On the first occurrence of *Xiphinema santos* in Brazil

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Summary

Based on morphology, measurements of juveniles and female specimens and sequences of the D2/D3 expansion 28S rDNA gene and ITS1 analysis by DNA barcode technique, a *Xiphinema americanum* group species associated with olive trees from state of São Paulo, Brazil was identified as *X. santos*. This is the first report of *X. santos* in Southern Hemisphere and outside the European and African continents, thus extending its geographic range.

Keywords: DNA sequence; Longidoridae; *Xiphinema americanum*-group; distribution; olive

Introduction

Xiphinema americanum sensu lato includes vectors of several important plant pathogenic viruses that cause significant damage to a wide range of agricultural crops. This group is considered to be a complex of many species (Lamberti & Bleve-Zacheo, 1979; Lamberti *et al.*, 2000) comprising 61 putative species (OEPP/EPPO, 2017). Recently, substantial progress has been achieved on the accurate determination of species belonging to the *X. americanum* group (Gutiérrez-Gutiérrez *et al.*, 2012; Archidona-Yuste *et al.*, 2016; Lazarova *et al.*, 2019), however the validity of some species is uncertain. Traditionally, morphological characters have been used to distinguish and identify different species of *X. americanum* group. However, some species like *X. brevicolle* and *X. diffusum* are distinguished by only minute morphometric or morphological differences (Brown & Halbrecht, 1997), and considerable taxonomic expertise is required for accurate determination of individual

species. As the number of skilled taxonomists decrease, development of molecular-based diagnostic protocols is increasingly a viable alternative to nematode identification (Roberts *et al.*, 2016). Although *Xiphinema* originated in Africa and Latin America (Taylor & Brown, 1997), few investigations on taxonomic studies of *X. americanum* group, has been done in Latin America (Oliveira & Neilson, 2016). For example, currently only five *X. americanum*-group species: *X. brevicolle*, *X. diffusum*, *X. luci*, *X. oxycaudatum* and *X. peruvianum* (Oliveira *et al.*, 2003; Silva *et al.*, 2008) has been recorded from Brazil.

Recently, during a survey for plant-parasitic nematodes in São Paulo State, Brazil, it was detected a population of *X. americanum*-group species from a root zone soil sample of olive trees, *Olea europea*. Thus, the objective of this study was to identify and characterize this *X. americanum*-group species based on both morphological characters of females and juveniles and DNA sequences of D2/D3 expansion segment of 28S rDNA.

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Materials and Methods

Longidorid specimens were obtained from three soil samples collected from olive tree plantation from Bom Sucesso de Itararé, SP Brazil (S22°38.738; W45°40.685; 1514m). Each sample consisted of approximately 1.5 – 2.0 kg of moist soil collected around actively growing young roots, to a depth of 40 cm. Soil was placed in a polythene bag, stored in a cooled box (15 – 20°C) and immediately transported to the laboratory.

Nematodes were extracted from a 1 kg sub-sample of soil by a modified decanting and sieving technique (Ploeg & Brown, 1997). Nematodes were examined under a stereoscopic microscope and longidorid specimens were removed for morphological study. Longidorid nematodes were heat-killed at 60 °C, fixed in a 1 % formalin/glycerol mixture, and processed to anhydrous glycerine using a slow method (Hooper, 1986). Species identification and measurements were made using a high power microscope. To identify the *X. americanum*-group species, taxonomic keys proposed by Lamberti *et al.* (2000) and OEPP/EPPO (2017) standard diagnostic protocol were used, as well as original descriptions of the species from the literature.

Principal component analysis of *X. santos* populations

Principal Components Analysis (PCA) was performed on the correlation matrix of the set of 11 measurements [length of body (L), length of both odontostyle and odontophore, tail length, body diameter at anus, largest body diameter, position of the vulva in relation to the anterior end of the body expressed as a percentage of the length of the body (V %), body length divided by largest body diameter (a), body length divided by oesophageal length (b), tail length divided by body diameter at the anus (c') and ratio of the length of the body and tail (c)] of females taken in the present study and data sourced from the literature of *X. santos* populations from Portugal, Egypt and Spain (Lamberti *et al.*, 1993; 1994; 1996; Gutiérrez-Gutiérrez *et al.*, 2012) using Community Analysis Package (PISCES Conservation Ltd, Lymington UK).

Molecular study

DNA from single female specimens was extracted with Lysis Buffer Holterman [(HLB) (800 ug proteinase K/ml, β -mercaptoethanol 1 % (v/v), 0.2M NaCl and 0.2M Tris HCl pH 8)] (Holterman *et al.*, 2006). A total of 25 μ L of HLB was diluted in 25 μ L of ultrapure water totaling 50 μ L in a 0.2 mL Eppendorf tube. A drop of this solution (5 μ L) was placed on a glass slide, where the nematodes were individually cut into three parts and placed in the same 0.2 mL tube. The 45 μ L remaining solution was used for wash the

Fig. 1. Light micrographs of *Xiphinema santos* from Brazil. Female anterior (A) and tail regions (B). Scale bars=10 μ m.

slide and added to tube with the sectioned nematode. Samples were submitted to PCR at 65 °C for 2 h, 99 °C for five minutes and stored at -20 °C (Consoli *et al.*, 2012). The universal primers D2A (5'-CAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'TCGGAA-GGAACCAGCTACTA-3') were used to amplify the D2/D3 expansion segment of 28S rDNA by PCR (Al-Banna *et al.*, 2004). A total of 12.5 µL of Gotaq Hot Start Green Master Mix (Promega, São Paulo State, Brazil), with the reagents necessary for reaction: 12.5 µL Go Taq®, 10 µL NFW (Nuclease Free Water), and 1 µL each primer [10 mM] and 0.5 µL of cDNA from the single female specimen, totaling 25 µL per reaction was submitted to PCR at 94 °C for seven minutes; followed by 35 cycles at 94 °C for 60 seconds, 55 °C for 60 seconds, 72 °C for 60 seconds; and 72 °C for 10 minutes (Mráček *et al.*, 2006). The internal transcribed spacer (ITS-1) region of the ribosomal DNA sequence (rDNA) was amplified using the forward primer BL58 (5'-CCCGTCGMTACTACCGATT-3') and reverse primer 5818 (5'-ACGARCCGAGTGATCCAC-3'). The PCR conditions were: denaturation at 94 °C for 5 minutes; followed by 40 cycles of 94 °C for 1 minute, 57 °C for 45 second, 72 °C for 2 min, and extension for 10 min at 72 °C. Five µL of PCR product were used for electrophoresis in TAE buffer on 1 % agarose gel, stained with ethidium bromide (0.02 mg/mL), visualized and photographed under UV light. The result of the amplification was compared to the molecular weight marker VIII (Roche Life Science). The amplified fragments of D2/D3 expansion 28S rDNA and ITS-1 region were sequenced with the Big Dye Terminator kit (Applied Biosystems). A reagent mix containing 2 µL Big Dye, 3.2 pmol

sense primer, 3.0 µL of amplified product containing 400 ng DNA and 2.0 mL of water was prepared for the product end of the PCR reaction. The reaction for sequencing was performed according to the manufacturer's protocol (Applied Biosystems) with further purification of the amplified product by precipitation with isopropanol. Samples were suspended in 10 µL of deionized formamide and denatured at 95 °C for three minutes. Electrophoresis was performed in a 3500xL Genetic Analyzer (Applied Biosystems). The obtained sequences were aligned and compared to nucleotide polymorphism identification with the aid of BioEdit Alignment Sequence Editor Program. The consensus sequence was compared with other species of nematodes in the database (GenBank, <http://www.ncbi.nlm.nih.gov>) for identification based on genetic similarity.

Ethical Approval and/or Informed Consent

The conducted research is neither related to human nor animals use.

Results and Discussion

Morphometrics (Table 1) of the specimens obtained here generally agree with those of the paratype specimens of *X. santos* (Lamberti *et al.*, 1993) except for a ratio and odontostyle length (36.5 – 46.8, 76 – 84 µm vs 54.0 – 59.0, 68 – 72 µm respectively). Nevertheless, these differences are within the range of intraspecific variation, as showed in different Portuguese, Egyptian and Spanish populations

Table 1. Morphometric data of female and four juveniles stages (J1, J2, J3 and J4) of *Xiphinema santos* from Brazil. Values are mean ± standard deviation (range).

Nematode	<i>Xiphinema santos</i>				
Stages	female	J1	J2	J3	J4
Morphometrics*					
n	24	1	1	1	14
L (mm)	1.7 ± 0.1 (1.6 – 1.9)	0.7	0.7	0.9	1.3 ± 0.1 (1.1 – 1.4)
Odontostyle (µm)	79.2 ± 2.4 (76 – 84)	32.0	42.0	51.0	64.9 ± 2.4 (61 – 69)
Replacement odontostyle (µm)	–	48.0	52.0	63.0	77.3 ± 2.9 (72 – 82)
Odontophore (µm)	51.3 ± 1.8 (47 – 55)	43.0	30.0	37.0	42.5 ± 1.7 (40 – 45)
Spear (µm)	130.5 ± 2.9 (126 – 136)	75.0	72.0	88.0	107.4 ± 2.7 (102 – 111)
Tail (µm)	34.4 ± 1.8 (30 – 37)	30.0	32.0	25.0	34.1 ± 2.2 (30 – 37)
Body diameter (µm)	40.1 ± 2.9 (36 – 45)	18.0	21.0	24.0	28.9 ± 2.6 (26 – 36)
V%	51.8 ± 0.9 (50.0 – 53.5)	–	–	–	–
a	42.5 ± 2.8 (36.5 – 46.8)	41.4	33.8	39.0	43.4 ± 3.2 (37.2 – 47.2)
b	5.9 ± 0.7 (4.7 – 7.6)	5.5	4.9	5.5	5.4 ± 0.9 (4.0 – 7.5)
c	49.5 ± 3.0 (44 – 53)	24.8	22.2	37.4	36.6 ± 3.2 (32.5 – 44.3)
c'	1.6 ± 0.1 (1.4 – 1.8)	2.5	2.5	1.8	1.8 ± 0.2 (1.2 – 2.1)

* length of body (L), position of the vulva in relation to the anterior end of the body expressed as a percentage of the length of the body (V %), body length divided by largest body diameter (a), body length divided by oesophageal length (b), tail length divided by body diameter at the anus (c') and ratio of the length of the body and tail (c).

Fig. 2. Principal component analysis of seven *Xiphinema santos* populations based on mean values of 11 morphometric characters (Table 1). Brazilian (XsBRA), Portuguese from Dao Region (XsPORDao), Azores (XsAzores), Braga (XsPORBraga) and Madeira (XsMadeira); Egyptian (XsEGP) and Spanish (XsESP) populations.

of this species (Lamberti *et al.*, 1994; 1996; Gutiérrez-Gutiérrez *et al.*, 2012). The alpha-numeric codes for the Brazilian population of *X. santos* to be applied to the polytomic identification keys for the *X. americanum*-group species by Lamberti *et al.* (2000) are: (A 2/3, B 2, C 3, D 1, E 2, F 1, G 2, H 2, I 3) and by OEPP/EPPO (2017) are: (A 2, B 2, C 1, D 2/3, E 2, F 3, G 1, H 2, I 3, J 3).

Female: The Brazilian population of *X. santos* was characterised by a body forming a close C-shaped. Lip region rounded anteriorly, slightly expanded and separated from the body by a weak depression (Fig. 1 A). Reproductive system amphidelphic, both branches apparently equally developed, opposed and reflexed, without any uterine differentiation. Ovaries with symbiont bacteria. Tail short, conoid, weakly curved ventrally with conoid rounded terminus (Fig. 1 B).

Juveniles: All four juvenile stages were identified using morphological characters such as body length, length of replacement and functional odontostyle (Table 1). Specifically, J1 was characterised by the position of replacement odontostyle just posterior to functional odontostyle. Subsequent stages (J2, J3 and J4) were identified following Halbrendt & Brown (1992) by establishing the range of corresponding replacement and functional odontostyle length.

Male: No male was detected.

The PCA separated the seven *X. santos* populations into four distinct clusters (Fig. 2). The Brazilian population (XsBRA) was morphometrically closer to the Portuguese populations from Azores (XsAzores) and Braga (XsPORBraga) and formed a distinct cluster. Also, in agreement with Lamberti *et al.* (1996), the Egyptian population (XsEGP) was almost identical to the type population from Dao Region, Portugal (XsPORDao). The Spanish (XsESP) and Madeira (XsMadeira) populations were both isolated from all other populations.

Amplification of D2/D3 expansion 28S rDNA gene and ITS1 of the Brazilian population of *X. americanum*-group species (XsBRA) produced, respectively, 628 bp and 706 bp fragments and the sequences were deposited in the GenBank under the accession codes MN318337 and MN318338. The technique of DNA barcode sequence showed that the expansion D2/D3 28S rDNA gene of XsBRA was identical to three *X. santos* populations (GenBank JQ990029.1; JQ990030.1; AY601587) from Spain and Portugal, *Xiphinema* sp. (MH558570) from Spain and *X. citricolum* (DQ285668) from USA, with a similarity of 100 %. ITS1 showed 98 % similarity with *X. santos* (JQ990046; JQ990047) from Spain and 97 % with several *X. americanum*-group species isolated from USA (KF748440.1; KF748389.1; KF748451.1; KF748442.1; KF748438.1) and *X. georgianum* (DQ299521.1).

Xiphinema santos was described from specimens collected from the rhizosphere of vineyards in Dão region, Portugal (Lamberti *et al.*, 1996), and has also been reported from vineyards in Nubaria, North Egypt (Lamberti *et al.*, 1996), vineyards and stone pine in Rociana del Condado (Huelva, Spain) (Gutiérrez-Gutiérrez *et al.*, 2012) and wild olive in Arcos de la Frontera (Cádiz, Spain) (Archidona-Yuste *et al.*, 2016). In this study, *X. santos* occurred around roots of cultivated olive from Bom Sucesso de Itararé (SP). This is the first record of the species in Brazil.

Combining the results of this study and of other previous reports, a total of 31 *Xiphinema* species have now been recorded from Brazil. These include six *Xiphinema americanum*-group species: *X. brevicolle*, *X. diffusum*, *X. luci*, *X. oxycaudatum* and *X. peruvianum* (Oliveira *et al.*, 2003; Silva *et al.*, 2008) and *X. santos* (present study). As mentioned by Brown & Halbrendt (1997), the taxonomic status of *X. americanum*-group nematodes is controversial and the species identification based on morphological characters is problema-

tic because several species are only distinguished by minor morphometric or morphological differences. However, the use of the polytomous keys published by Lamberti *et al.* (2000) and OEPP/EPPO (2017) facilitated the identification of *X. santos*. Additionally, the sequences of the D2/D3 expansion 28S rDNA gene and ITS1 analysis by DNA barcode technique was useful for the diagnosis of *X. santos* Brazilian population.

Based on both morphological, morphometrical and molecular analysis we concluded that the longidorid nematodes associated with olive trees from state of Sao Paulo, Brazil, belong to the species *X. santos*. This is the first report of *X. santos* in Southern Hemisphere and outside the European and African continents, thus extending its geographic range.

Conflict of Interest

Authors state no conflict of interest.

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