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# MOLECULAR AND MORPHOMETRIC IDENTIFICATION OF PLANT PARASITIC NEMATODES

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De Luca F., Agostinelli A. – Molecular and morphometric identification of plant parasitic nematodes.

During a nematode survey in vineyards of the Veneto region (Italy), several soil samples were collected. On morphological and morphometric studies, a population from Conegliano Veneto was preliminarily identified as *X. rivesi* Dalmasso 1969. To support the morphological diagnosis, a molecular characterization of *X. rivesi* from Italy using the D2-D3 expansion segments of the 28S rDNA and the ITS containing region was undertaken. PCR-RFLP analyses were also performed to determine species-specific patterns for the Italian population of the nematode. Neighbor-Joining, Minimum Evolution and Maximum Parsimony analyses were used to reconstruct phylogenetic relationships within *X. rivesi* and other *Xiphinema* species.

KEY WORDS: D2-D3 expansion domains, Internal Transcribed Spacers, phylogeny, *Xiphinema americanum*-group.

## INTRODUCTION

The species of the genus *Xiphinema* Cobb, 1913 are migratory ectoparasitic nematodes that cause direct damage to a wide range of cultivated plants by feeding on root cells. Also, many species of *Xiphinema* are putative vectors of nepoviruses of fruit and vegetable crops (TAYLOR and BROWN, 1997; BROWN *et al*., 2003).

*Xiphinema* species tend to be greatly conserved in gross morphology and this makes species identification a very difficult task. The accurate identification of *Xiphinema* species infecting vineyards is a prerequisite for designing effective management strategies and quarantine measures. The availability of molecular methods provides additional tools to differentiate species of nematodes and thus making their identification easier. Sequences of the D2-D3 expansion segments of the 28S rDNA and the ITS containing region have been used for molecular identification and phylogenetic reconstruction of many *Xiphinema* species (YE *et al*., 2004; HE *et al*., 2005; OLIVEIRA *et al*., 2005; GUTIERREZ-GUTIERREZ *et al*., 2010; KUMARI *et al*., 2010). The objectives of this study were: 1) to characterize morphometrically and morphologically the Italian population resembling *X. rivesi*; 2) to molecularly characterize this population by using the D2-D3 expansion segments of the 28S rDNA and the ITS1-5.8S-ITS2 region; 3) to determine species-specific patterns by PCR-RFLP for this population; and 4) to reconstruct the phylogenetic relationship of our population with the closest species of *Xiphinema*.

## MATERIAL AND METHODS

### NEMATODE POPULATION AND MORPHOLOGICAL IDENTIFICATION

Soil samples were collected from the rhizosphere of *Vitis vinifera* at Conegliano Veneto (Veneto region, Italy). Nematodes were extracted from the soil by the wet sieving method described by FLEGG (1967). Ten nematodes for biometric studies were fixed in hot 5% formalin, mounted in anhydrous glycerine and measured with the aid of a camera lucida.

### DNA EXTRACTION, PCR AMPLIFICATION

Specimens for molecular analysis were kept in NaCl 1 M. Genomic DNA was extracted from fifteen individual nematodes as described by DE LUCA *et al.* (2004). The crude DNA isolated from each individual nematode was directly amplified. The ITS1-5.8S-ITS2 regions were amplified using the forward primer 18S-Ext (5'-TGATTACGTCCCTGCCTTT-3') and the reverse primer 26S-Ext (5'-TTTCACTCGCCGTTACTAAGG-3') (VRAIN *et al*., 1992) and the D2-D3 expansion segments of 28S rDNA was amplified using the D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') primers (CASTILLO *et al*., 2003). PCR cycling conditions used for amplification were: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 50s, annealing at 55°C for 50s and extension at 72°C for 1 min and a final step at 72°C for 7 min (DE LUCA *et al*., 2004). The size of the amplification products was determined by comparison with the molecular weight marker ladder 100 (Fermentas, St. Leon-Rot, Germany) following electrophoresis of 10 µl on a 1% agarose gel.

### RFLP ANALYSIS

Ten µl of each PCR product from six individual nematodes was digested with five units of the following restriction enzymes: *Alu*I, *Ava* II, *Dde*I, *Nde* II, *Pst* I and *Rsa* I (Roche Diagnostics, Manheim, Germany) for the D2-D3; *Alu*I, *Bam* HI, *Dde*I, *Hin*fI, *Rsa*I and *Xba* I for the ITS region. Digested products were separated onto a 2.5% agarose gel by electrophoresis, stained with ethidium bromide, visualised on a UV transilluminator and photographed with a digital system.

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CLONING, SEQUENCING AND PHYLOGENETIC ANALYSIS

PCR products of the ITS region from two individual nematodes were purified for cloning and sequencing using the protocol listed by manufacturer (High Pure PCR elution kit, Roche, Germany). Purified ITS fragments were cloned in TA cloning vector (Invitrogen) and four clones were sequenced using an ABI Prism 377 sequencer (PE Applied Biosystem, Foster City, CA). The D2-D3 regions of rDNA from two individual nematodes of the Italian population were purified and used for direct sequencing.

A BLAST (Basic Local Alignment Search Tool) analysis was performed at NCBI (National Center for Biotechnology Information) using the here obtained sequences as queries to confirm their nematode origins and to identify the most closely related *Xiphinema* species which were then used in the phylogenetic analyses. Initial alignments were performed using ClustalW (THOMPSON *et al.*, 1994). Sequence alignment was manually edited using BioEdit (HALL, 1999) in order to improve the default parameters of the multialignment. The corresponding sequences of the plant parasitic nematode *Longidorus elongatus* were used as outgroup.

Phylogenetic trees, obtained for both ITS and D2-D3 datasets, were performed with Neighbour-Joining (NJ), Minimum Evolution (ME) and Maximum Parsimony (MP) methods using the MEGA version 4 software (TAMURA *et al.*, 2007). The phylograms were bootstrapped 1,000 times to assess the degree of support for the phylogenetic branching indicated by the optimal tree for each method.

## RESULTS

Ten adult females of the Italian *Xiphinema* population were measured. Morphometric data of the Italian population of *Xiphinema* from grapevine fitted with those of *X. rivesi* Dalmasso, 1969 from France, Iran, Portugal, Spain and Slovenia (Table 1) and therefore the Italian population was considered as *X. rivesi*, a nematode belonging to the *X. americanum*-group, whose non European populations are listed as a quarantine pest for Europe.

The amplification of D2-D3 expansion segments of the 28S rDNA and the ITS containing region produced a single fragments of approximately 800 bp and 1420 bp,

*Table 1* – Morphometric data of of *Xiphinema rivesi* populations from Italy, Iran, Portugal, Slovenia, France and Spain

| Locality Host | Conegliano Veneto (Loc. Cecchin) Grapevine | Ramsar, Mazandaran, Iran Citrus tree | Quinta da Alorna, Almeirim Grapevine | Bilje, Slovenia Peach-Prunus persica L. | Bordeaux, France Grapevine | Cordoba, Spain Grapevine |
|---|---|---|---|---|---|---|
| n | 10 | 13 | 5 | 25 | 10 | 8 |
| L (mm) | 2.0±0.12 | 1.7±0.08 | 2.0 | 1.86 | 1.96 | 1.7±0.09 |
| | 1.9-2.2 | 1.5-1.8 | 1.9-2.0 | 1.51-2.18 | 1.68-2.11 | 1.6-1.8 |
| a | 55±2.39 | 44.7±2.9 | 53 | 47.97 | 41.8 | 44.1±3.2 |
| | 50-58 | 38.5-50 | 50-55 | 37.61-55.68 | 37-49 | 37.7-48.1 |
| b | 6.4±0.61 | 6.4±0.5 | 7.0 | 5.93 | 6.28 | 6.8±0.5 |
| | 5.5-7.5 | 5.5-7 | 6.5-7.0 | 4.74-7.28 | 5.7-6.9 | 6.2-7.6 |
| c | 63.3±4.99 | 55.2±3 | 61 | 55.24 | 55.5 | 52.8±3.2 |
| | 56.9-69.8 | 49.5-60 | 59-63 | 44.76-69.32 | 51-59 | 47.6-57.5 |
| c’ | 1.4± 0.09 | 1.3±0.07 | 1.5 | 1.60 | 1.38 | 1.4±0.1 |
| | 1.2-1.5 | 1.2-1.4 | 1.4-1.6 | 1.40-1.76 | 1.3-1.5 | 1.2-1.5 |
| V | 52±0.85 | 52.2±0.7 | 52 | 53.5 | 55.5 | 53.3±0.7 |
| | 50-53 | 51-53.5 | 50-55 | 51.0-56.0 | 51-59 | 52-54 |
| Odontostyle µm | 93.6±1.60 | 81.5±2.8 | 91 | 90.9 | 96 | 89.7±7.7 |
| | 90.8-94.8 | 73.5-86 | 88-95 | 84.1-96.8 | 90-101 | 72-96 |
| Odontophore µm | 53.3±1.80 | 46.2±1.8 | 50 | 50.2 | 51 | 49.1±2.8 |
| | 50.6-55.7 | 42-48.5 | 47-53 | 42.0-53.5 | 48-57 | 45-53 |
| Oral aperture to guide ring µm | 75.4±1.14 | 66.2±2.0 | 73 | 80.0 | 76 | 71.9±2.8 |
| | 73.6-77.6 | 63-70 | 72-73.5 | 69.5-86.6 | 7 1-79 | 67-76 |
| Tail µm | 32.2±1.25 | 30.8±1.9 | 33 | 33.8 | 35 | 32±3.0 |
| | 30.5-35.0 | 28-33.5 | 31-34 | 29.0-39.6 | 30-40 | 28-36 |
| J (hyalin portion of tail) µm | 7.4±0.83 | 9.4±0.8 | 9 | 9.0 | – | – |
| | 6.3-8.6 | 8-1 1 | 8-10 | 7.2-11.1 | | |
| Body diam. at lip region µm | 10.3 ± 0.00 | 11.1±0.4 | 9 | 10.3 | 10 | – |
| | 10-10 | 10.5-12 | 9-9 | 9.8-11.2 | 10-1 1 | |
| Body diam. at guide ring µm | 26.2±0.62 | 25.6±0.9 | 26.5 | – | – | – |
| | 25.3-27.0 | 25-27 | 26-26.5 | | | |
| Body diam. at base of oesophagus µm | 33.2±1.11 | 33.151.5 | 34 | – | – | – |
| | 32.2-35.6 | 31-36.5 | 32-35 | | | |
| Body diam. at mid-body or vulva µm | 36.9±1.58 | 38.2±3.3 | 37 | 39.0 | 48 | – |
| | 34.5-38.5 | 33.5-46.5 | 36-40 | 32.1-46.8 | 40-54 | |
| Body diam. at anus µm | 23.6±0.81 | 23.2±1.1 | 22.5 | 21.2 | 26 | – |
| | 22.4-25.3 | 21.5-25 | 21-24 | 18.2-24.5 | 23-29 | |
| Body diam. at beginning of J µm | 10.2±0.47 | – | 10.9±0.8 | 12 | 11.0 | – |
| | 9.2-10.9 | – | 10-12.5 | 11-14 | 10.0-13.2 | |

respectively. PCR-RFLP analyses of both ribosomal regions allowed us to determine for the first time species-specific patterns for the Italian population of *X. rivesi* (figs. I and II) that clearly identified this species. Two clones for each individual were sequenced. There was little or no sequence variation between clones from the same or different individuals in either the ITS or D2-D3 regions that could be explained in terms of natural variation within population. BLAST at NCBI using D2-D3 and ITS sequences as queries revealed a close relationship with *X. americanum*-group species. In particular the D2-D3 region showed a 99% similarity with the corresponding region of *X. rivesi* from USA (AY210845) and Spain (HM921358) and 96-97% with other species of the *X. americanum*-group. The ITS containing region showed a 99% similarity with *X. rivesi* (HM921338) from Spain, a 98% similarity with *X. inaequale* (GQ231530) and *X. thornei* (AY430176) and only 94% similarity with *X. rivesi* (AY430186) from USA. These data reveal that the Italian population of *X. rivesi* is closely related to the Spanish population of this species. Inter-population variability was observed at the level of (TA) and (GAAT) microsatellites present in the ITS1 region.

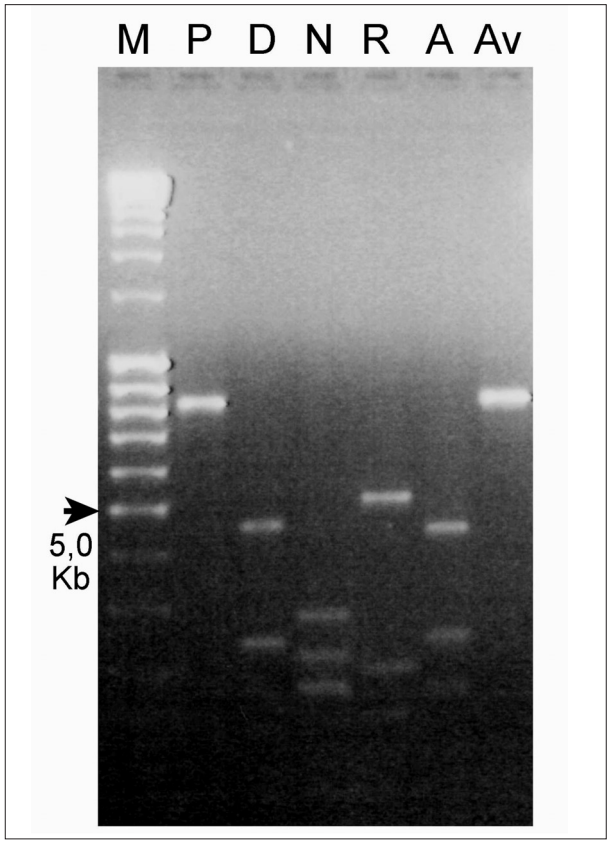


Fig. I – Restriction fragments of amplified D2-D3 expansion domains of *Xiphinema rivesi*. A: *Alu* I, Av: *Ava* II, D: *Dde* I, N: *Nde* II, P: *Pst* I, R: *Rsa* II and M: 100 bp ladder.

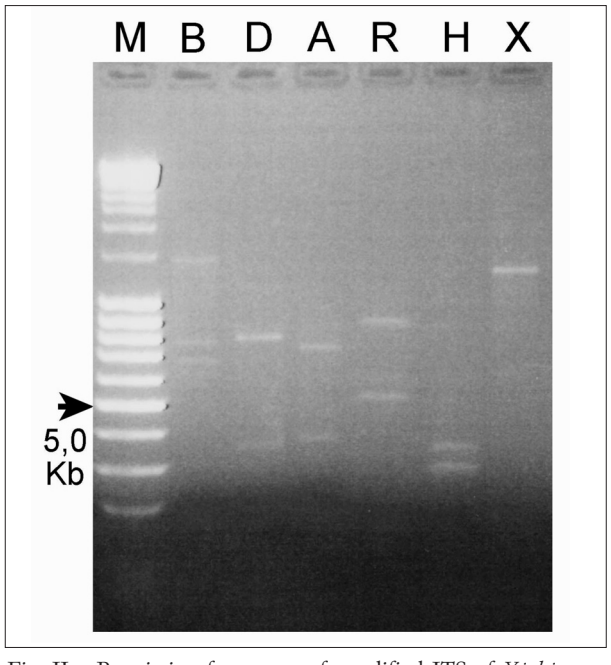


Fig. II – Restriction fragments of amplified ITS of *Xiphinema rivesi*. A: *Alu* I, B: *Bam* HI, D: *Dde* I, H: *Hinf* I, R: *Rsa* I, X: *Xba* I and M: 100 bp ladder.

The closest *Xiphinema* sequences to D2-D3 and ITS sequences of the Italian *X. rivesi* were aligned and included in the phylogenetic analyses. The alignment of the D2-D3 expansion domains included 13 *Xiphinema* sequences, whereas the ITS alignment included 14 *Xiphinema* sequences and the corresponding accession numbers are reported in Table 2. Both alignments resulted highly conserved among the species considered. The phylogenetic analysis among the *Xiphinema* species were carried out on D2-D3 and ITS alignments by means of Minimum Evolution (ME), Neighbor-Joining (NJ) and Maximum Parsimony (MP) methods using *Longidorus elongatus* as outgroup. ME/NJ tree resulted highly polytomic with nodes that give rise to more than two descendent taxa and three clusters are supported (fig. III). Cluster I grouped all populations of *X. californicum* and *X. floridae*. Cluster II grouped all populations of *X. georgianum*, *X. rivesi* from Spain, Italy and USA and *X. americanum*. Cluster III contained only one taxon, *X. brevicollum* located at the basal position of the tree. MP tree resolved the same major clades obtained by using ME/NJ analysis, only the grouping of *X. californicum* populations is not supported.

Topologies of ME, NJ and MP trees of the ITS sequences were identical and supported eight clades (fig. IV). Clade I grouped *X. americanum* populations. Clade II included *X. laevistriatum* and *X. georgianum*. Clade III grouped *X. californicum* and *X. georgianum*. Clade IV contained only one taxon, *X. californicum*. Clade V contained only *X. rivesi* from Italy that resulted sister species of clade VI containing *X. inaequale*, *X. thornei* and *X. rivesi* from Spain. Clade VII contained *X. tarjanense*. Clade VIII grouped the different populations of *X. rivesi* from USA.

## DISCUSSION

Accurate identification of species of the *X. americanum*-group is economically important due to their specificity with regard to plant hosts and virus transmission capability. Classical-morphometrical species identification of this group is hindered because of similarities among species and intra-specific morphometrical variability. In order to investigate morphological variation as component of species diversity in the *X. americanum*-group, different molecular approaches (PCR-RFLP, D2-D3 and ITS ribosomal DNA sequencing, phylogeny) have been used for nematode identification (Vrain *et al.*, 1992, He *et al.*,

*Table 2 – Xiphinema* sequences used in this study.

| Nematode species | Ribosomal region | Genbank accession number for ITS and D2-D3 | Collection codes for DNA sequences |
|---|---|---|---|
| *X. inaequale* | ITS | GQ231530 | Xin |
| *X. thornei* | ITS | AY430176 | Xt |
| *X. americanum UK* | ITS | AY430189 | Xa-UK |
| *X. americanum USA* | ITS | AY430188 | Xa-USA |
| *X. californicum* | ITS | GQ231532 | Xc |
| *X. georgianum G2* | ITS | DQ299521 | Xg-G2 |
| *X. georgianum G3* | ITS | DQ299522 | Xg-G3 |
| *X. laevistriatum L2* | ITS | DQ299529 | Xl-L2 |
| *X. rivesi USA1* | ITS | AY430186 | Xr-USA1 |
| *X. rivesi USA2* | ITS | AY430185 | Xr-USA2 |
| *X. rivesi Spain* | ITS | HM921338 | Xr-nem213 |
| *X. tarjanense T1* | ITS | DQ299536 | Xt-T1 |
| *X. citricolum C5* | ITS | DQ299519 | Xc-C5 |
| *X. rivesi IT* | ITS | FR878063-FR878066 | Xr-IT |
| *X. rivesi USA* | D2-D3 | AY210845 | Xr-USA |
| *X. rivesi Spain* | D2-D3 | HM921357 | Xr-nem226 |
| *X. rivesi IT* | D2-D3 | FR878067-FR878068 | Xr19-IT |
| *X. americanum* | D2-D3 | AY580056 | Xa |
| *X. brevicollum* | D2-D3 | AY580057 | Xb |
| *X. floridae F1* | D2-D3 | DQ299507 | Xf-F1 |
| *X. floridae F3* | D2-D3 | DQ299509 | Xf-F3 |
| *X. georgianum G3* | D2-D3 | DQ299497 | Xg-G3 |
| *X. georgianum G7* | D2-D3 | DQ299501 | Xg-G7 |
| *X. georgianum G8* | D2-D3 | DQ299502 | Xg-G8 |
| *X. citricolum C2* | D2-D3 | DQ299494 | Xc-C2 |
| *X. citricolum C4* | D2-D3 | DQ299491 | Xc-C4 |
| *X. citricolum C5* | D2-D3 | DQ299493 | Xc-C5 |

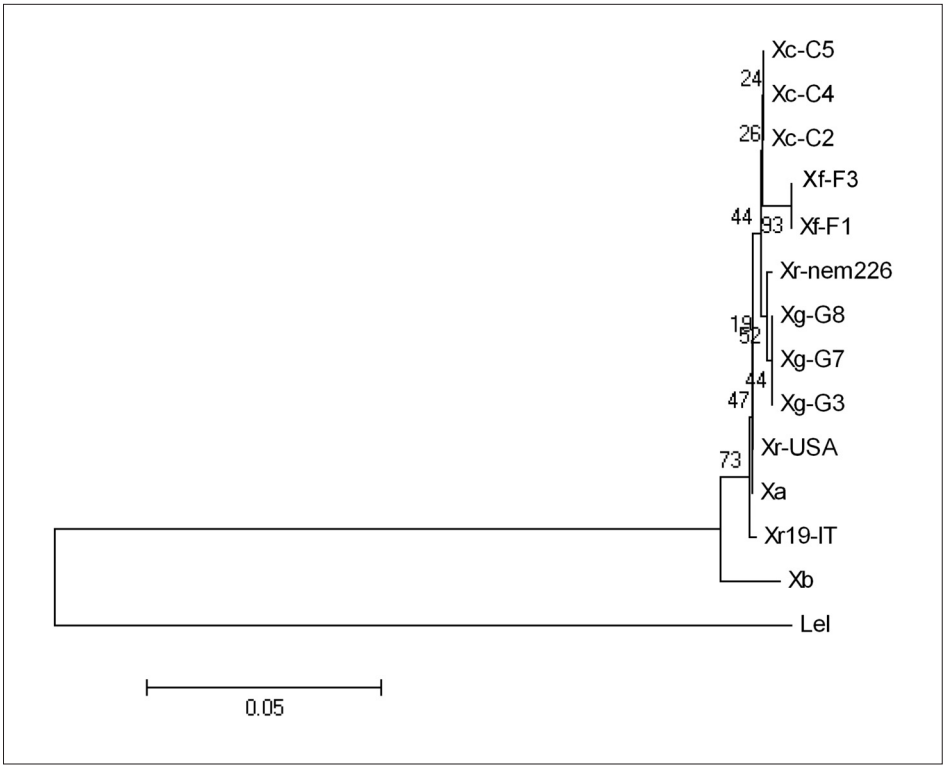


Fig. III – Phylogenetic tree of rDNA D2-D3 sequences of *Xiphinema americanum*-group species. Sequences were analysed using Neighbour Joining, Minimum Evolution and Maximum Parsimony methods. Numbers at nodes indicate bootstrap values.

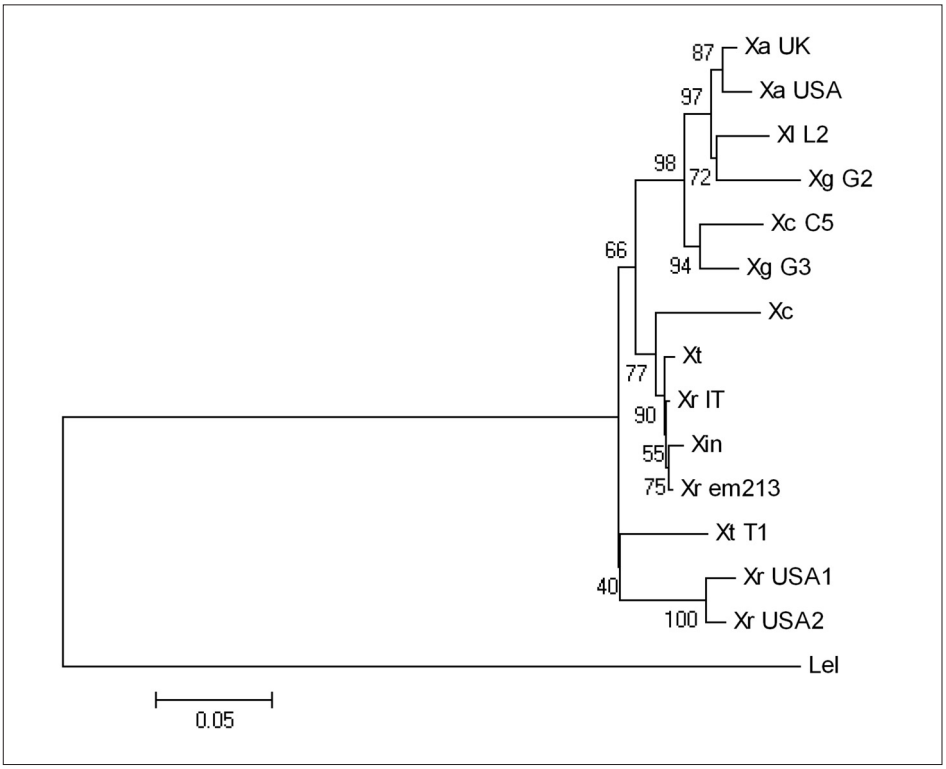


Fig. IV – Phylogenetic tree of the ITS sequences of *Xiphinema americanum*-group species. Sequences were analysed using Neighbour Joining, Minimum Evolution and Maximum Parsimony methods. Numbers at nodes indicate bootstrap values.

2005, GOZEL *et al.*, 2006). In our study, the Italian population of *X. rivesi* was characterized by using all four molecular approaches. PCR-RFLP analysis of D2-D3 and ITS ribosomal regions enabled us to determine, for the first time, specie-specific patterns that clearly identify this species (fig. I and II).

The D2-D3 sequences revealed very little interspecific variation among *X. americanum* group. The ITS sequences of *X. rivesi* from Italy showed high similarity with *X. rivesi* from Spain and lower similarity with that from USA suggesting that *X. rivesi* from Europe and USA have evolved independently or *X. rivesi* from USA was not correctly identified.

Phylogeny of D2-D3 region of 28S rRNA confirmed that *X. rivesi* from Italy is closely related to *X. rivesi* from Spain and USA, to *X. americanum, X. georgianum*, X. *floridae* and *X. californicum* as reported in other studies (HE *et al.*, 2005; CANTALAPIEDRA-NAVARRETE *et al.*, 2010; GUTIERREZ-GUTIERREZ *et al.*, 2011)

Phylogeny of the ITS sequences with ME, NJ and MP produced also identical trees. The clade grouping *X. rivesi* from Italy and Spain, *X. thornei* and *X. inaequale* revealed that these species were closely related. *X. rivesi* populations from USA, instead, formed a distinct and well supported clade confirming that the USA *X. rivesi* is a different species from *X. rivesi* from Europe.

In conclusion our study revealed that the ITS region is a good molecular marker, compared to the D2-D3, to differentiate species within the *X. americanum*-group. The ITS dataset allowed clear separation of European *X. rivesi* from *X. rivesi* from USA because of the high nucleotide variability (6% dissimilarity). Morphometrical data of Italian and Spanish *X. rivesi* are in agreement with those of *X. rivesi* from USA (GUTIERREZ-GUTIRREZ *et al.*, 2011) suggesting that *X. rivesi* could represent a cryptic species, morphologically similar but genetically different. Specialists are encouraged to study more *X. rivesi* populations, morphometrically and genetically, in order to improve our knowledge about this cryptic or complex species.

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