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Molecular Characterisation of 28S Region of *Xiphinema index* (Dorylaimida: Longidoridae) from Türkiye

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Abstract: *Xiphinema index* (Dorylaimida: Longidoridae) is a virus vector nematode and is highly distributed worldwide. In this study, the DNA of the *Xiphinema index* nematode was subject to Polymerase chain reaction (PCR) using D2A and D3B primers to analyse the 28S region. The *X. index* population in the study was recovered from a fig (*Ficus carica* L.) plantation in Tekirdağ, Türkiye. At first, the nematodes were identified morphologically and then molecularly. After PCR, amplified DNA was sequenced for molecular purposes, and data from the D2-D3 region of the 28S rRNA molecule of *X. index* were subjected to GenBank sequence comparison using BLAST (Basic Local Alignment Search Tool). The local sequence submitted to GenBank with accession number PQ165044 showed identity (98.26-99.81%) with several *Xiphinema index* hits. Local *X. index* sequence clustered close with Spanish (HM921406, HM921400, HM921399, HM921398, HM921363, HM921349, HM921347 and HM921364) and Crete/Greece (KJ802882) sequences in Neighbor-joining and Maximum likelihood analysis comparing the local sequence, 16 different *X. index* accessions and 14 accessions from various species.

Keywords: Dagger nematode, 28S rRNA, Ficus carica L., Türkiye

Türkiye'de *Xiphinema index* (Dorylaimida: Longidoridae)'in 28S Bölgesinin Moleküler Karakterizasyonu

Öz: *Xiphinema index* (Dorylaimida: Longidoridae) virüs vektörü nematod türü olup dünya genelinde oldukça yaygındır. Bu çalışmada, *Xiphinema index* nematodunun DNA'sı ile, 28S bölgesini analiz etmek için D2A ve D3B primerleri kullanılarak Polimeraz Zincir Reaksiyonu (PCR) yapılmıştır. Çalışmada *X. index* popülasyonu, Türkiye, Tekirdağ'daki bir incir plantasyonundan (*Ficus carica* L.) toplanmış, ve türün filogenetik analizini gerçekleştirmek için DNA'sı D2A ve D3B primerleri ile amplifiye edilmiştir. Nematodlar önce morfolojik olarak, daha sonra moleküler olarak tanımlanmıştır. PCR sonrası amplifike yapılan nematod DNA'sı sekansa tabi tutulmuştur ve *X. index*'in 28S rRNA molekülünün D2-D3 bölgesinden gelen veriler BLAST (Basic Local Alignment Search Tool) kullanılarak GenBank dizi karşılaştırmasına tabi tutulmuştur.Yerel sekans PQ165044 aksesyon numarası ile GenBank'a kaydedilmiş ve diğer *Xiphinema index* türleri ile (% 98.26 – 99.81) benzerlik göstermiştir. Yerel *X. index* dizisi, 16 farklı *X. index* aksesyonu ve farklı *Xihpinema* türlerine ait 14 aksesyon ile yapılan Neighbor-joining ve Maximum likelihood analizlerinde yerel sekans, İspanya (HM921406, HM921400, HM921399, HM921398, HM921363, HM921349, HM921347 ve HM921364 ve Girit/Yunanistan sekanslarıyla aynı dalda kümelenmiştir.

Anahtar kelimeler: *Kamalı nematod, 28S rRNA, Ficus carica L., Türkiye*

1. Introduction

The dagger nematode, *Xiphinema index* (Dorylaimida: Longidoridae), is one of 260 ectoparasitic nematodes of the genus *Xiphinema* belonging to family Longidoridae (Decraemer, 2007). This species was first described by Thorne and Allen in 1950 from the root zone soils of the fig tree (Ficus carica). Since then, it has been reported from various locations worldwide (Jawhar, 2006; Leopold et al., 2007). Severe damage caused by this nematode has been documented in Mediterranean countries, where dense populations result in root swelling, necrosis, wilting of above-ground plant parts, and general stunting (Van Zyl et al., 2011).

X. index plays a primary role in the spread of virus diseases, which are among the most dangerous plant

pathogens worldwide. More than 2.000 viruses infect plants, animals, and other microorganisms, with 1.200 of them parasitising plants (Bernardo et al., 2018). Unlike bacterial and fungal pathogens that can be controlled with pesticides, virus diseases pose the greatest threat to agricultural production due to the lack of effective control methods. The disease potential of viruses varies depending on the virulence of the isolate and the susceptibility of the cultivar (De Klerk & Loubser, 1988). *X. index* was initially identified as the vector of grapevine fanleaf virus (GFLV) (Hewitt et al., 1958), one of the most harmful nematode-borne virus diseases affecting vineyards. This virus is widely distributed across several countries, causing disruptions in carbohydrate metabolism and hormonal balances, significant decreases in photosynthesis, and increased grapevine respiration rates (Basso et al., 2017). Severe infections can lead to irregularly maturing clusters with non-uniform, poorly developed berries, resulting in yield reductions of up to 80%. The damage inflicted on infected plants varies based on the virus isolate, infection severity, and grape variety susceptibility (Martelli and Savino, 1990; Andret-Link, 2004). Nematodes can acquire virus particles within 15 minutes of feeding on young root tips and can simultaneously transmit these particles to healthy plants. Nematodes that carry virus particles can maintain their ability to transmit the virus for up to 9 months, complicating efforts to eradicate viruses when nematode populations persist (Taylor and Raski, 1964). Moreover, nematodes can survive in the soil for several months even after the host plant is removed, posing ongoing risks for future infections.

Morphology and morphometric observation have been mostly preferred to identify *Xiphinema* species (Kumari and Liskova, 2009). However, many species have very similar characteristics, which make them difficult to distinguish under mixed populations (Sırca 2007). Besides, this kind of identification is only possible using adult nematodes and can only be performed by experts. (Kumari et al., 2010). Because of these limitations, several researchers developed molecular techniques for rapid and reliable species determination. Again, genetic similarities between populations of different countries and species can be easily revealed with molecular techniques (Vrain et al., 1992; Wang et al., 2003). The D2-D3 segments of the 28S region can be successfully applied to separate different *Xiphinema* species. (Blaxter et al., 1998; Powers, 2004; Daramola et al., 2019; Fayaz et al., 2022). This method identifies nematodes in every developmental stage (Hübschen et al., 2004a; Hübschen et al., 2004b).

The nematode species *Xiphinema index*, has been detected by several researchers in our country, particularly in vineyards. It has been found in the several provinces including Şanlıurfa, İzmir and Manisa, (Yıldız & Elekcioğlu, 2011; Mistanoğlu et al, 2015; Öztürk et al., 2017; Kasapoğlu et al., 2018).

This study aimed to molecularly characterize the *Xiphinema index* by amplifying the D2-D3 region of 28S rRNA with general primers and to determine the closeness using local sequence data and other GenBank records.

2. Material and method

2.1. Collection of soil samples, extraction, and identification of *Xiphinema index*

In our previous studies, the *Xiphinema index* has been detected in various plants in Thrace, whereas in our survey in PhD thesis, it was found in high populations at depths of 30-60 cm in fig orchards. In this study, soil samples were collected in March 2017 from a fig orchard where the *Xiphinema index* was previously identified. A molecular study was conducted with nematodes isolated from these soil samples. Female nematodes were extracted from a 200 gr subsample using Cobb's sieving and centrifugal flotation method (Jenkins, 1964; Brown & Boag, 1988). To confirm the *X. index*, slides were prepared from heat-killed, fixed (in a double strengthen formalin-triethanolamine (7 ml formaldehyde+2 ml triethanolamine+ 71 ml distilled water) solution, and on a slide-mounted female (Thorne and Allen, 1950; Seinhorst, 1959; Hooper, 1961). Species were identified by comparing morphometric and morphologic parameters of female nematodes with descriptions of Thorne & Allen and keys of Loof and Luc, 1990, 1950 and other researchers. The images of the females were taken with a Celestron microscope. The females, confirmed to be *X. index,* were picked and transferred to PCR tubes for DNA extraction and further molecular studies.

2.2. 28S Phylogenetic analysis with *Xiphinema index*

To isolate the *Xiphinema index,* The Sigma Aldrich Extract-N-Amp™ Tissue PCR Kit was used, and the manufacturer's procedure was followed in the extraction process. Individuals were hand-picked under the microscope to extract DNA and placed into 0,2 ml microcentrifuge tubes containing 2.5 µl tissue preparation and 10 µl extraction solutions. Following

processing at 55°C for 10 minutes and 95°C for 3 minutes, a neutralisation solution was added to the extract, and the DNA concentration (A260/280 A260/230) was measured using a spectrophotometer. The extracted DNA was stored at -20 °C until molecular studies.

Amplification of nematode DNA was performed with D2A (5′ ACAAGTACCGTGAGGGAAAGT 3′) and D3B (5′ TCGGAAGGAACCAGCTACTA 3′) primer pair (Nunn, 1992). The PCR reaction was carried out with the following program: 95 °C for 3 min, followed by 39 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min, ending with one cycle at 72 °C for 5 min. The Amplification product was separated on a 1.5 % agarose gel in 1×TAE (Tris-acetate-EDTA) buffer.

The PCR product was sequenced following UV visualisation. The sequencing was performed in the Central Research Laboratory at Namık Kemal University using the Beckman Coulter GenomeLab GeXP Genetic Analysis System. Sequence data from the D2-D3 region of the 28S rRNA molecule was edited with Bioedit 7.2.5 (Biological sequence alignment editor) software and subjected to Blast (Basic Local Alignment Search Tool) sequence comparison in NCBI (National Center for Biotechnology Information). Neighbour-joining (1000 Bootstrap) and maximum likelihood were performed on Mega 7 software, comparing the *X. index* (16 accessions) and other *Xiphinema* sequences (14 species/accessions) from 10 countries (Table 1).

Table 1. The list of NCBI GenBank accessions used in the phylogenetic analysis
Çizelge 1. *Filogenetik analizlerde kullanılan NCBI GenBank aksesyonları*

ACCESSIONS	SPECIES	LOCALITY
HM921406; HM921400; HM921399; HM921398; HM921363; HM921349; HM921347; HM921348; HM921364;	*Xiphinema index*	Spain
KX244910	*Xiphinema index*	Spain
HG969307	*Xiphinema index*	Hungary
AY601628	*Xiphinema index*	Argentina
KM283422; MF996703	*Xiphinema index*	Iran
KJ802882; KJ802881	*Xiphinema index*	Crete, Greece
JQ780362	*Xiphinema diversicaudatum*	Czech Republic
KJ802879	*Xiphinema cretense*	Crete, Greece
MT271611	*Xiphinema hyrcaniense*	Iran
KX244900	*Xiphinema cadavalense*	Spain
KX244915	*Xiphinema pseudocoxi*	Spain
KY623487	*Xiphinema tica*	Costa Rika
GU549474	*Xiphinema globosum*	Spain
KY131240	*Xiphinema japonicum*	Spain
KU052864	*Xiphinema bakeri*	Japan
AY601629	*Xiphinema basiri*	Cuba
KC567170	*Xiphinema belmontense*	Spain
KC567166	*Xiphinema baetica*	Spain
KX244887	*Xiphinema andalusiense*	Spain
KT308868	*Longidorus intermedius*	Spain

3. Results

The *Xiphinema index* specimens used in the molecular studies had elongated bodies [3±0.06 (2.97-3,11)] mm] that formed an open spiral shape when at rest or deceased, with the lip region continuous with the neck contour. The a=60.4±2.91 (56-66), the c=78.52±1.52 (76.2-80.8), and the c′=0.83±0.01 (0.82-0.84). The oesophagus had a narrow anterior part that widened towards the posterior. The posterior part of the oesophagus was cylindroid. The oesophagus-intestinal valve was small and conoid-rounded, and the stylet was long. A conical-shaped cardia was located at the junction of the oesophagus and intestine. Stylet 203.4±5.6 (195-213) µm long with odontophore 79.1±2.16 (76,6-80,0) µm and odontostylet 123.4±3.6 (116.4-127.9) µm. Guiding ring 120.4±1.28 (118.0-122.3) µm from anterior end. Vulva was a depressed transverse slit located anterior to mid-body. Reproductive system amphidelphic, with reflexed ovary. The vulva appeared as a depressed transverse slit situated anterior to the mid-body [38.6±2.4 (36-40.6)]. The reproductive system was amphidelphic, with a reflexed ovary. The tail was convex-conoid, with greater curvature dorsally and a small peg called mucro. The tail was 0.87±0.22 times as long as the anal body width (Thorne and Allen, 1950). Mucro length was 8.11±002 (7.8-8.30) (Figure 1).

The morphometric values of individuals were similar

to those of the Spanish, Chilean, Serbian and Lebanese populations (Body length 2.7-3.65 mm; Stylet=193-217 µm; Tail=32-48.6µ; V=36-43.9%) (Barsi and Lamberti, 2000; Jawhar et al, 2006; Gutierrez et al. 2011; Meza et al., 2011).

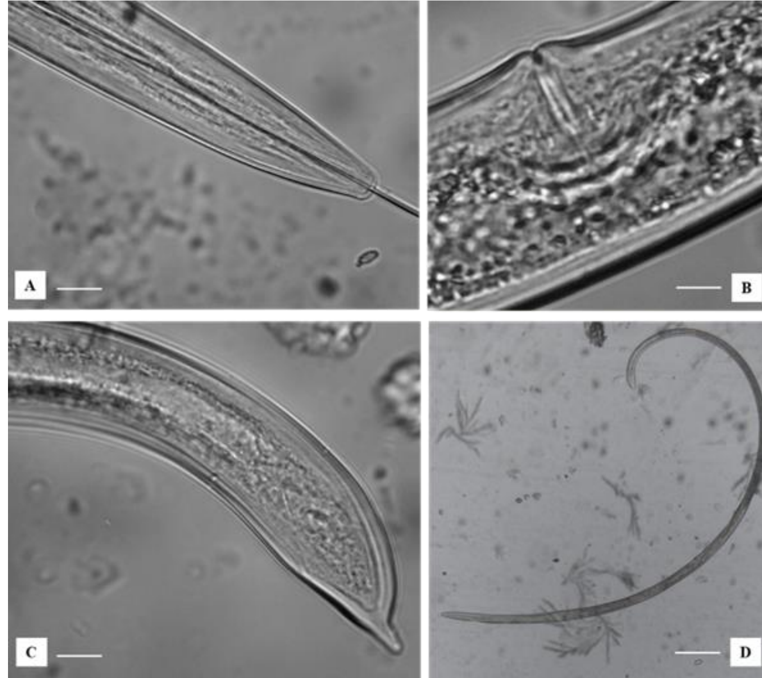

Figure 1. *Xiphinema index* female A. Head with odontostylet B. Vulval region C. Tail and mucro (Scale bar: 20 µm) D. Female entire body
Şekil 1. *Xiphinema index dişi A. Baş bölgesi ve odontostilet B. Vulva bölgesi C. Kuyruk ve mucro D. Dişi vücut genel görünüm*

The partial sequence of the *X. index* from Tekirdağ (PQ165044) was compared with records from other countries, revealing over 98% identity with 16 hits of the 28S ribosomal RNA gene partial sequence of the *X. index* in the NCBI GenBank. According to Blast (Basic Local Alignment Search Tool), the sequence showed 99.81% identity (519-520 nt/1 gap) with sequences from Greece (KJ802882) and 99.41% identity (560-563 nt/1 gap) with sequences from Spain (HM921364). Spanish accessions HM921406, HM921400, HM921399, HM921398, HM921363, HM921349, HM921347, and HM921364 exhibited a 99.3% identity score. The lowest identity was observed with the Iranian accession KM283422 (98.78%; 567-574 nt/2 gaps). Nucleotide differences ranged from 1 to 7 in closely related sequences, with gaps between all sequences ranging from 1% to 3%.

The closest match was *X. diversicaudatum* MG994934 (92.08%) when comparing identity with other *Xiphinema* species. Other close matches include *X. pyrenaicum* AY601626 (97.52%), *X. cretense* KJ802880 (91.89%), *X. cadavalense* KX244900 (91.73%), *X. hyricaniense* MT271611 (91.73%), *X. pseudocoxi* KX244915 (91.70%), *X. tica* KY623487 (91.57%), *X. japonicum* KY131240 (91.36%), *X. bakeri* KU052864 (91.36%), *X. belmontense* KC567170 (91.35%), *X. basiri* AY601629 (91.35%), *X. coxi* AY601631 (91.30%), *X. baetica* KC567166 (91.23%), *X. andalusiense* KX244887 (91.21%), *X. abrantinum* AY601625 (90.83%), and *X. turdetanense* KX244920 (91.21%).

Figures 2 and 3 represent the Neighbor-joining and Maximum likelihood trees generated by comparing the Tekirdağ sequence and 31 different *Xiphinema* accessions from NCBI. In both trees, the local *X. index* clustered in the nearest clade with sequences from Spain, Hungary, and Greece. All *X. index* species were found in the same clade as other non-*X. index* species were grouped in other clades.

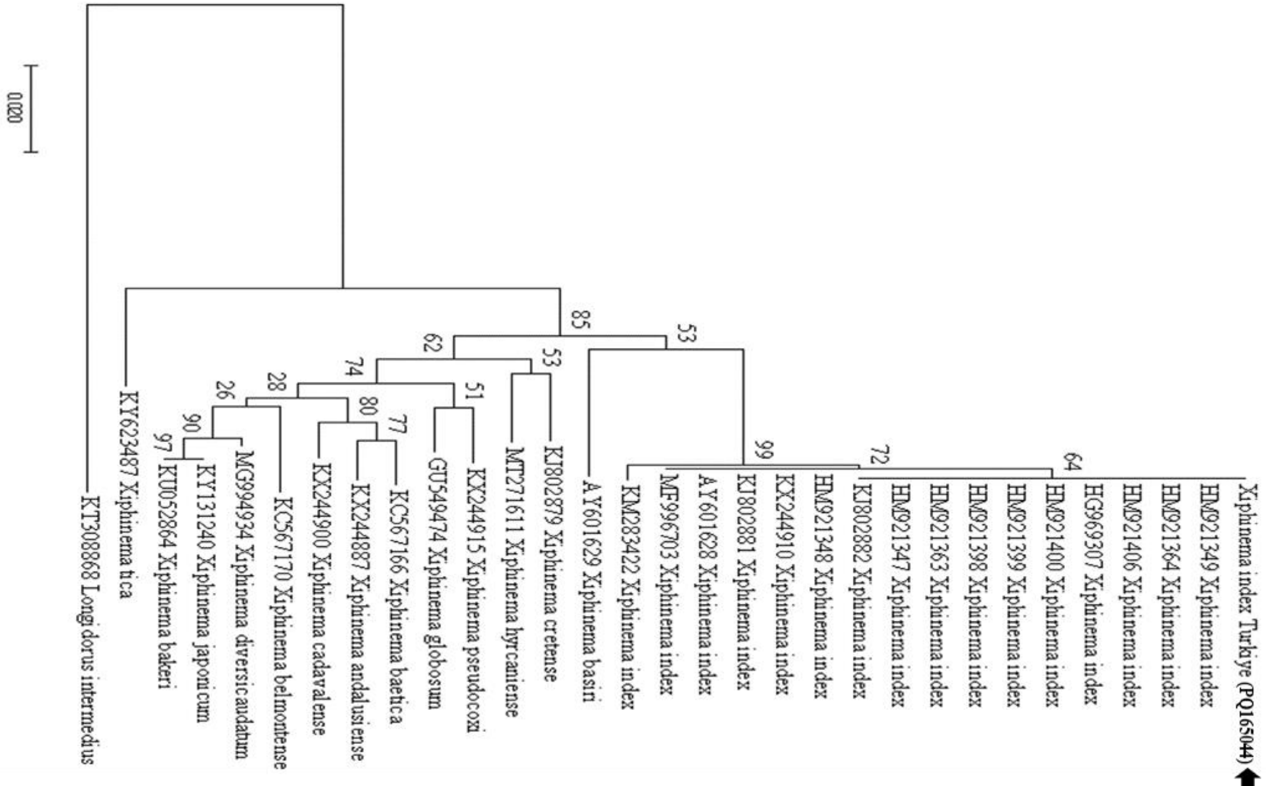

Figure 2. Neighbor-joining tree generated by comparing 28S rRNA gene D2-D3 regions.
Şekil 2. *28S rRNA geni D2-D3 bölgelerinin karşılaştırılmasıyla oluşturulmuş Neighbor joining ağacı*

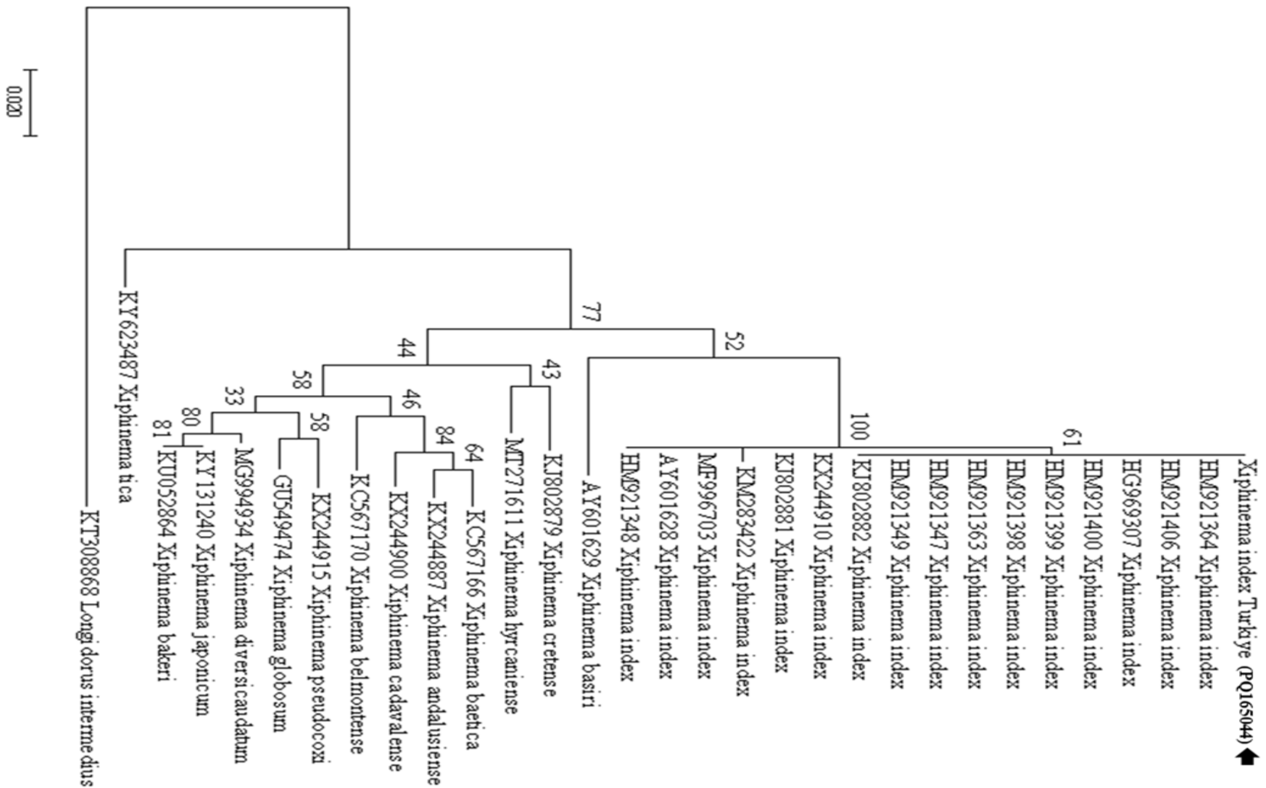

Figure 3. A maximum likelihood (Kimura-2 parameter model) tree generated by comparing 28S rRNA gene D2-D3 regions.
***Şekil 3**. 28S rRNA geni D2-D3 bölgelerinin karşılaştırılmasıyla oluşturulmuş Maximum Likelihood (Kimura-2 Parametre modeli) ağacı*

4. Discussion

In previous studies conducted in Thrace, *Xiphinema index* nematode has been vineyard areas (Ozturk et al., 2017). Although this nematode species can parasitize many cultivated plants, it is reported to reproduce best on fig trees, acting as its primary host. Research has determined that *X. index* has a lifespan of 60 to 64 weeks on *Ficus carica*, with a total reproductive capacity ranging from 140 to 160 progeny (Brown & Coiro, 1985). Fig trees, which grow along roadsides and various other locations, are commonly found between rows of vines or along vineyard edges, especially in districts like Şarköy in Thrace. The presence of these host plants, particularly in vineyards where viral infections are prevalent, can facilitate the proliferation of vector nematodes, thereby exacerbating the spread of epidemics. To prevent the spread of *grapevine fanleaf virus* (GFLV), one of the most significant viruses affecting vineyards, it is essential for new vineyard sites to be free of nematode infestations. Soil samples from these areas are analyzed to assess the presence of vector nematode infestations.

Identifying *X. index* mostly relies on observing morphologic and morphometric characteristics under a microscope. *X. index* shows identical morphological features with many species, such as *X. vuittenezi* and *X. italiae* (Van Zyl, 2011). Its closest relative is *X. diversicaudatum* Micoletzky, 1927, and *X. index* is distinguished by the more anterior position of the vulva, four pairs of caudal pores compared to six pairs in *X. diversicaudatum*, and the smaller body structure. Morphological identification is difficult, and misidentification is sometimes possible. For this reason, researchers are using molecular methods for diagnosis. Many species-specific primers were designed based on the sequence divergence of the DNA region of species and closely related species (Esmenjaud & Bouquet, 2009). Several studies have previously achieved molecular confirmation of cyst nematodes, lesion nematodes, stunt nematodes, and root-knot nematodes. The internal transcribed spacer (ITS), region of ribosomal DNA, cytochrome c oxidase subunit I (COI) and some other genetic markers were generated and successfully applied in identifications. (Blaxter et al., 1998; Powers, 2004). (Vrain et al., 1992; Wang et al. 2003; Hübschen et al., 2004). Ribosomal RNA is divided into two subgroups in eukaryotic organisms such as nematodes: the large subunit covering the 60S, 5S, 5.8S, and 28S regions and the small ribosomal unit containing the 18S region (Lafontaine and Tollervey, 2001). The D2 and D3 segments of 28S rRNA are frequently used in phylogenetic studies because it is easy to design diagnostic primers from this region, and the gene encoding 28S rRNA is more variable than 18S (Subbotin et al., 2008).

In this study, the 28S region was used in the molecular characterisation and phylogenetic studies of the *Xiphinema index*. The results revealed that the local population was highly identical to those in Spain, Crete, and Greece. Furthermore, Blast analysis showed a 98% or more identity with Iranian and Hungarian accessions. These significant findings contribute to *the* existing body of knowledge on the phylogeny of the *X. index* in different countries.

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Author contributions

Lerzan ÖZTÜRK carried out molecular studies, and İbrahim Halil ELEKÇİOĞLU approved the nematode identification, reviewed the paper, and approved.

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