernema* sp.) tespit edilmiştir. Filogenetik analizler, *H. bacteriophora* izolatları içinde düşük genetik değişkenlik olduğunu gösterirken *Steinernema* türleri arasında ise daha fazla çeşitlilik olduğunu göstermiştir. Bu çalışma, Şırnak ilindeki EPN biyoçeşitliliği hakkında ilk bilgileri sunmakla birlikte sürdürülebilir tarımsal uygulamalar için yerel izolatların önemini vurgulamaktadır. Bu izolatların, bölgenin kendine özgü tarımsal ve ekolojik koşullarına uygun entegre zararlı yönetimi stratejilerindeki etkinliklerini belirleyebilmek için farklı böcek türleri üzerindeki etkinliklerini belirleyen çalışmalara ihtiyaç bulunmaktadır.

Anahtar kelimeler: 18S rDNA, COXI, entomopatojen nematod, *heterorhabditis*, *steinernema*, Şırnak.

INTRODUCTION

Recently, there has been a growing global concern about the impact of pesticides on the environment and ecology. This concern has led to increased interest in using biological methods to control pests. One such method is the use of entomopathogenic nematodes (EPNs), which are parasitic nematodes belonging to the Steinernematidae and Heterorhabditidae families. EPNs are known for their ability to parasitize insects and eliminate them rapidly, due to their symbiotic relationship with specific bacteria. This unique characteristic makes EPNs valuable tools for controlling various economically significant insect pests (Hazır et al., 2003b).

In Turkey, there has been a growing focus on researching the effectiveness of EPNs in combating economically important pest groups. This research aligns with the global effort to find sustainable and environmentally friendly alternatives to traditional pesticide use. Some attempts to control pests have involved introducing non-native EPNs into major crops worldwide (Lacey et al., 2015; Abate et al., 2019). Unfortunately, these introduced EPN strains have not been successful in parasitizing the local insect populations, likely due to environmental factors or the presence of soil contaminants (Lacey et al., 2015; Lankin et al., 2019).

Therefore, it is essential to isolate and study indigenous EPNs, which may have adapted to the local insect species. These native EPNs may be more effective in infecting and parasitizing both adult and larval insects (Labaude and Griffin, 2018; Sun et al., 2021). Particularly promising are the strains that can thrive in soils that have been modified for agricultural purposes, as these EPNs hold potential for on-site pest control applications.

When conducting applied biological control studies using entomopathogenic nematodes (EPNs), it becomes imperative to not only identify the native isolates within the species but also to delineate their distribution and assess their efficacy. The investigation of the effectiveness of various EPN isolates in Turkey against diverse insect groups has unveiled compelling findings. Studies by Özdemir et al., (2020a,b), Kepenekci and Susurluk, (2006), Kepenekci et al., (2013), Tülek et al., (2015), Yılmaz et al., (2010), and Yüksel and Canhilal, (2019) have collectively demonstrated significant variations in the activities of different isolates belonging to the same EPN species. This underscores the need for a nuanced understanding of the specific isolates' performance, as opposed to generalizing the effectiveness of an entire species. These detailed investigations contribute not only to our comprehension of the intricate dynamics between EPNs and various insect pests but also offer valuable insights for the development of targeted and efficient biological control strategies.

Currently, approximately 100 species within the Steinernematidae family and 26 within the Heterorhabditidae family have been identified globally, with the exception of the Antarctic region (Cimen et al., 2016; Hominick, 2002). Steinernematids are generally encountered more frequently and widely than heterorhabditids, suggesting a broader distribution in soil habitats across various regions (Hominick, 2002). In Turkey, extensive research efforts to identify local EPN species have been successful, resulting in the notable discovery of *Steinernema anatoliense* in Eastern Anatolia (Hazır et al., 2003a). To date, Turkey has been recognized for 12 species of entomopathogenic nematodes, cataloged in various studies (Susurluk, 2007; Unlu et al., 2007; Kepenekci, 2014; Gökçe et al., 2013; Gökçe et al., 2015; Canhilal et al., 2017; Özdemir et al., 2020b). Among these, the species list includes: *Heterorhabditis bacteriophora*, *H. indica*, *H. marelatus*, *H. megidis*, *Steinernema affine*, *S. anatoliense*, *S. bicornutum*, *S. carpocapsae*, *S. feltiae*, *S. litorale*, *S. weiseri*, *S. websteri*, and *S. krausseri*.

This study is aimed to determine the entomopathogenic nematode fauna in Şırnak Province. Şırnak covers a land area of 7,172 square kilometers and has an average elevation of 1,400 meters. The western part of this province, notably elevated above sea level, lies within the Tigris section of the Southeastern Anatolia Region, while its other half extends into the Eastern Anatolia Region. It is bordered by Mardin to the west, Siirt to the north, Van and Hakkari to the northeast, and Iraq and Syria to the south. Şırnak is characterized by two distinct agro-ecological subregions. The first encompasses the Cizre, Silopi, and İdil Districts, known for their expansive plains at altitudes of 300-500 meters. The second features rugged terrain with steep slopes and high mountains, rising 1000 meters and above, offering limited agricultural land. This subregion includes the Central, Beytüşşebap, Güçlükonak, and Uludere Districts, where forests and pastures are abundant. Şırnak Province holds significant biological and ecological importance owing to its unique geographical features and diverse ecosystems. Şırnak is characterized by a rich biodiversity that encompasses various plant and animal species adapted to its distinct climatic conditions. The province is home to several endemic and rare species, contributing to the overall biodiversity of the region. The diverse landscapes, including mountainous terrains, valleys, and river basins, provide essential habitats for a variety of flora and fauna. Therefore, conducting entomopathogenic nematode research in Şırnak is not only scientifically valuable but also holds practical significance for pest management in the region.

MATERIAL AND METHOD

Production of Galleria mellonella L. (Lepidoptera: Pyralidae) larvae: The last instar larvae of *Galleria mellonella* are used in the isolation and production of entomopathogenic nematodes through the White's trap method (Bedding & Akhurst, 1975; Griffin et al., 2000). In this study, mass production of *G. mellonella* larvae was ensured for the new generation nematode production in obtaining entomopathogenic nematodes from the soil and diagnosing the obtained entomopathogenic nematodes. For culturing *G. mellonella*, 2000 ml wheat bran, 250 ml glycerin, 200 ml honey, and 100 ml distilled water were mixed thoroughly in a container until homogenized, and then 200 ml of bee-wax was added to the mixture in small pieces (Kaya & Stock, 1997). Mass production of *G. mellonella* larvae was carried out at 25 °C in artificial diet prepared in this way. The last instar larvae were used in the production of entomopathogenic nematodes, while others were allowed to develop into pupae and adults for the continuity of the *G. mellonella* culture.

For the purpose of obtaining entomopathogenic nematodes from cotton fields in Şırnak Province, a total of 256 soil samples were taken in the fall of 2023. Soil sampling was carried out at specific points representing the fields, depending on the size of the fields (Griffin et al., 2000). The collected soils were put into polyethylene bags, and they were labeled with the necessary information (Stock et al., 1999). The labeled soil samples were stored in coolers with ice until they were brought to the laboratory.

Isolation of entomopathogenic nematodes from the soil: Soil samples brought to the laboratory from the fields were cleaned of foreign materials such as stones and plant residues and then thoroughly mixed before being placed into 500 ml plastic containers. Subsequently, five last instar *G. mellonella* larvae were placed in small wire cages and introduced into the soil samples in plastic containers (Griffin et al., 2000). Since the optimum temperature for infecting insects with entomopathogenic nematodes is generally 22-25 °C, the prepared soil samples were kept under these temperature conditions (Stock et al., 1999). This ensured that entomopathogenic nematodes in the soil samples could easily infect *G. mellonella* larvae. Samples were checked every 2-3 days to detect infected larvae. Dead larvae that were identified as infected were removed from the soil and placed in White's Trap (White, 1927), where entomopathogenic nematodes could be obtained after multiplying and leaving the host. After obtaining entomopathogenic nematodes, they were placed in 250 ml tissue culture flasks filled with distilled water and stored in climate cabinets at 15 °C (Kaya & Stock, 1997).

Table 1. The obtained entomopathogenic nematode isolates from Şırnak.

No	Isolate Number	Location	pH	Temperature (°C)	Altitude
1	*Steinernema feltiae* SLP1	Silopi	7.15	23	510
2	*Steinernema feltiae* SLP94	Silopi	7.20	23	523
3	*Steinernema affine* SLP 37	Silopi	7.50	24	542
4	*Steinernema carpocapsae* SLP44	Silopi	7.50	26	505
5	*Steinernema* sp. CZR29	Cizre	7.46	25	365
6	*Steinernema* sp. GK121	Güçlükonak	6.90	19	895
7	*Heterorhabditis bacteriophora* SLP218	Silopi	7.15	23	508
8	*Heterorhabditis bacteriophora* SLP25	Silopi	7.15	21	510
9	*Heterorhabditis bacteriophora*	İdil	7.10	19	750
10	*Heterorhabditis bacteriophora* CZR21	Cizre	7.46	24	367
11	*Heterorhabditis bacteriophora* CZR14	Cizre	7.50	24	370

In vivo production of entomopathogenic nematodes: To determine whether the isolates obtained from soil samples were entomopathogenic nematodes and to propagate those isolates with infectivity, inoculation was performed on *G. mellonella* larvae. For the production of EPN isolates, last instar *G. mellonella* larvae were placed on 6 cm diameter plates containing Whatman paper, and the isolates obtained from soil samples were applied to them. These prepared samples were kept at the optimum temperature for infecting insects with entomopathogenic nematodes, which is 22-25 °C (Stock et al., 1999). Dead larvae identified as infected were transferred to the White's Trap (Koppenhofer, 2000). Thus, in this environment, the next generation infective larvae were produced on *G. mellonella* larvae, which served as the host (Koppenhofer, 2000). The infective juveniles that moved into the water were transferred to flasks and stored in climate cabinets at 15 °C (Kaya & Stock, 1997).

Morphology-based identification of EPNs: The process for identifying entomopathogenic nematodes at the genus level involved creating temporary slides using ten infective juvenile specimens from each sample. These juveniles were terminated at 60°C and stored in TAF solution, which consists of 7 ml of 40% formalin, 2 ml of Triethanolamine, and 91 ml of distilled water. Following preservation, specimens underwent processing in anhydrous glycerin using the rapid method developed by Seinhorst, (1959) and later improved by De Grisse, (1969). Permanent microscope slides were prepared using the ring method introduced by Hooper, (1986). Morphological examinations were conducted using a Leica DM 3000 light microscope. Genus level identification was carried out

according to guidelines established by Poinar, (1990) and expanded upon by Liu and Berry, (1996).

of entomopathogenic nematodes: Total genomic DNA was extracted from freshly harvested nematodes by using a Genematrix Tissue and Bacterial DNA Purification Kit (EURx). following by manufacturer's instructions. PCR amplification and sequencing of the both gene regions were used to identify and determine the phylogenetic relationship between the isolates. The 18S rDNA region was amplified, using 26R and 28A primers (Nguyen & Hunt, 2007). COXI region was amplified using the universal primers LCO 1490 and HCO 2198 (Folmer et al., 1994).

The cycling parameters for 18S rDNA region consisted of initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min; and a final extension at 72 °C for 10 min. For the COXI region, the PCR program was as follows: one cycle of 94°C for 2 min followed by 37 cycles of 94°C for 30 s, 51°C for 45 s, 72°C for 2 min, and a final extension at 72°C for 12 min. The presence of DNA fragments and yield were measured by agarose gel electrophoresis (1.5% in 1X TAE buffer).

DNA sequencing and analysis: PCR products were directly sequenced in both directions at BM Labosis (Turkey). Chromas 2.6.6 was used for editing the generated sequence. Afterwards, it was compared with the sequences present in the National Centre for Biotechnology Information (NCBI) by means of a BLAST (Basic Local Alignment Search Tool) search. A cut of ≥99% identity was considered for the same species. An alignment of our samples together with sequences of related heterorhabditid and steinernematid species were produced for both regions using default ClustalW (Thompson et al., 2003) parameters in MEGA X (Kumar et al., 2018), and optimized manually in BioEdit (Hall, 1999). The phylogenetic tree was generated using the maximum likelihood (ML) method based on the Kimura 2 parameter model for both gene regions.

RESULTS AND DISCUSSION

Entomopathogenic nematodes obtained from soil samples: A total of 11 entomopathogenic nematode isolates were successfully obtained from 256 soil samples collected in Şırnak Province. The overall rate of obtaining entomopathogenic nematodes from soil samples was determined to be 4.30%. Upon confirmation of positive results for 11 isolates, sequence analyses of the 18S and COI regions indicated that 5 isolates belonged to the genus *Heterorhabditis* (45.5%), and 6 belonged to the genus *Steinernema* (54.5%) (Figure 1). Soil properties such as moisture level, pH, organic matter content, and texture affect the distribution of EPNs and their potential for finding hosts (Stuart et al., 2015). The environmental parameters of each collection site, as shown in Table 1, indicate a range of pH levels (6.90 to 7.50), temperatures (19°C to 26°C), and altitudes (365m to 895m). These variations suggest the adaptability and resilience of EPNs in different environmental conditions within Şırnak Province. The pH range determined for EPNs in this study is consistent with the pH values identified in the literature. In previous studies, the pH ranges for EPNs have been determined as 4.6-8 (Hara et al., 1991; Griffin et al., 1991), 5.71-7.24 (Uribe-Lorio et al., 2005), and 5.0-7.2 (Stock et al., 1999). EPNs can maintain their viability within pH values ranging from 4 to 8. It is known that soil pH values above 8 have a particularly adverse effect on the viability of *Steinernematids* (Kung et al., 1990; Stuart et al., 2006; Stuart et al., 2015).

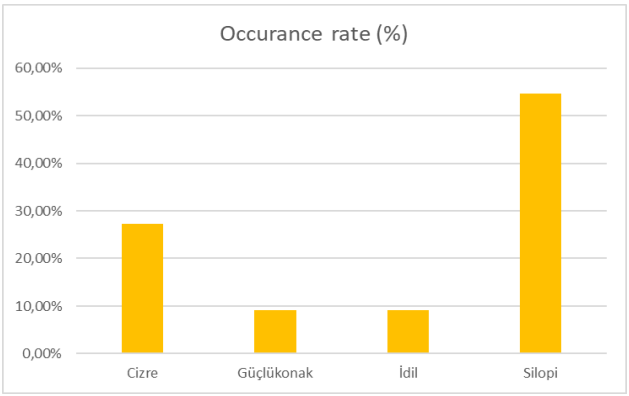

Figure1. Occurance rates of EPNs according to provinces.

The identification and characterization of the isolates were carried out based on both mtCOI and 18S rDNA gene regions. Infected larvae were sorted according to color and shape, and subsequently, molecular diagnostic methods were applied to all isolated species from the soil samples. Following the analysis, the sequences and DNA were compared against the NCBI database in GenBank, revealing that all isolated species belonged to either *Heterorhabditis* or *Steinernema*.

The molecular identification for both gene regions disclosed that five isolates were identified as *Heterorhabditis bacteriophora*, two as *Steinernema feltiae*, one as *S. carpocapsae*, and one as *S. affine* and two as *Steinernema* sp. (Table 1).

The presence of species from both the *Steinernema* genus and the *H. bacteriophora* species was observed in the three districts where the soil samples were collected. Notably, it was determined that the majority of the isolated entomopathogenic nematodes belonged to the species *H. bacteriophora*, suggesting its prevalence as the most common entomopathogenic nematode in Şırnak Province.

Thriving in the soil, their natural habitat, entomopathogenic nematodes prove effective in controlling soil-dwelling insect pests. Given that many insect pests undergo part of their life cycles underground, the interaction and survival dynamics between nematodes and their hosts become crucial. This study, focusing on local isolates from Şırnak, suggests that these entomopathogenic nematodes could hold promise in future biological control efforts against local pests.

Moleculer identification of entomopathogenic nematodes: The survey conducted in Şırnak Province yielded 11 distinct entomopathogenic nematode (EPN) isolates out of 256 soil samples, translating to a recovery rate of 4.3%. The isolates consisted of 5 *Heterorhabditis bacteriophora*, 2 *Steinernema feltiae*, 1 *Steinernema carpocapsae*, 1 *Steinernema affine*, and 2 unidentified *Steinernema* spp. (Table 2). The identification was based on molecular diagnostics employing both the mitochondrial COI and the 18S rDNA gene regions, which are widely recognized for their utility in delineating nematode phylogenies.

The molecular phylogenetic analysis, conducted using the Maximum Likelihood method based on the Kimura 2-parameter model, revealed insights into the evolutionary relationships of the EPNs within the local context and in comparison to their nearest neighbors as per GenBank entries.

In the phylogenetic trees constructed from the 18S rDNA region, the isolates of *H. bacteriophora* clustered tightly, indicating a low genetic variability within this species in the Şırnak province (Figure 2). This also suggests a strong genetic stability of *H. bacteriophora* across different geographic locations. This finding is consistent with previous studies suggesting that *H. bacteriophora* is among the most widespread and adaptable EPN species, capable of thriving in a variety of climatic conditions from tropical to temperate regions (Hominick et al., 1996; Susurluk et al., 2001; Grewal et al., 2005). In Şırnak Province, which has a temperate climate, isolates of this species were observed to have a broad distribution. Similar studies conducted in different geographical regions in our country have also detected these species (Hazır et al., 2003c; Güneş & Gözel, 2011; Gözel & Güneş, 2012).

Conversely, the tree generated from the COI gene region showed a greater degree of genetic diversity among the isolates (Figure 3). Notably, the local isolates, marked by distinct colored dots, interspersed with GenBank sequences, indicating a complex relationship with both local and global populations. The two unidentified *Steinernema* species (CZR29 and GK121) presented a unique opportunity to possibly discover new taxa or to learn more about the genetic diversity present within known species.

The clustering patterns observed in the trees have several implications. Both phylogenetic trees confirm the monophyletic nature of *H. bacteriophora*, a finding that underscores the genetic stability of this species across different geographic regions. The COI gene tree, in particular, illustrated a higher resolution in distinguishing between closely related *Steinernema* species, suggesting that this marker may be more sensitive for detecting intraspecific variation. The comparison between the 18S rDNA and COI gene trees demonstrates the complementary nature of these genetic markers. While 18S rDNA provides a broad overview of species relationships, COI offers a finer scale resolution that can be crucial for identifying and characterizing local isolates.

The presence of diverse EPN species within Şırnak Province is promising for biological control applications. The identification of local isolates that cluster closely with known effective biological control agents suggests that these isolates may possess similar pest control properties.

In conclusion, the evolutionary analysis has highlighted the rich EPN biodiversity in Şırnak Province and provided a genetic basis for the selection of promising isolates for biocontrol purposes. The findings from this study add valuable knowledge to the global EPN database and could guide future efforts in sustainable agricultural practices within the region.

Table 2. GeneBank accession information for entomopathogenic nematodes **(Accession numbers will be provided after acceptance).**

No	Isolate Number	Accession Number (18S rDNA)	Accession Number (mtCOI)
1	*Steinernema feltiae* SLP1	PP538015	PP537408
2	*Steinernema feltiae* SLP94	PP538016	PP537409
3	*Steinernema affine* SLP 37	PP538017	PP537406
4	*Steinernema carpocapsae* SLP44	PP538021	PP537407
5	*Steinernema* sp. CZR29	PP538013	PP537410
6	*Steinernema* sp. GK121	PP538014	PP537411
7	*Heterorhabditis bacteriophora* SLP218	PP538022	PP537404
8	*Heterorhabditis bacteriophora* SLP25	PP538023	PP537405
9	*Heterorhabditis bacteriophora* IDL36	PP538019	PP537402
10	*Heterorhabditis bacteriophora* CZR21	PP538018	PP537401
11	*Heterorhabditis bacteriophora* CZR14	PP538020	PP537403

CONCLUSION

This study on the entomopathogenic nematodes in Şırnak Province, Turkey, offers valuable contributions to our understanding of EPN biodiversity and their role in sustainable agriculture. The research demonstrated the presence of a diverse range of EPN species, predominantly *Heterorhabditis bacteriophora*. The molecular characterization and phylogenetic analysis highlight the genetic diversity and relationships of these nematodes, emphasizing their potential as biological control agents. The findings from this study enrich the global database of EPNs and underline the importance of utilizing local EPN isolates in developing effective biological control

strategies. This research paves the way for future sustainable agricultural practices, promoting the use of EPNs as an environmentally friendly alternative to chemical pesticides.

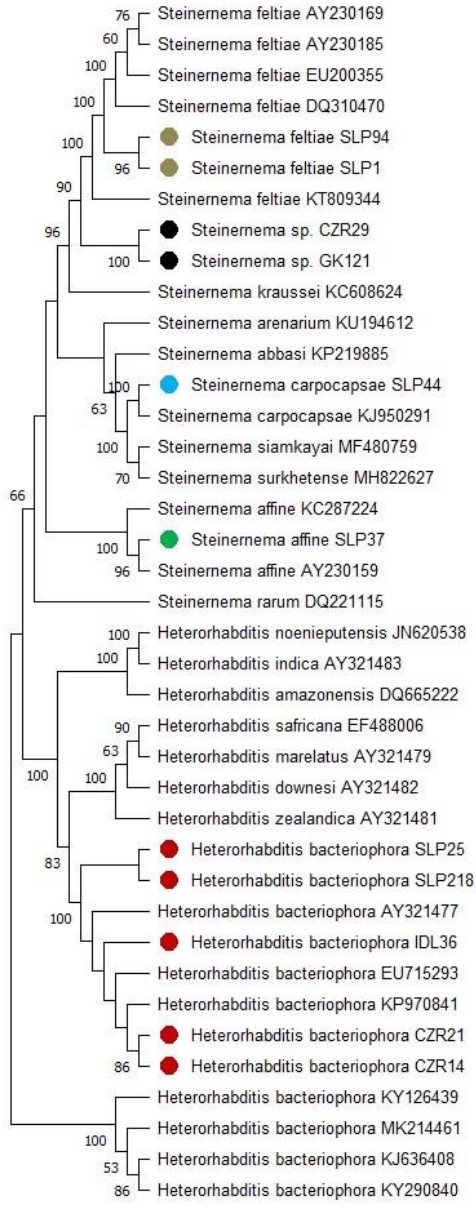

Figure 2. Phylogenetic relationships of *Heterorhabditis* and *Steinernema* species in Şırnak based on 18S rDNA analysis.

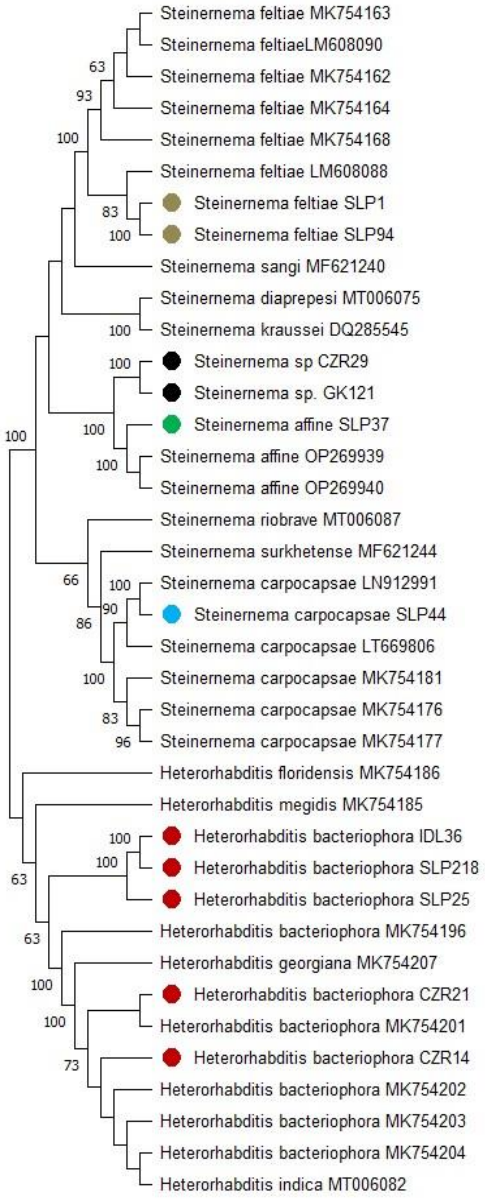

Figure 3. Phylogenetic relationships of *Heterorhabditis* and *Steinernema* species in Şırnak based on COXI analysis.

AUTHOR CONTRİBUTİONS

All authors contributed to the study conception and design. EE and MR collected the samples and performed the molecular analysis. EE wrote the original draft. All authors read and approved the final manuscript.

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