MORPHOLOGICAL AND MOLECULAR CHARACTERISATION OF *XIPHINEMA INDEX* THORNE AND ALLEN, 1950 (NEMATODA: LONGIDORIDAE) ISOLATES FROM CHILE

Pablo Meza[1]*, Erwin Aballay[2] and Patricio Hinrichsen[3]

[1]Center for Advanced Horticultural Studies (CEAF), CONICYT-Regional, GORE O´Higgins R08I1001. Avenida Salamanca s/n Los Choapinos, Rengo, Chile; [2]Faculty of Agronomy, Universidad de Chile, Avenida Santa Rosa 11315, Santiago, Chile; [3]Biotechnology Laboratory, INIA La Platina, Avenida Santa Rosa 11610, Santiago, Chile. *Corresponding author, e-mail: pmeza@ceaf.cl

ABSTRACT

Meza, P., E. Aballay, and P. Hinrichsen. 2012. Morphological and molecular characterisation of *Xiphinema index* Thorne and Allen, 1950 (Nematoda: Longidoridae) isolates from Chile. Nematropica 42:41-47.

Xiphinema index is a major plant parasitic nematode for vineyards, both as a root pathogen and as a vector for Grape Fanleaf virus. In Chile it has a wide distribution, causing considerable damage to *Vitis vinifera*. The main morphological and morphometric features that have been used for its identification are related to the vulval position and the presence of a mucro on the tail. However, these features have a limited discrimination capacity, as identification is more complicated in a highly complex matrix such as the soil, where a mixture of species coexists. This situation has motivated the search for and the application of molecular techniques with increased resolution power. This investigation shows the results of morphological and molecular characterization of *X. index* isolates from Chile. The complete ITS regions (ITS1 and ITS2) were sequenced revealing very low intraspecific diversity, less than 1% or the most divergent available sequences. This coincided with the low level of differences detected at the morphometric level.

Key words: internal transcribed spacers, molecular, morphology, morphometrics, *Xiphinema index*.

RESUMEN

Meza, P., E. Aballay, and P. Hinrichsen. 2012. Caracterización morfológica y molecular de *Xiphinema index* Thorne and Allen, 1950 (Nematoda: Longidoridae) en Chile. Nematropica 42:41-47.

Xiphinema index es uno de los nemátodos fitoparásitos de mayor relevancia para el cultivo de la vid, tanto por el daño directo que provoca en las raíces cómo por su capacidad de transportar a Grape Fanleaf virus. En Chile presenta una amplia distribución, causando considerable daño sobre *Vitis vinifera*. Los principales rasgos morfológicos y morfométricos utilizados para su identificación están relacionados con la posición de la vulva, la presencia de un mucrón en la cola, entre otros. Sin embargo, estas características muestran una limitada capacidad discriminatoria frente a coexistencia de especies en el suelo, lo que ha motivado la búsqueda, desarrollo y aplicación de técnicas moleculares con un mayor poder resolutivo. Está investigación muestra los resultados de la caracterización morfológica, morfométrica y molecular de *X. index* en Chile. La región ITS (ITS1 e ITS2) fue clonada y secuenciada mostrando un baja diversidad intraespecífica, menor al 1%, en relación a las secuencias disponibles en GenBank. Una situación similar se observó a nivel morfométrico y morfológico.

Palabras clave: espaciadores transcritos internos, molecular, morfología, morfometría, *Xiphinema index.*

INTRODUCTION

Grapes are the most important fruit crop in Chile, with table and wine grape cultivars grown in several areas of the country. Growth and yield of this crop depends on healthy root systems and ability to extract and transport water and nutrients from the soil. However, many diseases and pests may damage or completely destroy the new roots initiated in spring and after fruit harvest in late summer. Among the phytopathological problems affecting vineyards, the presence of plant-parasitic nematodes is one of the main limiting factors for grape production (Valenzuela *et al.* 1992; San Martin and Magunacelaya, 2005), with *Xiphinema index* (Thorne and Allen 1950) being a major pathogen (Aballay *et al.*, 2009). The earliest records of *X. index* in Chile

were reported by González and Valenzuela (1968). The most recent survey showed its presence in over 85% of the vineyards between the administrative regions of Atacama and Maule (25ºS to 37ºS, approximately; González, 2007). Xiphinema index is a migratory ectoparasite and a natural vector for *Grapevine Fanleaf Virus (GFLV)*, which is widespread along the central valley (the main grape growing region) of Chile (Fiore *et al.*, 2008).

The identification of this species is mainly based on the morphological and morphometric characterization of adult females. However, variability of these features has been poorly studied. The use of traditional taxonomic studies is the only way to analyze the modifications of these features and to identify the emergence of morphogroups, a situation reported in closely related species (Taylor and Brown, 1997; Brown and Topham, 1985). In countries where *X. index* coexists with other *Xiphinema* species, discrimination between species by traditional methods has been limited. This situation has driven the use of molecular techniques with increased resolution power, mainly through the characterization of the internal transcribed spacer (ITS) of ribosomal genes (Kumari *et al.*, 2004; Leopold *et al.*, 2007; Demangeat *et al.*, 2005). Thus, specific primers have been designed to identify *Xiphinema index, X. italiae, X. vuittenezi* and *X. diversicaudatum* (Wang *et al.*, 2003).

The goal of this work was to complete a morphological and molecular characterization of a Chilean population of *X. index* isolated from local vineyards. Cloned and sequenced fragments could be used to develop a rapid method of identification through techniques such as RFLPs (Restriction Fragment Length Polymorphism), which would be useful to distinguish very similar species such as *X. vuittenezi* reported in Chile (Lamberti *et al.*, 1988), and the *X. americanum* group species *X. inaequale* and *X. peruvianum*, recently characterized in this country (Meza *et al.*, 2011).

MATERIALS AND METHODS

Females of *X. index* were collected in 2010 from the rhizosphere of grapevines located in the Metropolitan region of Chile, at 33ºS 70ºO. Nematodes were extracted using the Cobb's wet sieving technique (Southey, 1986). For morphological and morphometric analysis, nematodes were relaxed and fixed in TAF solution (2 ml of 37% formaldehyde and 5 ml of triethanolamine in 93 ml of distilled water). Specimens were photographed and measured using a light microscope connected to a digital camera. Measurements were based on morphology and morphometrics of 25 females and were compared with those of the original description (Thorne and Allen, 1950) and other descriptions of the species reported in the literature, i.e., Gutiérrez *et al.* (2011) in Spain, Barsi and Lamberti (2000) in Serbia; Jawhar *et al.* (2006) in Lebanon and Lamberti *et al.* (1987) in Peru.

Nematode DNA was extracted from single females as described by Meza *et al.* (2011). Two primers that amplify the complete ITS region (ITS1, 5.8S and ITS2), as well as short sections of the 18S and 28S ribosomal genes, were used for PCR amplification. These primers bind to the 3' portion of the 18S small ribosomal subunit (forward primer, named ITS1-S: 5'-TTGATTACGTCCCTGCCCTTT-3') and 5' end of the 28S subunit region (reverse primer, named P28S: 5'-TTTCACTCGCCGTTACTAAGG-3') (Vrain *et al.*, 1992). The PCR reaction was done in a solution of 20 µl containing 1.5 mM MgCl2, dNTPs (0.1 mM each), 1 µl of each primer (10 pM), 0.2 µl of Taq DNA polymerase, 2 µl of 10X buffer (composed of 500 mM KCl plus 100 mM Tris-HCl adjusted to pH 8.3), 6 µl of DNA solution and 8.4 µl of sterile distilled water. The amplification was carried out in a Eppendorff X-6 thermocycler under the following conditions: an initial denaturation of 94ºC for 2 min, followed by 40 cycles at 94ºC for 1 min, 58ºC for 1 min and 72ºC for 70 s, and a final incubation at 72ºC for 10 min. Amplified products were separated in 1% agarose gels in 0.5X TAE buffer (composed of 0.04 M Tris-base, 20 mM acetic acid and 1 mM EDTA) containing ethidium bromide and visualised under UV light.

The DNA bands were excised and purified using a QIAEXII kit (Qiagen, Germany). The purified PCR products were ligated to the pGEM-T easy vector (Promega, Madison, WI, USA) according to the protocol suggested by the supplier. The recombinant plasmids were introduced into *E. coli* JM-109 competent cells. The transformants were grown in LB medium supplemented with carbenicillin, IPTG and X-Gal. The positive white colonies were confirmed by PCR using the M13 (-20) forward and reverse universal primers and ITS1-S plus P28S primers. Plasmid DNA was purified from the bacterial culture using a standard miniprep protocol (Sambrook *et al.*, 1989). DNA was sent to an external sequencing service. Five nematodes were sequenced. The sequences were processed with the GENEIOUS software. The ITS rDNA region sequence of *X. index* was deposited in GenBank under accession number JF437918 and was aligned with other similar sequences also reported in GenBank. In these analyses a global alignment with free end gaps was used, with a cost matrix of 65% similarity, a gap open penalty of 12 and a gap extension penalty of 3.

RESULTS

Morphological and morphometric characterization

Table 1 shows the morphometric characterization of *X. index* from Chile, and its comparison with those from Spain (Gutiérrez *et al.*, 2011), Serbia (Barsi and Lamberti, 2000), Lebanon (Jawhar *et al.*, 2006), Peru (Lamberti *et al.*, 1987), and with morphometric values of the original description (Thorne and Allen, 1950), done on isolates from the United State of America.

In general, the morphometric characterization of *X.*

Table 1. Morphometric characterisation of adult females of *Xiphinema index* from Chile, Spain, Serbia, Lebanon, Peru and the original description. All measurements in µm (unless stated otherwise) and in the form: mean ± s.d. (range).

Characters	Chile	Spain[v]	Serbia[w]	Lebanon[x]	Peru[y]	Original[z] description
N	25	12	29	18	2	
L (mm)	3.0 ± 0.1 (2.6-3.4)	3.0 ± 0.21 (2.7-3.4)	3.05 ± 0.19 (2.71-3.49)	3.39 ± 13 (3.19-3.65)	(3.3-3.5)	3.4
a	57.6 ± 3.9 (50.3-65.2)	56.5 ± 6.9 (45.7-68.6)	61.4 ± 2.34 (57.0-67.9)	39 ± 5.3 (36-43)	(50-54)	58
b	6.9 ± 0.6 (4.9-7.8)	6.1 ± 0.8 (5.1-7.3)	6.4 ± 0.41 (5.8-7.5)	6.9 ± 4.2 (6.3-7.8)	(6.9-7.4)	7.6
c	91 ± 9.4 (78.2-113.5)	74.9 ±7.7 (64.5 – 89)	71.9 ± 5.97 (61.0-83.5)	94 ± 5.2 (86-99)	(77-83)	76
c`	0.9 ± 0.07 (0.77-1.0)	1.1 ± 0.12 (1.0-1.2)	1.13 ± 0.08 (0.96-1.25)	0.83 ± 0.1 (0.75-1.0)	(1-1.1)	-
V	39.4 ± 1.4 (36.2-42.9)	41.4 ± 1.6 (39-45)	41.6 ± 0.96 (39.8-43.9)	39 ± 2.2 (36-43)	38	38
Odontostyle	123.13 ± 3.4 (116.8-129.5)	131.3 ± 5.9 (122-142)	127.5 ± 4.38 (118.8-136.3)	131 ± 4.3 (125-139)	(135-136)	90
Odontophore	68.8 ± 4.0 (56.3-74.5)	72.4 ± 6.4 (60-83)	73.6 ± 2.11 (68.8-77.5)	74 ± 3.5 (68-78)	71	-
Oral aperture to guide ring	111.9 ± 4.5 (100.7-121)	113 ± 12.8 (92-127)	116.9 ± 5.19 (108.8 -128.8)	121 ± 8.8 (105-129)	(123-125)	-
Anterior end to end of pharynx	428.5 ± 37.5 (374.9-538.7)	-	-	-	-	-
Tail length (excluding terminal mucro)	32.9 ± 2.84 (27.1-39.4)	40.3 ± 2.9 (35-45)	42.5 ± 3.06 (35.7-48.6)	36 ± 2.3 (32-38)	(42-43)	-
Body diam. at lips region	11.5 ± 0.3 (11-12.3)	13.2 ± 0.7 (12-15)	13.7 ± 0.25 (13.1-14.2)	-		-
Body diam. at guide ring	35.2 ± 1.4 (32.1-38)	-	37.6 ± 1.17 (35.0-40.0)	-		-
Body diam. at pharynx base	45.6 ± 3.2 (40.9-54.6)	-	46.1 ± 2.18 (41.7-51.3)	-		-
Body diam. at vulva	51.3 ± 3.8 (44.2-58.9)	-	49.6 ± 2.39 (44.4-53.8)	-		-
Body diam. at anus	36.7 ± 2.3 (33-41.5)	-	37.7 ± 1.89 (32.9-41.3)	-		-
Body diam. at beginning of J	15.7 ± 2.5 (11-20.2)	-	19.4 ±1.97 (15.0-23.1)	-		-
Max. body diam.	51.7 ± 4.1 (44.2-59.2)	-	-	-		-
Tail terminal mucro length	9.9 ± 1.1 (7.7-12.7)	-	-	-		-

[v] Gutiérrez *et al.*, 2011.
[w] Barsi and Lamberti, 2000.
[x] Jawhar *et al.*, 2006.
[y] Lamberti *et al.*, 1987.
[z] Thorne and Allen, 1950.

index from Chile was similar to specimens from other origins. Their main morphological features, commonly used for species identification, are as follows: elongated and cylindrical body (2.6 - 3.4 mm long) tapering at both ends; habitus ventrally arcuate usually in an open C shape when relaxed by gentle heating; lips region continuous with the neck contour (Figure 1, A); spear typical of the genus with the usual extension and flanges; oesophagus Dorylaimoid (the nucleus of dorsal oesophageal gland is indicated by a black arrow in Figure 1, B); vulva location approximately at 40% of the body length, seen as a transverse slit in ventral view (Figure 1, C); reproductive system amphidelphic, with ovary reflexed; convex-conoid tail with a definite ventral mucro (Figure 1, D).

The main difference between Chilean and European populations (Serbian and Spanish) was the body diameter at the lip region. Also differences with both populations were observed in tail length, maybe due to differences in mucro measures. Additionally, Chilean,

Spanish, and Serbian populations have a total body length slightly shorter than the other three described, including the original description, this shorter length coinciding with one found in Italy (Catalano, 1991) and is within the range described for the species (2.6-3.3 mm) by Dalmasso (1969). All the populations, including the Chilean, have an odontostyle longer than the original description.

Molecular characterization of X. Index

The ITS consensus sequence obtained for the Chilean isolate of *X. index* was reported to GenBank with the accession number JF437918. Intra-specific differences were not found in our population. This sequence had 2,008 bp if 18S and 28S terminal portions are considered or 1,708 bp if just ITS1 and ITS2 plus the 5.8S gene are counted. Their breakdown in coding and non-coding regions is presented in Table 2.

The consensus sequence obtained in this work was aligned with other *X. index* ITS sequences deposited in GenBank. In every case the percentage of divergence between sequences was lower than 1%. Eleven points of divergence (transitions, transversions and indels) were detected in ITS-1, whilst six were identified in ITS-2, listed in Table 3. Most likely, this intraspecific variability corresponds to microheterogeneity of ITS, which has been reported in other nematodes of agricultural interest (Szalanski *et al.*, 1997; Subbotin *et al.*, 2003; Chen *et al.*, 2005; Meza *et al.*, 2011).

DISCUSSION

Although *X. index* is a major parasitic nematode for viticulture around the world, few morphometric characterizations have been reported. The comparative analysis between the characterized populations, including our research, shows a low intraspecific morphological and morphometric variability. Principal features that identify this species remained practically unchanged. According to Taylor and Brown (1997), morphometric differences between populations or specimens have been reported for many species of Longidoridae and Trichodoridae, and such differences

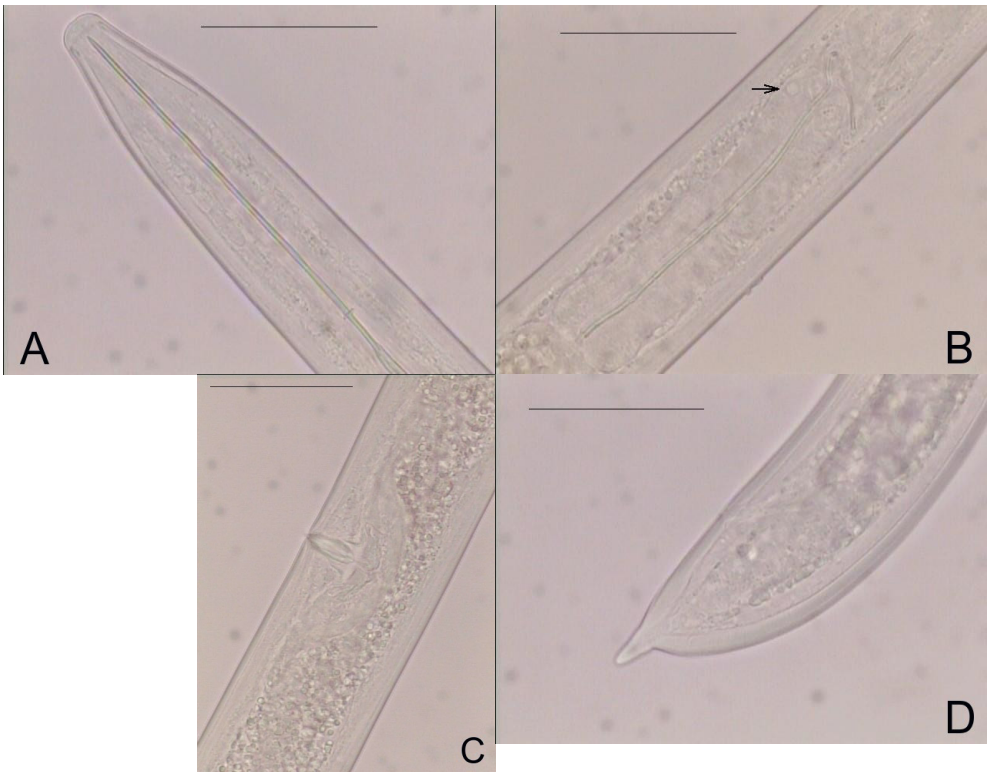

Figure 1. *X. index* from Chile. A) Female anterior region. B) Posterior oesophagus base. C) Vulval region. D) Tail showing a typical ventral mucro. (Scale bar = 50 µm).

should be considered when identifying specimens. A study performed with *X. diversicaudatum* found significant morphometric differences between populations, related to biotopic changes, e.g., change in geographical location, plant host, soil type, climate, etc. (Brown and Topham, 1985). Traditional taxonomic studies are necessary to establish intraspecific morphological variability in *X. index*, and are the only way to identify features that can be used to differentiate it from closely related taxa or to establish morphogroups.

On the other hand, almost 100% homology was obtained in the alignment of the ITS sequences of the Chilean isolates with those reported in GenBank. This confirms that ITS fragment study is a powerful tool for diagnosis and identification of the *Xiphinema* genus species. This homogeneity allowed Wang *et al.* (2003) to design specific primers for the discernment of very close species, such as *X. index, X. diversicaudatum, X. vuittenezi* and *X. italiae*. The specific primer pair designed by Wang *et al.* (2003) to identify *X. index* was named I27 and binds to ITS1, allowing the amplification of a specific 340 bp fragment. We observed that primer binding sites are not affected by the nucleotide divergence presented in Table 3.

Our work combines a morphological, morphometric and molecular characterization of *X. index*. Correspondence of these characterizations with previous ones reported in the literature and GenBank, suggest that it is feasible to identify this species through both methodologies. Recent records of *X. index* in the world have shown various results; for instance, Demengeat *et al.* (2005) identified *X. index* in France using both classical and molecular methodologies, but these authors did not characterize the population because it was not the goal of their work. Nevertheless, the study undertaken by Demengeat *et al.* (2005) demonstrated

Table 2. Size of ribosomal genes (or gene fragments), and intervening ITS-1 and ITS-2 regions, and GC content from Chilean isolates of *Xiphinema index*.

Fragment/gene	Size (pb)	% GC
18S[z]	193	47.7
ITS1	926	45.1
5.8S	160	56.9
ITS2	622	39.7
28S[z]	107	39.3

[z] Partial sequences of ribosomal genes that begin in the annealing site of the specific primers.

Table 3. Intraspecific variation sites in the *X. index* ITS-1 and ITS-2, deduced from sequences reported in GenBank. The position corresponds to the number of bases considering the first nucleotide of 18S gene sequence AY430175 reported to GenBank as number 1. (-), identical nucleotide sequence; (*), nucleotide deletion.

ITS-1 intraspecific variation sites											
Position	337	398	545	669	761	805	866	876	996	1012	1050
AY430175[v]	T	T	C	T	A	A	T	A	C	G	A
JF437918 (this work)	-	-	-	*	-	-	A	-	T	A	C
AY584243[x]	-	-	-	*	A	-	-	-	T	A	-
AJ437026[y]	-	-	-	*	-	-	-	-	T	-	-
HM921333[w]	*	*	A	*	-	T	-	G	T	-	-

ITS-2 intraspecific variation sites						
Position	1438	1591	1619	1670	1746	1789
AY430175[v]	C	C	T	T	C	G
JF437918 (this work)	-	T	A	*	T	A
AY584243[x]	T	T	-	*	-	A
AJ437026[yz]						
HM921333[wz]						

[v] From He, Y.; Lamberti, F.; Brown, D. and Moens, M. (2004). Diversity of internal transcribed spacer in *Xiphinema* (Nematoda: Longidoridae). Unpubl.
[x] From Finetti-Sialer, M. and Ciancio, A. (2005).
[y] From Wang, X.; Bosselut, N.; Castagnone, C.; Voisin, R.; Abad, P. and Esmenjaud, D. (2002). PCR multiplex identification of single individuals of the nematode *Xiphinema index, X. diversicaudatum* and *X. italiae* using specific primers from ribosomal. Unpubl.
[w] From Gutiérrez et al. (2011).
[z] ITS-2 was no reported for this sequence accession.

the usefulness of using both methodologies, especially in a soil sample that most probably contains a mixture of nematode species. By contrast, Jawhar *et al.* (2006) characterized *X. index* in Lebanon only through classical taxonomic methods. Leopold *et al.* (2007) identified *X. index* for the first time in Austria using molecular techniques, but lacking the morphological identification of the species.

The results of our investigation show that the identification and characterization of *X. index* by molecular tools is equally or more powerful than traditional morphological methods. Considering the potential of molecular technologies, it is likely that they will be applied to most plant-parasitic nematodes. However, it is important to encourage the application of classical techniques since these have laid the groundwork for the development of nematology. It should also be considered that the taxonomic status of major plant parasitic nematode genera are still ambiguous and molecular techniques are not yet routinely used in developing countries. This means that only with the combination of molecular and classical techniques can a comprehensive diagnosis of plant parasitic nematodes be achieved, and for this reason both methods should work in synergy.

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