Review Article
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# Morphologic and molecular identification of *Xiphinema americanum* associated with pine trees

## Abstract

*Xiphinema* spp. member *Xiphinema americanum*, isolated from soils around roots of pine tree (*Pinus* spp.) were identified by morphological and molecular methods. Morphologic identification of species was carried out under microscope by observing featuring characteristics of heat killed and TAF fixed females. On the other hand a polymerase chain reaction protocol has been developed for the specific molecular identification of *Xiphinema americanum.* A primer sets amlifying ITS1 region were designed (*X.*americanum F- *X.* americanumR) and used in diagnostic tests. After running %1 agarose gel, DNA from a single *Xiphinema americanum* female yielded amplification products with expected lenght of 183 bp. *Xiphinema americanum* was succesfully identified with species-specific primers developed from ITS-1 region of rDNA.

**Keywords:** *Xiphinema americanum*, morphology, molecular identification, PCR

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## Introduction

The *Xiphinema americanum*-group is a large group containing 55 nematode species occurring around roots of several plants and trees.[1] The genus consists of migratory ectoparasitic nematodes which cause root galls in young roots by feeding which result in hypertrophy in cell and necrosis.[2] Susceptible roots show symptoms of stunting, swellings and dark lesions. Nematode feeding damage causes water and nutrient stress leading to reduction of plant vigor and yield. The major economic impact of *X.americanum* group is that 14 species vectors several nepoviruses: *Xiphinema californicum* and *Xiphinema rivesi* transmit *Cherry rasp leaf virus* (CRLV), *Tobacco ringspot virus* (TRSV) and *Tomato ringspot virus* (ToRSV); *Xiphinema bricolense* vectors *Tomato ringspot virus* (ToRSV), *Peach rosette mosaic virus* (PRMV); *Xiphinema tarjanense* and *Xiphinema intermedium* vector *Tobacco ringspot virus* (TRSV) and *Tomato ringspot virus* (ToRSV) and *Xiphinema inaequale* vector *Tomato ringspot virus* (ToRSV).[3,4] *X.americanum* members can acquire virus particles within 1h of feeding on roots of infected plants and transfer within 1h to healthy plants.[5] Thus essential issue for prevention of virus infection of healthy plantlets is planting in soils free of vector nematodes. Furthermore according to agricultural regulations of several countries, plant propagation materials must be grown in soils free from virus-transmitting nematodes. This restriction is aimed for the prevention of nematode borne virus infections. On this purpose nematode analysis is recommended to clearly distinguish between vector and non-vector *Xiphinema* species in newly established planting areas. Identification of *Xiphinema* species are generally made based on morphological and morphometric characteristics. But due to presence of several species with similar characteristics, mis-identification is always possible with *Xiphinema americanum* group members.[6] Furthermore if only juveniles of more than one species occur in the same soil sample reliable identification is difficult. Techniques based on DNA detection provide an attractive solution to problems associated with identification. Ribosomal DNA, basically from internal transcribed spacer (ITS) regions mitochondrial DNA, esophageal gland proteins and cytochrome c oxidase subunit I (COI) have been used for identification of several nematode species.[7] Many researchers designed species-specific primers based on sequence divergence of DNA region in many populations of the same species and in closely related species. Clear identification of cyst nematodes, lesion nematodes, stunt nematodes, dagger nematodes and root knot nematodes was achieved with this method.[8–11] This method allows the correct determination of all kind of individuals even at juvenile stage.[12]

The American dagger nematode, *Xiphinema americanum* Cobb, is one of the most commonly encountered nematodes occurring mostly around many plants.[1] *X. amerianum* was reported to infect cherry, walnut, almond, apricot, peach, alfa alfa, grapevine, maize, pear, strawbrry and several crop plants. *X.americanum* is an important virus vector nematode that has the ability to vector more than one strains of viruses.[13] Transmission of *Tobacco Ringspot Virus* (TRSV), *Tomato Ringspot Virus* (ToRSV), *Peach Yellow Bud Mosaic Virus* (PYBMV) and *Prunus necrotic ringspot virus* (PNRSV) and grape vein clearing viruses was reported in several studies.[5,14,15] *Xiphinema americanum* was identified for the first time in Turkey by Bora[16] in Northern Turkey. Becides in our recent studies nematode was extracted from soils around roots of pine tree (*Pinus* spp.) and rose (*Rosa* spp.) and ToRSV virus was detected in pear, cherry and walnut trees. Because of occurance of viruses and nematode in planting areas, a morphologic and molecular identification of common dagger nematode *Xiphinema americanum* study was carried out. For molecular studies a PCR based method with specific primer was applied.[17]

## Materials and methods

### Nematodes isolation

In this study individuals of *X.americanum* were isolated from soil samples taken from 0-60 depth around roots of pine tree (Pinus spp).[18] Sugar centrifuge flotation method was performed to extract nematode individuals from 200 g sub samples. In this method Approximately 200 gr soil and water suspension was washed into two sieves in order of 200 and 400 mesh, nematode suspension in the last sieve were collected and was centrifuged for 5 minutes at 1750 rpm and 1 minute at 500 rpm after addition of 50% sugar solution. The isolated nematode individuals were heat killed at 60ºC for one minute, fixed in double strenghten TAF solution and mounted on slides by wax-

ring method.[19] The identification of species were carried out based on morphology and morphometrics of female individuals.[20]

### Molecular confirmation of species

A total nematode DNA extraction was performed by hand-picking single individuals under microscope and place into micro centrifuge tubes containing 2.5µl tissue preparation and 10µl extraction solution. Tubes were then incubating at 55°C for 10 minute followed by 95°C for 3 minutes. After incubation for 13 minutes, 10µl neutralization solutions was added and extracted nematode DNA was stored at +4°C until use. DNA amplification of *Xiphinema americanum* was carried out by using designed specific primer pair *X.* americanum F (GTCGCCACTGTGAATGAATG) and *X.*americanum R (GATCCCGAACGTCTGAGATAA). Species specific primers were generated by comparison of different sequences of specimens at BIOEDIT Sequence Aligment Editor Software and primers were designed with PRIMER 3 software. The specificity of these primers was checked on NCBI Blastn Nucleotide Sequence Database.[21–24] PCR reaction of both species were carried out with the following cycles: 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 1 cycle at 72°C for 5 min. Amplification products were separated on a 1.5% agarose gel in 1 x TAE buffer.

## Results

### Xiphinema americanum

**Description:** (Figure 1) Female body forming an open spiral when killed. Labial region rounded, 5µm high, set off from rest of body by slight depression. Amphidial pouches stirrup-shaped with slitlike aperture. Oesophagus dorylaimoid with posterior part enlarged and occupying 1/3 to 1/4 of its total length. Esophagus-intestinal valve large, heart-shaped. Vulva, a transverse slit with slightly prominent lips, located approximately equatorially; vagina occupying 1/3 of corresponding body diameter. Gonads opposed, reflexed, with short uteri, more or less one body-width long. Tail conoid, well curved dorsally, with 2-3 caudal pores. Tail tip more or less rounded.[25]

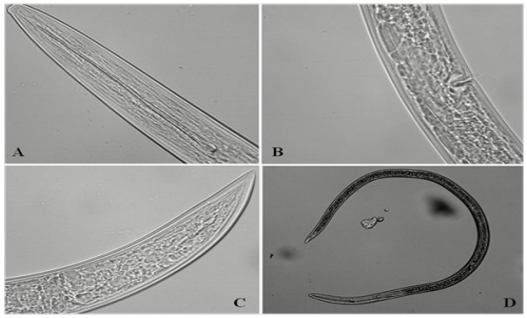


**Figure 1 A:** *X.americanum* lip region **B:** *X.americanum* vulva **C:** *X.americanum* tail. **D:** *X.americanum* entire body.

### Molecular confirmation of X.americanum

Molecular confirmation of *X.americanum* was performed with generated primers. Visible DNA bands were obtained after Agarose Gel Electrophoresis of PCR products (Figure 2). Nematode was being able to identified at all developmental stages by this molecular method. Furthermore the specificity of primers was tested with non target nematode species to determine primer specificity and specific bands were not generated.

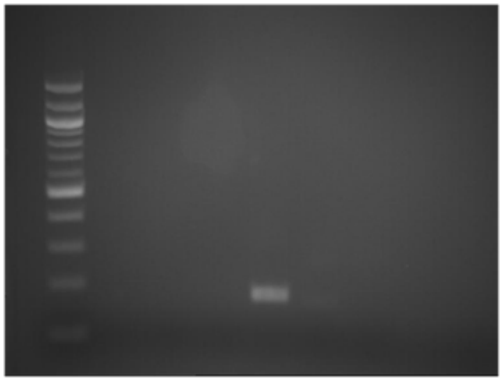

**Figure 2** Electrophoresis of the amplified products of *Xiphinema americanum*

## Discussion

*Xiphinema americanum* is widespread nematode distributed mostly in North America and other continents. Nematode is reported infecting coconut, rice, grapevine, coffeae, medicinal, aromatic and spice plants, wide range of fruit trees and ornamental plants.[26–29] This nematode is considered as one of the major virus vectoring nematode by ability to acquire more than one viruses from a doubly infected host or from two separate hosts.[30]

Classical indetification method requires knowledge of family and diagnostic characteristics of all species and can only be performed by experienced taxonomists. *Xiphinema americanum* identification relying on observation of morphologic and morphometric characteristics under microscope is quiet difficult. There are several species such as *X. pachtaicum, X.californicum* and *X.rivesi* with slightly similar characteristics with minor differentiations. Moreover generally juveniles of *Xiphinema* species has almost same morphometric measurements and morphologic characteristics. Furthermore it is nearly impossible to distinquish species when they exist in the soil as mixed populations. Furthermore populations of nematodes is affected with climate conditions and in cold weathers or very dry soils it may not be possible to isolate efficient number of females for morphologic observations and nematodes may be in juvenile form. Reliable specimen identification are mandatory for choosing adequate control strategies and for prevention of virus spread. Based on these difficulties molecular technique studies became important in recent years. Being one of the most conserved gene region, Ribosomal DNA (rDNA) is is used for accurate nematode identification.[12] Three nuclear rDNA regions 18S, internal transcribed spacer (ITS) region, 5.8 gene and 28S are widely used.[25] Molecular identification allows identification of species at any developmental stage. DNA multiplication can be performed by single nematode specimen. There are several published molecular diagnostic studies of molecular identification of *Xiphinema* species. Sets of primers from ITS sequences were designed to identify four species of *Xiphinema. Xiphinema index, X.diversicaudatum, X.vuittenezi,* and *X.italiae* and some *X.americanum* group species were studied with several specific primers.[5,8,10]

## Conclusion

In summary, in this study highly distributed *Xiphinema americanum* nematode was identified by PCR based molecular method and by observation of morphologic characteristics. A primer pair developed

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in this study yielded the expected products for *Xiphinema americanum* population. This PCR protocol and primer pair can be used in rapid identification of local populations. The molecular identification will allow us the identification of individuals from mixed populations and at juvenile stage as well.

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## Conflict of interests

Authors declare that there is no conflict of interest.

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