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Original article (Orijinal araştırma)

Genetic diversity of *Steinernema feltiae* Filipjev, 1934 (Rhabditida: Steinernematidae) and *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) in potato production areas of Türkiye[1]

Türkiye'de patates üretim alanlarında, *Steinernema feltiae* Filipjev, 1934 (Rhabditida: Steinernematidae) ve *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) genetik çeşitliliği

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Abstract

Entomopathogenic nematodes (EPNs) are one of the most important biological control agents and have proved their biocontrol success against a variety of insect pests. However, limited knowledge exists regarding the genetic structure of various species and populations of EPNs. Thus, this study was conducted to isolate and elucidate the EPN's phylogenetic diversity sourced from potato (*Solanum tuberosum* L.) (Solanales: Solanaceae) crops in 2020 at Bolu Abant Izzet Baysal University. Through ribosomal DNA sequencing, we investigated genetic variability within and among isolates of *Steinernema* and *Heterorhabditis* species. Widespread sampling across Afyonkarahisar, Bolu, İzmir, Sivas, Niğde, Kayseri, and Konya provinces, covering a total area of 795 hectares, led to the recovery of two EPN isolates, constituting 10% of the samples. Molecular characterization involved ribosomal DNA sequencing, which, upon integration with sequences from 41 populations, confirmed the identification of *Steinernema feltiae* Filipjev, 1934 (Rhabditida: Steinernematidae) and *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae), displaying monophyly in most *Steinernema* and *Heterorhabditis* clades, respectively. This survey emphasizes the common occurrence of these EPNs in key potato-growing areas in Türkiye, highlighting their biocontrol potential against arthropod pests of agricultural importance.

Keywords: Beneficial nematodes, genetic variability, ribosomal DNA sequencing

Öz

Entomopatojen nematodlar (EPN) en önemli biyolojik mücadele ajanlarından biridir ve çeşitli böcek zararlılarına karşı biyolojik savaşta başarılarını kanıtlamıştır. Ancak çeşitli türlerin ve EPN popülasyonlarının genetik yapısına ilişkin bilgiler sınırlıdır. Bu nedenle, bu çalışma, patates alanlarından elde edilen EPN'lerin filogenetik çeşitliliğini ortaya koymak ve tel kurtlarının, özellikle *Agriotes* spp. (Coleoptera: Elateridae) türlerinin mücadelesi için alternatif mücadele yöntemlerini belirlemek amacıyla 2020 yılında Bolu Abant İzzet Baysal Üniversitesi'nde yapılmıştır. Ribozomal DNA dizileme yoluyla, *Steinernema* ve *Heterorhabditis* türlerinin izolatları arasındaki genetik farklılıklar araştırılmıştır. Afyonkarahisar, Bolu, İzmir, Sivas, Niğde, Kayseri ve Konya illerini kapsayan geniş bir örnekleme ile, toplamda 795 hektarlık bir alanı kapsayarak, örneklerin %10'unu oluşturan iki EPN izolatının elde edilmiştir. Moleküler karakterizasyon, ribozomal DNA dizileme içermekte ve 41 popülasyonun dizileriyle incelendiğinde, *Steinernema feltiae* Filipjev, 1934 (Rhabditida: Steinernematidae) ve *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae)'nın varlığını doğrulayarak, çoğu *Steinernema* ve *Heterorhabditis* kladlerinde yer almıştır. Bu araştırma, bu EPN'lerin Türkiye'deki önemli patates yetiştirme alanlarında yaygın olarak bulunduğunu göstermekte ve bunların tarımsal öneme sahip arthropod zararlılara karşı biyolojik savaşta potansiyellerini vurgulamaktadır.

Anahtar sözcükler: Faydalı nematodlar, genetik çeşitlilik, ribosomal DNA sekans

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Introduction

The potato (*Solanum tuberosum* L.) (Solanales: Solanaceae) is a vital food and a significant industrial commodity globally. It holds promise in reducing hunger and poverty worldwide. With an output of 388 million tons across 162 nations, potatoes rank as the fourth most crucial main food following maize, rice, and wheat (FAOSTAT, 2021). However, potato cultivation faces numerous challenges from pathogens and pests, including nematodes. Among these pests, wireworms, specifically *Agriotes* spp. Eschscholtz, 1829 (Coleoptera: Elateridae), pose a substantial threat, causing substantial economic losses in various crops, notably potatoes (Furlan & Tóth, 2017). Wireworms predominantly reside underground during their larval stage, feeding on subterranean plant parts of potato, causing a significant reduction in tuber yield and tuber quality (Furlan et al., 2021). Moreover, the feeding punctures on tubers by wireworm larvae make plants susceptible to other soil-borne pathogens (Keiser et al., 2012). As a result, damaged potato tubers lose their commercial value, and the profitability of potato production drops dramatically (Keiser et al., 2012). Traditionally, chemical insecticides with broad-spectrum compounds like carbamates, organophosphates, and organo-chlorine have been primary tools for wireworm control among most growers. Yet, due to environmental and health concerns, numerous synthetic chemicals face regulatory restrictions and bans. Consequently, researchers have been prompted to investigate eco-friendly alternatives for controlling wireworm populations (Reddy & Tangtrakulwanich, 2014).

In recent years, entomopathogenic nematodes (EPNs) from the families Heterorhabditidae and Steinernematidae have garnered significant attention for their potential in biological pest control, targeting various economically significant insect pests (Bhat et al., 2020; Peçen & Kepenekci, 2022). Taxonomists have described over 100 species of *Steinernema* and 21 of *Heterorhabditis* (Bhat et al., 2020). These nematodes have shown remarkable efficacy in controlling agricultural pests, particularly those belonging to the orders Lepidoptera, Diptera, and Coleoptera, across a wide range of crops (Garriga et al., 2020; Özdemir et al., 2021; Yüksel, 2022; Wakil et al., 2023). Notably, EPNs have formed symbiotic relationships with insect pathogenic bacteria of the genera *Photorhabdus* and *Xenorhabdus* (Boemare, 2002). During the infective juvenile (IJ) stage, these nematodes, residing in the soil, actively seek out insect hosts, penetrating their bodies through natural openings or by breaching the cuticle directly. Upon locating a suitable host, they release their bacterial symbionts upon detecting chemical cues in the insect's hemolymph. These bacteria then proliferate, generating virulence factors and toxins that incapacitate the host (Boemare, 2002). Additionally, the bacteria release exoenzymes that break down insect tissues and generate metabolites essential for the growth, development, and reproduction of nematodes (Forst et al., 1997). Moreover, they produce potent secondary metabolites with antibiotic properties, deterring scavenging arthropods. Upon exhausting resources, the succeeding generation of nematodes disperses to seek out new hosts (Dreyer et al., 2018). The soil-dwelling nature of EPNs, coupled with their effective host-searching strategies, makes them ideal candidates to suppress pest populations that live in the soil environments (Hazir et al., 2003a).

Accurate identification of EPN species is essential for devising effective control strategies. However, relying solely on morphological characteristics for nematode diagnosis is challenging and time-consuming, requiring substantial expertise. Consequently, molecular techniques are increasingly favored for disease diagnosis, offering precise and swift identification, along with insights into population origins and introduction pathways. Consequently, genomic and ribosomal DNA analysis has emerged as the preferred method for nematode identification (Hashmi et al., 1996). In a prior investigation, a comprehensive field survey was conducted in key potato cultivation regions of Türkiye to assess the genetic diversity of EPN species for controlling significant potato pests. Here, the current study aims to (i) employ molecular data, specifically sequencing of the ITS-rDNA expansion segments, to identify various isolates of the genera *Steinernema* and *Heterorhabditis*, and (ii) explore the genetic relationships among EPN species.

Materials and Methods

Sampling area and EPN Isolation

The sampling area encompassed potato fields from 407 locations spread across 7 provinces (Afyonkarahisar, İzmir, Bolu, Sivas, Konya, Niğde and Kayseri) situated in various regions of Türkiye, which are significant centers for potato production (Figure 1).

Figure 1. The survey of entomopathogenic nematodes conducted in potato areas of Türkiye

A grand total of 407 soil samples were gathered, with each farm contributing nine samples, covering a combined area of 325.7 hectares (as indicated in Table 1). Using a hand shovel, the rhizosphere of potato plants was sampled. The samples were taken at a depth of 0-20 cm, labeled, and put in plastic boxes in bags. All samples were transferred to laboratory for isolation process of EPNs.

Table 1. The sampled location for entomopathogenic nematodes in potato growing area in Türkiye

No	Province	Production areas (decare)	Samples
1	Bolu	30	22
2	İzmir	112	55
3	Afyonkarahisar	129	65
4	Konya	139	70
5	Sivas	60	30
6	Kayseri	90	45
7	Niğde	235	120
Total		795	407

After eliminating plant debris and stones in the soil, samples were subjected to the Galleria trap technique (Akhurst & Bedding, 1986). Samples were poured into clean plastic containers (8×8×10 cm) containing eight last-instar larvae of *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae). These containers were covered and inverted every 24 hours to facilitate interaction between the wax moth larvae and the infective juveniles (IJs) of nematodes. After a week of incubation in darkness at 25°C, the containers were examined every three days to check for dead larvae. Any deceased larvae found were individually transferred to modified White traps to collect the emerging infective-stage juveniles. During the initial week, the emerging infective juveniles (IJs) were washed with distilled water, and each nematode isolate underwent pathogenicity assessments on 10 *G. mellonella* larvae to validate Koch's postulates (Kaya & Stock, 1997). Subsequently, the juveniles coming out of the cadavers were reproduced on *G. mellonella* larvae by re-inoculating the IJs to larvae. All these processes were conducted at the Plant Protection Department of Bolu Abant Izzet Baysal University.

Molecular studies

The collected isolates of *Steinernema* and *Heterorhabditids* were cultured *in vivo* using the last instar larvae of *Galleria mellonella*. Subsequently, DNA extraction was performed from a single F1 female of each isolate using the Quiagen® DNeasy blood and tissue kit, according to the manufacturer's guidelines. The DNA samples containing a concentration of approximately 20 ng/µL DNA were used for further molecular analyses. Following this, DNA pattern and phylogenetic analyses were run using two standard barcoding loci from the nuclear genome, namely the ITS and LSU rDNA regions.

For each locus, PCR amplifications were conducted by combining 2 µL of DNA (20 ng/µL), 2.5 µL of 10× PCR Buffer (NH4)2SO4, 2 µL of 25 mM MgCl2, 1 µL of 20 mM dNTPs, 0.4 µL of each forward and reverse primer (10 mM), 0.5 µL of 5× BSA, 1 µL of 10% Trehalose, 0.16 µL of Taq DNA polymerase (5 U/µL), and 15 µL of ultrapure water, resulting in a total volume of 25 µL. The primer sets utilized are listed in Table 2. The PCR protocol began with an initial denaturation step at 95°C for 600 s and this step was followed by 32 cycles consisting of 45 s denaturation at 55°C, annealing at 55°C for 45 s, and extension at 72°C for 60 s, with a final extension step at 72°C for 60 s. To determine the length of the PCR products, amplified DNA of isolates was subjected to electrophoresis in 1.5% agarose gels and run at 120 V for 45 minutes. All PCR products were purified, and their concentrations were verified by electrophoresis using 1 µL of the purified product.

Table 2. The primer sets used for both PCR amplification and sequencing reactions targeting entomopathogenic nematodes (EPNs)

Primer	Region	Primer Sequence	Orientation	
AB28	ITS rDNA	ATATGCTTAAGTTCAGCGGGT	Forward	Joyce et al. (1994)
TW81	ITS rDNA	GTTTCCGTAGGTGAACCTGC	Reverse	

Genetic diversity quantification among EPN species was conducted using Mega 7 (Kumar et al., 2018). Moreover, the calculation of segregating sites was conducted by assessing the average number of polymorphic nucleotides between sequences and the G + C content, employing DnaSP 5.1 (Librado & Rozas, 2009). Notably, all nucleotide sequences of EPN isolates have been archived in the GenBank NCBI database (refer to Table 3). Maximum Likelihood (ML) analyses were executed with 1000 replicates of bootstrap support, utilizing the General Time Reversible model (Tavaré, 1984), incorporating invariable sites (I) (Shoemaker & Fitch, 1989), and accounting for variations across sites (G) (Yang, 1994). A concatenated analysis was also conducted, incorporating sequences from 41 species reported for ITS (Tavaré, 1984). The Maximum Likelihood (ML) analysis comprised 1000 bootstrapped sequence alignments, which underwent global rearrangement with random replications. The phylogenetic relationship between *S. feltiae* and *H. bacteriophora* populations was compared to international isolates, with *Caenorhabditis elegans* (Maupas, 1900) (Rhabditida: Rhabditidae) serving as an outgroup to root the phylogeny. Reference sequence *Steinernema carpocapsae* (Weiser, 1955) (Rhabditida: Steinernematidae) *(*AY944007) (Nadler et al., 2006) was employed to identify all nucleotide-level substitutions.

Table 3. Sequenced entomopathogenic nematode samples that were collected from surveyed areas

No	Code	Species	Accession Number
1	Bolu-1	*Steinernema feltiae*	OR979106
2	Bolu-2	*Steinernema feltiae*	OR979107
3	Bolu-7	*Steinernema feltiae*	OR979108
4	Bolu-8	*Steinernema feltiae*	OR979109
5	Bolu-9	*Steinernema feltiae*	OR979110
6	Bolu-10	*Steinernema feltiae*	OR979111
7	Bolu-14	*Steinernema feltiae*	OR979112
8	Bolu-22	*Steinernema feltiae*	OR979113
9	Bolu-24	*Steinernema feltiae*	OR979114
10	Bolu-26	*Steinernema feltiae*	OR979115
11	Konya-3	*Steinernema feltiae*	OR979123
12	Konya-4	*Steinernema feltiae*	OR979124
13	Konya-6	*Steinernema feltiae*	OR979125
14	Konya-7	*Steinernema feltiae*	OR979126
15	Konya-8	*Steinernema feltiae*	OR979127
16	Konya-10	*Steinernema feltiae*	OR979128
17	Konya-12	*Steinernema feltiae*	OR979129
18	Konya-14	*Steinernema feltiae*	OR979130
19	Konya-15	*Steinernema feltiae*	OR979131
20	Konya-16	*Steinernema feltiae*	OR979132
21	Konya-18	*Steinernema feltiae*	OR979133
22	Konya-19	*Steinernema feltiae*	OR979134
23	Konya-20	*Steinernema feltiae*	OR979135
24	Konya-22	*Steinernema feltiae*	OR979136
25	Kayseri-4	*Steinernema feltiae*	OR979116
26	Kayseri-5	*Steinernema feltiae*	OR979117
27	Kayseri-10	*Heterorhabtidis bacteriophora*	OR979118
28	Kayseri-14	*Steinernema feltiae*	OR979119
29	Kayseri-16	*Heterorhabtidis bacteriophora*	OR979120
30	Kayseri-18	*Steinernema feltiae*	OR979121
31	Kayseri-20	*Heterorhabtidis bacteriophora*	OR979122
32	Afyonkarahisar-1	*Heterorhabtidis bacteriophora*	OR979098
33	Afyonkarahisar-2	*Steinernema feltiae*	OR979099
34	Afyonkarahisar-4	*Heterorhabtidis bacteriophora*	OR979100
35	Afyonkarahisar-8	*Steinernema feltiae*	OR979101
36	Afyonkarahisar-12	*Heterorhabtidis bacteriophora*	OR979102
37	Afyonkarahisar-14	*Heterorhabtidis bacteriophora*	OR979103
38	Afyonkarahisar-35	*Steinernema feltiae*	OR979104
39	Afyonkarahisar-54	*Steinernema feltiae*	OR979105
40	Sivas-6	*Steinernema feltiae*	OR979137
41	Sivas-8	*Steinernema feltiae*	OR979138

RESULTS and DISCUSSION

Sampling area and EPN isolation

Through an extensive sampling effort in potato cultivation areas across seven provinces (Niğde, Bolu İzmir, Konya, Kayseri, Sivas and Afyonkarahisar) in Türkiye (Figure 1), forty-one out of 407 soil samples (10%) tested positive for EPNs. These isolates were predominantly recovered from Konya, Afyonkarahisar, Bolu, Kayseri, and Sivas provinces. Among the positive samples, the majority of nematode isolates were from the *Steinernema* genus, with 34 (82.4%) out of the 41 samples testing positive. *Steinernema feltiae* was the most frequently encountered species, found in 7 soil samples (16.6%), followed *by Heterorhabditis bacteriophora*.

This study represents the first comprehensive assessment demonstrating the widespread presence of entomopathogenic nematodes in potato fields across seven provinces in Türkiye, a key region for potato production. Among the 407 soil samples collected from various districts within the provinces, 41 entomopathogenic nematode isolates were obtained, indicating a recovery rate of 10%. This recovery rate (17.9%) aligns closely with findings from Karaman province (19.2%) (Yavuzaslanoglu et al., 2016), and it notably surpasses rates reported in other surveys, such as 4.71% in Rize (Keskin et al., 1995), 9% in Adana and Kahramanmaras provinces (Canhilal et al., 2016), and 2.03% across Türkiye (Hazır et al., 2003b). Similarly, recovery rates in subtropical regions of other European countries were 13.8% in Southern Italy (Tarasco & Triggiani, 2016), 4.6% in Spain (Del Pino & Palomo, 1996), and 9.5% in Egypt (Shamseldean & Abd-Elgawad, 1994).The relatively high recovery rate in this study may be attributed to meticulous sampling from a confined land area at optimal times, particularly after rainfall, as soil moisture and temperature are crucial factors influencing the survival of entomopathogenic nematodes in the soil environment (Wright, 1992; Ehlers, 1996). Among the isolates obtained, *S. feltiae* was the most prevalent species, accounting for 84.6% of the isolates, while the occurrence of *H. bacteriophora* was less common, at 16.4%. This observation aligns with previous studies in Turkey where *S. feltiae* was consistently identified as the most common entomopathogenic nematode species, followed by *H. bacteriophora* (Laznik et al., 2009; Canhilal et al., 2016; Yuksel & Canhilal, 2019).

Molecular Identification

The rDNA ITS regions from all 41 nematode populations were effectively amplified using specific primers. This region, which includes the ITS1-5.8S-ITS2 segments covering flanking regions of the 18S and 28S genes, yielded a consistent fragment approximately 859 base pairs in length across all populations. Importantly, no PCR products were observed in the negative control lacking DNA template, confirming the specificity of the amplification. Subsequently, sequencing efforts produced 42 sequences from the sampled nematode populations, identifying them as belonging to *Steinernema feltiae* and *Heterorhabditis bacteriophora* species. For each species, a single consensus sequence was generated from the obtained sequences. Alignment of these sequences with corresponding 18S rRNA gene sequences from nematode isolates revealed matches with 41 nematode species cataloged in the GenBank database (Table 1).

The rRNA sequence was discovered to be less efficient in resolving taxonomic conflicts at the species level, mainly because it represents fewer taxa, which is linked to its slower evolutionary pace (Stock, 2009). However, this trait has been leveraged to distinguish the monophyletic origins of nematode groups (Peat et al., 2009). Additionally, the 5.8S rRNA region within the ITS is comparatively shorter and more conserved than the ITS-1 and ITS-2 regions, yet it evolves more rapidly than the 18S and 28S genes, rendering it suitable for taxonomic and population genetic studies of entomopathogenic nematodes (EPNs) at the species (population) level (Stock, 2009). Specifically, the ITS-1 region has been demonstrated to be adequate for species differentiation and assessment of *Heterorhabditis* spp. evolutionary relationships (Peat et al., 2009; Stock, 2009). On the contrary, the 28S rRNA gene displays a quicker rate of variation

compared to the 18S rRNA gene and presents fewer uncertainties in alignment than the ITS region (Stock, 2009). Despite this, it's considered more informative and appropriate for evaluating phylogenetic relationships, defining terminal taxa, and fulfilling diagnostic roles within *Steinernema* spp. (Stock & Hunt, 2005; Stock, 2009). The identification approach employed in this study aligns with previous findings (Liu et al., 1999).

A phylogenetic tree was constructed based on genetic distance, clustering populations at various levels using ITS sequence alignment (Figure 2).

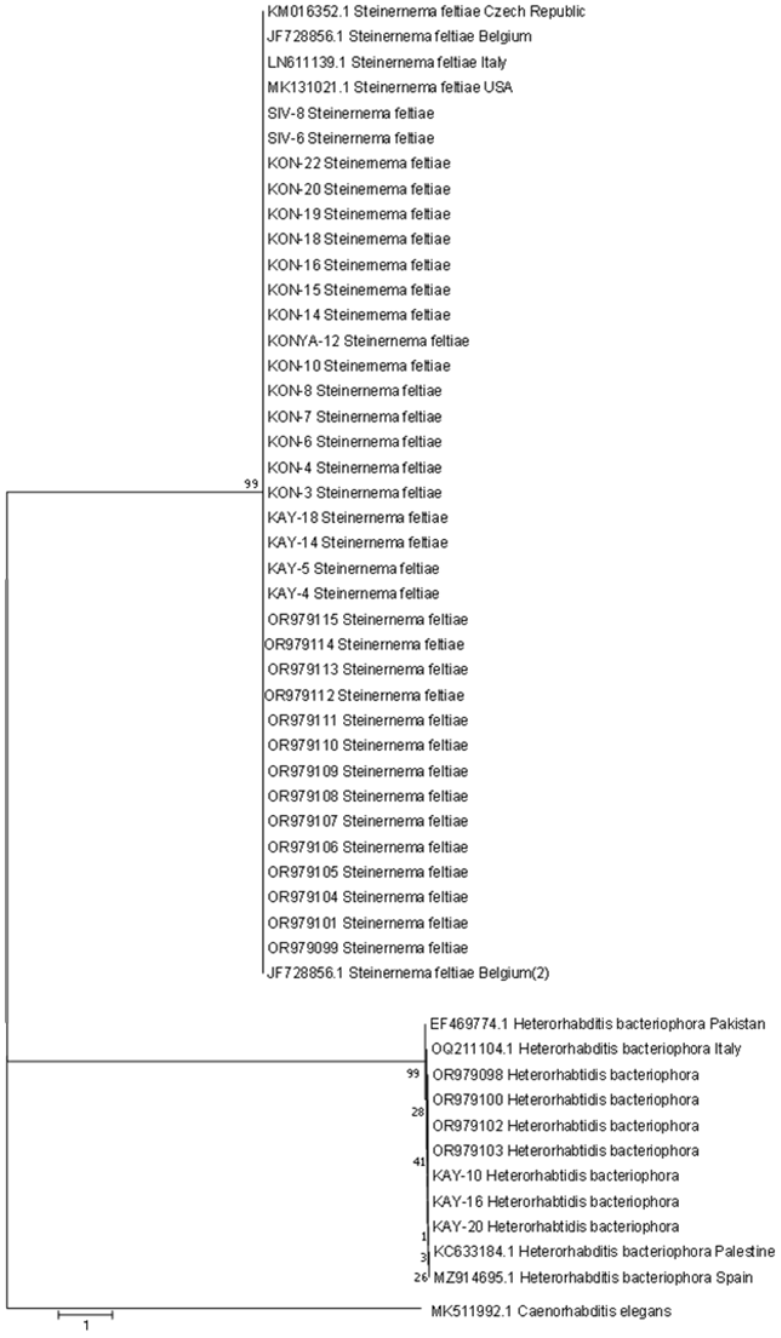

Figure 2. A phylogenetic tree (Neighbour-joining) was generated using the ITS sequence alignment derived from 41 populations of *Steinernema feltiae* and *Heterorhabtidis bacteriophora*. Bootstrap values are provided for the relevant clades to indicate their statistical support. Accession numbers for the population codes are listed in Table 1.

This analysis evaluated samples from five geographically distant sites, including 34 populations of *Steinernema feltia*e, 7 populations of *Heterorhabditis bacteriophora,* and one outgroup, *Caenorhabditis elegans* (MK511992.1). The resulting tree exhibited a distinct separation between the outgroup and *S. feltiae* and *H. bacteriophora* populations. Species with bootstrap values exceeding 99% were considered well-supported. The analysis revealed differences in ribosomal DNA sequences among the 41 isolates of *H. bacteriophora and S. feltiae*, indicating the presence of intraspecific polymorphism among the nematode populations. For the ITS region, a 859 bp fragment was obtained for the isolate of *Steinernema feltiae*. A BLAST analysis conducted against sequences archived in GenBank revealed a striking similarity ranging from approximately 96% to 99% with sequences belonging to the same species. The consensus tree derived from Bayesian inference prominently displayed a well-supported cluster (100% bootstrap) comprising sequences of *S. feltiae* originating from diverse geographical locations, including Belgium (JF28856.1), Czech Republic (KM016352.1), Italy (LN611139.1), and the USA (MK131021.1), which were sequenced in this study (Figure 4). Similarly, a 859 bp fragment was obtained for the *Heterorhabditis bacteriophora* isolate, which exhibited approximately 98–99% similarity with sequences of the same species in GenBank. The Bayesian inference consensus tree also displayed a well-supported group (100% bootstrap) consisting of sequences of H. bacteriophora from different countries, including Pakistan (EF469774.1), Italy (OQ211104.1), Palestine (KC633184.1), and Spain (MZ914695.1), which were sequenced in this study (Figure 2). Overall, the topology of our phylogenetic tree closely resembles that of previous studies (Liu et al., 1999; Emelianoff et al., 2008).

Historically, species determination within the Steinernematid nematode group has relied on crossbreeding experiments, morphometrics, and morphological characterization (Hominick et al., 1997). Morphometric differences in nematodes could be attributed to intra-specific variability (Poinar, 1992; Stock & Hunt, 2005). However, molecular tools may offer a solution to this issue and provide a novel approach for evaluating species boundaries in this group. Adams (1998) established several criteria for species description in nematology, emphasizing the importance of identifying autapomorphies (unique derived characters) as a primary requirement for establishing a new species.

Entomopathogenic nematodes, particularly *H. bacteriophora* and *S. feltiae*, have demonstrated significant potential for biological control of insects (Bhat al., 2020). Field tests have shown that *H. bacteriophora* effectively controls various target pests such as white grubs, cucumber beetles, black vine weevil, potato beetle, strawberry root weevil, among others (Grewal, 2012). While efforts have been made to assess the efficacy of these nematodes against foliar pests, challenges including desiccation, sunlight exposure, and high temperatures, which can be fatal to exposed nematodes, limit such applications (Grewal, 2012). The current survey indicates that *S. feltiae* and *H. bacteriophora* are frequently found in key potato-growing regions in Türkiye, suggesting they may hold promise for insect pests' biological control.

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