Morphological and molecular characterisation of *Xiphinema index* populations in vineyards from Southwestern Iran

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Summary. In a survey in the vineyards of Southwestern Iran, Chaharmahal and Bakhtiyari province, *Xiphinema index* populations were collected during 2019-2020. These nematodes are described based on morphological characters. Identification of this species was done based on the morphological and morphometrical features and analysis of the D2-D3 segments of 28S rRNA gene sequences. Females are similar morphologically to the original and other descriptions of *X. index* from different countries. Comparison of our sequences with those from the GenBank database showed Iranian *X. index* had 99-100% identity and 4-10 bases pair differences with sequences of the other populations of *X. index*. Phylogenetic analysis based on sequences of the partial 28S rRNA gene and using Neighbour Joining method placed our population within other populations of *X. index*.

Key words: 28S rRNA gene, dagger nematode, Chaharmahal and Bakhtiyari province, *Vitis vinifera*.

Grapes are one of the most important horticultural products in the world (FAO, 2017). Among fruit trees, the highest number of fertile orchards in the world belongs to grapes, after coconut and olive. The area under cultivation of this crop has been constantly increasing during recent decades (Porika *et al.*, 2015). Iran is the eighth largest producer in the world with an area of 308,000 ha and an annual production of 3.2 million tons (FAO, 2017). Various diseases including parasitic nematodes affect the vineyards, and reduce the quantitative and qualitative grape production (Askary, 2017). Among the plant-parasitic nematodes associated with grape, the dagger nematode, *Xiphinema index*, is most important (Sasser & Freckman, 1987). It is especially important because in addition to feeding on grape roots, it transmits the Grapevine fan leaf virus (GFLV). Eventually it causes the decline and death of grapevines (McKenry & Bettiga, 2013). All stages of *X. index* are capable of transmitting the virus (McKenry & Roberts, 1985), and it has been reported that the virus can remain in the adult nematodes for at least 4 years (Esmenjaud *et al.*, 2014). The species of *Xiphinema* are found in all types of soils including most vineyards in the world (Taylor & Brown, 1997).

Xiphinema index has been recorded from Africa, America, Australia, Asia and Europe. It was first reported by Thorne (1939) and was first identified and described by Thorne & Allen (1950). In Iran, this species was first observed by Mojtahedi *et al.* (1980) in cultivated soils and in natural woodland, and was subsequently reported from different regions of Iran. According to Ghaderi *et al.* (2018), 26 species of the genus *Xiphinema* have been reported from Iran. Already some populations of *Xiphinema* have been reported in Chaharmahal and Bakhtiari province (Fadaei *et al.*, 2003). Accurate identification of nematodes at the species level is essential for effective control, especially for virus vector species. (Zeng *et al.*, 2016). Chaharmahal and Bakhtiari province is one of the most important provinces in south-western Iran in terms of grape production.

Identification of this species is usually done using the morphological and morphometric characteristics of females, but the diversity of these features has been less studied (Brown & Topham, 1985). In addition to the traditional methods used to identify nematodes, molecular techniques are powerful tools for more accurate identification (Kiewnick *et al.*, 2014). Nevertheless, recorded information from *X. index* in the world varies. In France, *X. index* has been identified using both morphological and

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molecular methods (Demengeat *et al.*, 2005). By contrast, Leopold *et al.* (2007), using only molecular techniques, identified *X. index* for the first time in Austria. This species has also been identified in Lebanon using only classical classification methods (Jawhar *et al.*, 2006). The application of molecular identification, especially the use of D2-D3 segments of the 28S rRNA gene is useful for detection and separation of different species of *Xiphinema* (Ye *et al.*, 2004). In this study, the objectives were *i*) to characterise *X. index* morphologically, and molecularly by using the D2-D3 segments; *ii*) to investigate phylogenetic relationships of the Iranian *X. index* populations with other populations of the same species and related species using the D2-D3 expansion segments of 28S rRNA gene; and *iii*) to provide morphological identification of different populations of *X. index* in Chaharmahal and Bakhtiari province and their comparison with molecular identification methods.

MATERIAL AND METHODS

Soil sampling. 350 soil samples from the rhizosphere of *Vitis vinifera* were collected, from five localities of vineyards in Chaharmahal and Bakhtiyari province during 2019-2020 years. All five localities were positive for the presence of *X. index*. The age of the vineyards sampled in this study was about 40 to 70 years.

Nematode extraction. Females of *X. index* were extracted from soil samples using the centrifugal-flotation method (Jenkins, 1964) and tray method (Whitehead & Hemming, 1965), and then killed and fixed according to De Grisse (1969). Nematodes were identified based on morphological and morphometric characters. Three populations were selected based on climatic conditions and their characteristics were measured (Shahrekord region with cold climate, Saman region with temperate climate and Lordegan region with warm climate). To study the characteristics of the uterus, adult females were photographed in a drop of water (temporary slides). Measurements were compared with those of the original description (Thorne & Allen, 1950). Photographs of permanent slides were taken with an ocular micrometer of Olympus CX31 microscope. The digital images were made using an Olympus DP50 digital camera attached to an Olympus BX41 microscope powered with differential interference contrast.

DNA extraction. For the molecular studies, nematode DNA was extracted from single adult females as described by Pedram *et al.* (2011). To prevent any possible mistakes in different populations, one live nematode from each sample was picked out, studied individually on temporary slides, transferred to a small drop of AE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0) on separate clean slides and each was squashed using a clean cover glass. The suspension on each slide was collected by adding 10 µl AE buffer, each regarded as an independent DNA sample and stored at –20°C until PCR amplification.

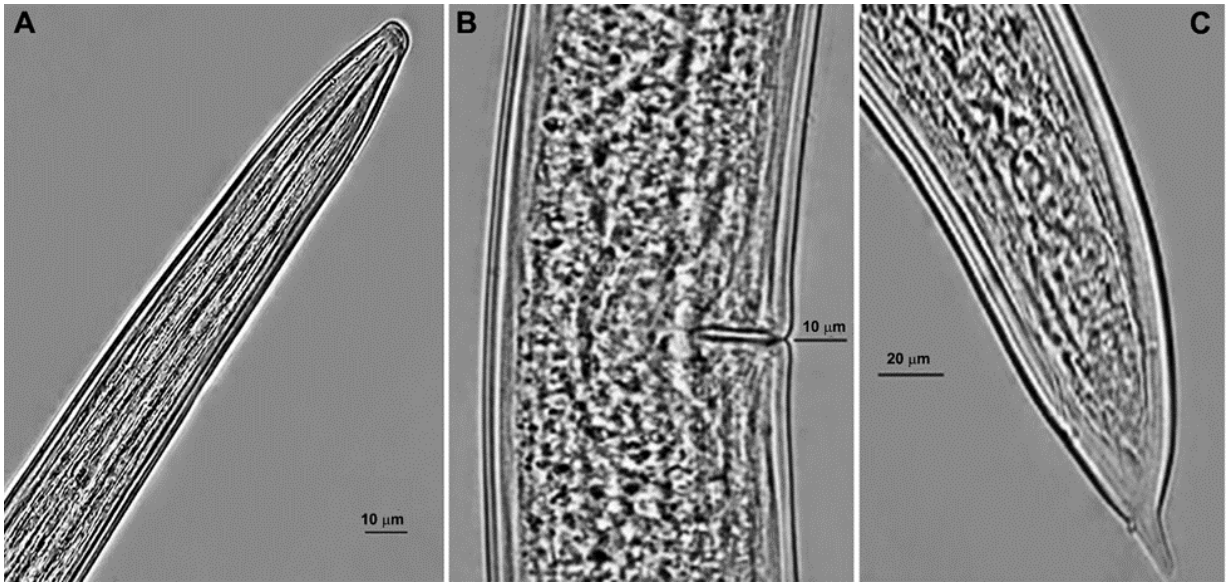

Fig. 1. *Xiphinema index* from Southwest Iran, Chaharmahal and Bakhtiyari province A: Female anterior region; B: Vulval region; C: Tail showing a typical ventral mucro.

PCR. Primers used for the amplification of the D2-D3 segments of the 28S rRNA gene were: forward D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3') and reverse D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) (Nunn, 1992; Subbotin *et al.*, 2006; Palomares-Rius *et al.*, 2008). The PCR mixture (25 µl) contained 12.5 µl of Master mix (DENA ZIST ASIA, Mashhad, Iran), 1 µl of each primer (10 pmol μl^{-1}), (SINA CLON Tehran, Iran, 5 U μl^{-1}), and 3 μl^{-1} of DNA template and nuclease-free water 7.5 µl. The thermal cycling program employed an initial denaturation at 95°C for 6 min, followed by 35 cycles with a denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. A final extension was performed at 72°C for 10 min. Amplification success was evaluated electrophoretically on 1% agarose gel.

Sequencing and phylogenetic analysis. PCR products were sent to Macrogen Corporation (South Korea) for purifying and sequencing and then the sequences were used in phylogenetic studies. The sequence chromatograms were checked using BioEdit software (Hall, 1999). Out of five submitted sequences, four sequences were read successfully. The newly produced sequences in this study were submitted in the GenBank database under accession numbers MZ677013, MZ677014, MZ677015, MZ677016. Twenty sequences of the 28S rRNA gene were also recorded from GenBank and aligned with four sequences produced in this study using Clustal X software (Larkin *et al.*, 2007) with default parameters. In this analysis *Longidorus intermedius* (JX445117) was used as the outgroup (Gutiérrez-Gutiérrez *et al.*, 2012). Drawing the phylogenic tree was done using MEGA 7 program (Kumar *et al.*, 2016) with 1000 bootstrap replicates. The phylogenic tree was obtained with the Neighbour Joining method.

RESULTS AND DISCUSSION

Distribution. During the present study, *X. index* was found in the 138 soil samples collected from different vineyards of Chaharmahal and Bakhtiyari province (Shahrekord, Saman, Farokhshahr, Lordegan and Naghan) in southwestern Iran. Abundance of this nematode in this study was 38%. Grape infestation with *Xiphinema* was expected to be higher than the data obtained. Unfortunately, Iran suffered from drought during these years, and it was the reason for the nematode infestation reduction in this study. The morphological characteristics of most populations were close to each other and only slight differences were observed between them. The measurements were completely consistent or overlapping. Morphological studies obtained from extracted nematodes from the Nagan region agreed with samples obtained from the Lordegan region. These two regions also have similar climates.

Xiphinema index Thorn & Allen, 1951 (Fig. 1)

Measurements. Morphometrics of females of three populations of *Xiphinema index* are presented in Table 1.

Female. Body forms an open spiral on death. The lip region is hemispherical and almost continuous along the body. Cuticle with two distinct layers of thin outer layer and its thickness is almost the same throughout the body. Odontostyle needle-like, averaging 121.5-136 µm long. Odontophore with

Table 1. Morphometrics of three populations of *Xiphinema index* used in this study and the original description. All measurements are in µm except L in mm.

Location \ Reference	Shahrekord	Saman	Lordegan	Thorne & Allen (1950)
Characters \ stage	♀	♀	♀	♀
n	7	7	7	–
L	3.3 (3.2-3.4)	3.0 (2.4-3.3)	3.5 (3.3-4.4)	3.4
a	59.0 (56.5-60.2)	50.8 (42.3-59.3)	52.7 (46.2-59.3)	58
b	7.7 (7.2-8.3)	7.7 (5.8-8.1)	7.5 (7.1-7.9)	7.6
c	81 (74.7-83)	71.8 (52.6-92.1)	76.3 (68.4-82.6)	76
c'	1 (1-1.2)	1 (1-1.2)	1 (1-1.2)	–
V	37 (34-39)	43 (41-45)	36.5 (36-38)	38
Stylet	219.2 (121.8-228)	174.1 (152-188.7)	207.8 (182.4-220)	–
Odontophore	83.6 (68.4-97.2)	87 (76-98.1)	77.7 (60.8-86.6)	–
Odontostyle	136 (114-156.5)	121.5 (91.2-152)	131.6 (118.5-144.4)	90
Tail length	40.6 (36.4-45.6)	42.6 (39.6-45.6)	43.8 (41.0 -45.6)	–
BW	56.6	56.6	63.7 (56.6-69.1)	–
Diam. at anus	36.8 (34.9-38)	32.5 (30.4-36.4)	39.5 (36.4-41)	–
Diam. at lip region	14.82 (13.6-15.2)	13.04 (12.4-13.6)	14.4 (13.6-15.2)	–
Diam. at mid body	56.6	56.6	63.7 (56.6-69.1)	–
Terminal peg	9.3 (7.6-10.6)	9.9 (9.1-12.4)	9.5 (9.1-10.6)	–

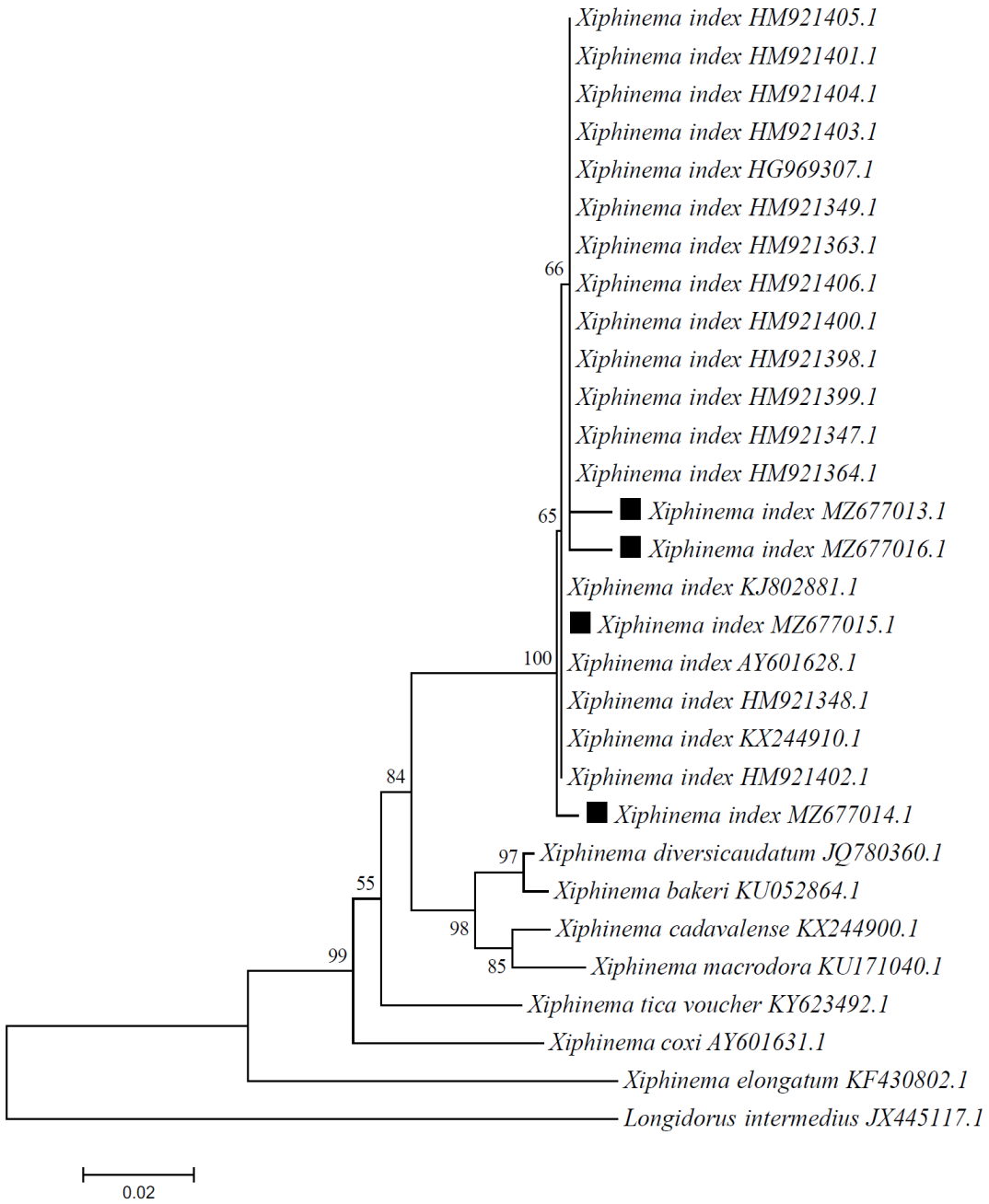

Fig. 2. Phylogenetic tree of *Xiphinema* species obtained using Neighbour Joining method with 1000 replicates for bootstraps as inferred from analysis of the partial 28S rRNA gene sequences. The newly isolates are indicated with black squares.

three large basal flanges, average length 77.7-87 µm. Pharynx in two parts anterior slender and posterior bulbar part. The dorsal pharyngeal gland cell extends along the length of the basal pharyngal bulb on the dorsal side and the anterior half of the ventral side. The cell has a system of six ducts formed by deep in folds of the limiting cell membrane. Two ducts extend almost the entire length of the bulb on the dorsal side and four extend half-way on the ventral side. Ovaries paired, opposed, reflexed. Z-organ absent. Tail is hemispherical and has a dorsal convex, it is about as long as anal body width, usually with a terminal peg 7.6-12.4 µm long.

Male. Not found.

Morphometric characterisation of *X. index* from Chaharmahal and Bakhtiyari province was similar to that from the original description by Thorne & Allen

(1950). *Xiphinema index*, *X. diversicaudatum*, *X. vuittenezi* and *X. italiae* are closely related taxonomically and therefore difficult to distinguish using morphological and morphometrical characteristics. *Xiphinema index* differs from *X. diversicaudatum* in having lower value of a, b and V ratios (50-59 *vs* 72; 7 *vs* 10 and 37-43 *vs* 47, respectively) and absence of Z organ in the uterus. *Xiphinema index* differs in having a longer terminal peg, absence of Z organ in the uterus and lower V values (37-43 *vs* 46-56.5%). *Xiphinema index* differs from *X. italiae* in tail shape; longer body size and a smaller index a (50-59 *vs* 88).

The present data confirm that the Iranian populations do not show any specific morphological diversity in the studied traits and intraspecific diversity is low. One of the reasons could be the uniformity of climatic conditions in the study area. Also, the host of the studied nematodes was the same and is probably another reason for the lack of morphological differences.

Molecular characterisation using the D2-D3 segments of 28S rRNA gene sequences. The partial sequences of the 28S rRNA gene for *X. index* were in 758-822 bp. The D2-D3 expansion segments of the 28S rRNA gene provided rather homogeneous results for *Xiphinema* species and apparently is a useful tool for species differentiation (He *et al.*, 2005). BLAST (Basic Local Alignment Search Tool) at NCBI (National Center for Biotechnology Information) revealed that the 28S rRNA gene sequences of Iranian *X. index* gave 99-100% similarities with other *X. index* sequences. The phylogenetic relationships of the studied and other populations of *X. index* are shown in Figure 2. The closest sample to the Iranian population was considered the Spanish one.

The available information indicates that most of the grape cultivars cultivated in Iran are imported mainly from Europe and the Caucasus. It is possible that the *Xiphinema* was imported with the seedlings. However, this issue requires further study. In the phylogenic tree reconstructed based on 28S rRNA gene sequences, *X. index* are distributed into several different clades, although no morphological differences were observed between the different populations studied. However, the results of molecular studies showed that the *X. index* was not genetically homogeneous and there was some intraspecific diversity. The results of molecular studies were performed using the ITS rRNA gene consensus sequence of the Chilean populations and showed that there were no intraspecific differences among the studied populations (Meza *et al.*, 2012).

This is the first complete study on the occurrence, abundance and determination of intraspecies morphometric and molecular variation of *X. index* from the vineyards of Chaharmahal and Bakhtiari province of Iran. This nematode is widespread in most of vineyards of the studied areas and is one of the dominant plant-parasitic nematodes in these regions. Our work combined morphological, morphometric and molecular characterisation using integrative taxonomy and support identity of *X. index*. *Xiphinema index* is one of the most damaging plant-parasitic nematodes in vineyards and proper management of these nematodes increases the production and better yield of grapes. Future data on resistant varieties to different populations of these nematodes may improve our knowledge of the management of grape-related plant-parasite nematodes.

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M. Fayaz, E. Mahdikhani Moghadam and A.A. Fadaei Tehrani. Морфологическая и молекулярная характеристика популяций *Xiphinema index* из виноградников юго-запаного Ирана.

Резюме. В ходе обследования виноградников юго-западного Ирана в 2019-2020 годах популяции *Xiphinema index* были обнаружены в остане (провинции) Чехармехаль и Бахтиария. Дается морфологическое описание этих нематод. Определение до вида основывается на морфологических и морфометрических признаках и анализе D2-D3 сегмента 28S rRNA гена. Обнаруженные самки морфологически сходны с оригинальным и другими описаниями *X. index* из различных стран. Сравнение нуклеотидных последовательностей с данными из NCBI GenBank, показало, что иранские *X. index* на 99-100% сходны меду собой и отличаются на 4-10 пар оснований от последовательностей от других популяций *X. index*. Филогенетический анализ полученных частичных последовательностей 28S rRNA гена с использованием метода присоединения ближайшего соседа позволяет выявить связи иранских *X. index* с другими популяциями вида.