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Full Length Article

Morphological and Molecular Characterization *of Xiphinema krugi* from Argentina Associated with Silk Floss Tree (*Ceiba speciosa*) Intercepted in China

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Abstract

A population of dagger nematode from Argentina was intercepted by Quarantine Bureau in Shenzhen, China. *Xiphinema* sp. was recovered from imported silk floss tree (*Ceiba speciosa*). Morphometric studies of the intercepted *Xiphinema* agreed with the description of *X. krugi* from Argentina and Brazil with slight variations in morphometric values of c' (1.1 µm vs. 1.3 µm vs. 1.2 µm), odontostyle length (114 µm vs. 112 µm vs. 116 µm), ratio of anterior genital branch as expressed to diameter of vulva (1.8 µm vs. 1.6 µm vs. 2.2 µm). Phylogenetic analyses of *X. krugi* (KY011940) using ITS1 rDNA region indicated a well-supported (PP=0.98) clade to *X. krugi* putative species from Brazil classified as genotypes profile B (DQ017149-DQ017151), all these specimens shares morphologically a conoid tail and distinct ventral peg. This is the first interception report of *X. krugi*, from Argentina in China associated with *Ceiba speciosa*. © 2017 Friends Science Publishers

Keywords: Argentina; *Ceiba speciosa*; China; Quarantine; *Xiphinema krugi*

Introduction

Genus *Xiphinema*, known as the "dagger nematode" are ectoparasitic migratory that feds on wide range of herbaceous and woody plants which are found prevalent in all continents (Taylor and Brown, 1997). This group is economically important because of its virus vector ability resulting to phytosanitary risk of some plant species (Decraemer *et al.,* 1998). *Xiphinema krugi* are reported to be widely distributed in tropical and subtropical climates (Luc and Hunt, 1978). The species is originally described in the rhizosphere of natural vegetation in Piracicaba, Sao Paulo (Maximiano *et al.,* 1998) a pseudomonodelphic species most prevalent in Brazil (Lamberti *et al.,* 1978; Germani, 1989; Oliveira *et al.,* 2006) and relatively widespread in USA (Doucet *et al.,* 1998).

X. krugi were also recorded in Argentina (Luc and Doucet, 1990), Paraguay (Luc and Hunt, 1978), Colombia (Volcy, 1990), Surinam (Loof and Maas, 1972), Martinique (Luc and Coomans, 1992), Uruguay (Jacob and Loof, 1996), Trinidad (Bala, 1984), Venezuela (Crozzoli *et al.,* 2001), Senegal (Luc and Hunt, 1978) and Hongkong (Shen *et al.,* 1999). A pantropical distribution of this species was reported by Coomans *et al.* (2001). The common occurrence of *X. krugi* is probably explained by its wide host range (e.g. grassland, ornamentals and natural vegetation) (Costa Manso *et al.,* 1994). The taxonomic status of *X. krugi* has been questioned due to morphological and morphometric heterogeneity (Luc and Hunt, 1978) and was considered to be synonym of *X. denoudeni* and *X. loosi* (Loof and Maas, 1972; Southey and Luc, 1973) This is because existing a resemblance of female genital branch and tail shape. Studies by Luc and Hunt (1978) including groups with distinct populations of *X. krugi*, group one was a population previously described as *X. loosi* having a short hemispheroid tail (29.3 µm) and short anterior genital branch (60.5 µm), a second group was an American population with long conoid tail and distinct ventral peg, a third group including two populations one from Paraguay and the other from Surinam both populations with mean tail of 35 µm and anterior genital branch of 72 µm, and a fourth group contained a putative population of *X. krugi* having a tail length of 31.8 µm and long anterior genital branch of 97.5 µm (Lordello, 1955).

In this study, a population of *Xiphinema* sp. was recovered from soils around the roots of silk floss tree (*Ceiba speciosa*) imports from Argentina intercepted by Shenzhen Entry-Exit Inspection and Quarantine Bureau. Studies of the intercepted *Xiphinema* sp. were conducted using a combination of morphology and molecular approach. Characterization of diagnostic characters for *Xiphinema krugi* and nucleotide sequences of ITS1 and 28S region of rDNA were used to compare to the intercepted *Xiphinema* and are presented in this paper.

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Materials and Methods

Morphological Identification

Samples of *Xiphinema* sp. used in this study were isolated and collected from soil and rhizosphere of silk floss tree imported from Argentina. Nematodes were extracted using modified method of Cobb's decanting and sieving (Brown and Boag, 1988). Nematodes were handpicked from the suspension. Killed over the flame for further ocular inspection, and for photographic documentation. Twelve adult females were kept for fixation and for preparation of permanent slides (Ye *et al.,* 2004). Photomicrographs were obtained using Zeiss compound microscope (Stem 2000-C) with attached digital camera. Morphometric were carried out using an installed software from Zeiss. All morphometric values are in µm and are expressed as mean ± SD.

Molecular Characterization

Extraction of DNA was made using a single adult nematode. The nematode was placed to temporary glass slide containing 13 µL ddH_2O and cut into fragments using a scalpel pre-heated over the flame. Fragments of nematode were pipetted up to 10 µL and transferred to Eppendorf tube and added with 8 µL Mg + free buffer and 2 µL proteinase K (Ye *et al.,* 2004). DNA extracts were centrifuged at 12000 rpm for 2 min and overnight stored at minus 70°C. The following day, each Eppendorf tube was incubated in a PCR machine using the following thermal protocol 65°C for 3 h, 75°C for 60 min and 95°C for 10 minutes. Afterwards, DNA suspensions were cooled down at 8°C and stored at minus 20°C until further use. A volume of 25 µL PCR mix consisting 2.5 µL LA buffer, 2 µL dNTP, 1.5 µL each primers (synthesized by Takara Company, Shanghai, China) and 3 µL DNA templates, 0.3 µL LATaq and 14.2 µL distilled water. All PCR reactions were conducted in the S1000 thermal cycler (BIO-RAD).

Generations of PCR products were conducted as previous described (Oliveira *et al.,* 2004). Fragments of ITS1 and 28S region were amplified using two sets of primers. First set: forward primer V1 (5'-TTG ATT ACG TCC CTG CCC TTT-3') and reverse primer 5.8S (5'-ACG AGC CGA GTG ATC CAC CG-3') (Gutierrez *et al.,* 2010) and, the second set: forward primer D2A (5' ACA AGT ACC GTG AGG GAA AGT TG 3') and reverse primer D3B (5' TCG GAA GGA ACC AGC TAC TA 3') (De Ley *et al.,* 1999), After DNA amplification, 2.5 µL aliquots of PCR products were analyzed by gel electrophoresis in 1% agarose gel (100V, 400 mA, 30 minutes) stained with DuRed 10,000x stain (Cat#D009-500) and DNA were visualized under UV illumination. Amplified DNA were purified according to TaKaRa DNA fragment Purification kit version 4.0 (catalogue No. 9761) of TaKaRa Clontech Bio Inc., China. Purified DNA were ligated to pUCM-T vector and transformed in to DH 5alpha competent cells and transformants were screened on an ampicillin agar LB plates at 37°C overnight. White colonies were selected and transferred to 5 mL LB containing 100 µg μL^{-1} ampicillin and incubated at 37°C for 16–24 h. PCR amplification was confirmed using the primer insertion and expected band. Sequencing was done at the SANGON Biotechnology Co., Ltd. Obtained sequences were submitted to GenBank for further comparison of closely related species. Sequences were analyzed and aligned using Clustal W program (Thompson *et al.,* 1994) of the Mega 5.0 (Tamura *et al.,* 2011).

Results

Xiphinema krugi Lordello, 1955

Measurements and distribution see Tables 1 and 2

Female: Body appears open C-shaped upon heat relaxed (Fig. 1A). Cuticle smooth with obscure transverse body striations. Head rounded, labial region offset by slight constriction from body profile (Fig. 1B–E). Body size averaging 1.8–2.2 mm, odontostyle (n = 12) long (116–120 µm), odontophore flange well-developed. Vulva transverse slit (Fig. 2B) and positioned 31–34% from anterior end (Fig. 1F) while vagina is about 40% of body width (Fig. 2E). Total oesophageal length of 377 µm, the basal bulb of esophagus measuring 92 µm long and 26.5 µm wide. Reproductive system consists of two branches with anterior reduced 67 µm (61–94 µm) without ovary and short uterus, while posterior branch is normally developed 234 µm (228–242 µm) consisting of ovary, oviduct and uterus (Fig. 2A). Tail conoid rounded with a slight depression in the dorsal side at the beginning of peg (Fig. 1G–J). Ventral peg distinct to all specimens. Cuticle at tail terminus with very faint oblique lines (Fig. 2C). Caudal pores present (Fig. 2F–G).
Male: Not found

Differential Diagnosis

Xiphinema krugi can be recognized from other *Xiphinema* species by lack of anterior ovary. Morphologically constitute similar structure to *X. filicaudatum*, *X. longicaudatum*; *X. surinamense* and *X. variegatum*, all belonging to taxonomic group II which characterized by only developed posterior genital branch. However, *X. filicaudatum* and *X. longicaudatum* can be easily distinguished in the difference of long tails having 363–545 µm and 154–241 µm, respectively. *X. krugi* is closely similar to *X. surinamense* having conoid-hemispheroid tail but has much a longer anterior genital branch (240 vs. 51–93 µm) and apparently posterior vulva (V=36–42 vs. 31–34 µm). Whereas *X. variegatum* has female genital branch shorter (45–52 vs. 51–93 µm) than *X. krugi*.

Molecular Characterization and Phylogenetic Relationships

The amplification product of partial D2-D3 expansion

Table 1: Morphometric of *Xiphinema krugi* from Argentina intercept in China including comparisons to previous studies using populations from different origin and crops

Origin Host	In this study Silk floss tree	Brazil (Lordello, 1955) Natural vegetation	Paraguay (Luc & Hunt, 1978) Sugar cane	Argentina (Chaves & Mondino, 2013) Potato	Surinam (Loof & Mass, 1972) Citrus	Senegal (Luc & Hunt, 1978) Citrus	USA (Ye & Robbins, 2010) Hardwood tree	Hongkong (Shen *et al*., 1999) Elm tree	Sri Lanka (Southey & Luc, 1973) Easter Lily
	12	5	20	4	50	15	4	20	11
L (mm)	1.9±0.21 (1.8-2.2)	2.12-2.22	2.07-2.56	2.0-2.34	1.93-2.41	1.91-2.32	1.9-2.2	1.88±0.69	1.78-2.18
a	41.2±1.8 (40.5-42.5)	37.9-43.8	31.2-56.9	42.5-50	39-49	49.3-59.5	31.3-51.1	36.9±9.2	32.9-37.5
b	5.4±0.65 (4.9-6.7)	5.2-5.6	4.5-6.4	4.9-5.4	4.8-6.5	4.2-5.8	4.7-5.2	4.8±0.3	-
c	64.2±5.2 (63.2-72)	66.3-69.6	54.2-80.5	52-60	55-74	62.6-73.1	51.1-61.8	64.4±4.2	66-90
c'	1.1±0.11 (0.9-1.1)	1.2-1.4	0.8-1.1	1.3-1.4	1.2-1.3	1.0-1.2	0.9-1.2	0.9±0.1	0.57-0.80
V	32.8±1.3 (31.8-34)	33.4-34.2	33.6-35.9	33-35	32-36	31.9-35.9	33.5-36.1	33.28±1.2	28.6-33.3
Odontostyle	114±5.6 (111-120)	116-120	102-123	106-111	114-126	111-124	113-120	115±6.6	118-127
Odontophore	72.8±1.9 (67-75)	68-72	70-84	71-73	69-79	63-72	70-75	73±2.0	68-74
Total stylet	186.8±6.2 (182-193)	184-192	176-207	178-182	186-205	180-195	184-194	188±6.9	186-195
Tail length	35.4±2.7 (31-38)	-	30-44	39-41	28-34	29-34	34-38	29.4±1.9	24-29
a.g.b	67±4.9 (61.4-93.7)	-	49-102	-	85-107	90-113	75-99	-	51-74
a.g.b/v.d	1.8±0.26 (1.6-2.1)	2.2	0.9-1.5	1.6-2.4	1.9-2.3	2.2-2.9	1.5-1.9	-	0.8-1.1
p.gb	234±6.5 (226-242)								

N= number of specimens; L= body length; a= body length/body width; body length/distance from head to pharynx; c= body length/diameter at anus; V= distance from head to vulva/body length x 100; agb=anterior genital branch; agb/vd= anterior genital branch/diameter at vulva; pgb=posterior genital branch (All measurements in µm unless noted otherwise)

Table 2: Distribution and occurrence of *Xiphinema krugi* populations

Species	Host	Local name	Origin	Reference
X. krugi	-	natural vegetation	Piracicaba, Sao Paolo, Brazil	Lordello (1955)
X. krugi	*Vitis* sp	grape	Garibaldi, Brazil	Oliveira *et al.* (2006)
X. krugi	*Eugenia uniflora*	cherry	Florianopolis, Brazil	Oliveira *et al*. (2006)
X. krugi	*Mangifera indica*	mango	Sao Jose de Rio Preto, Brazil	Oliveira *et al*, 2006
X. krugi	*Cucurbita* sp.	gourd	Jaranjal de Jari, Brazil	Oliveira *et al*. (2006)
X. krugi	*Solanum* sp	potato	Concepcion, Argentina	Chaves and Mondino (2013)
X. krugi	*Allium sativum*	garlic	Medanos, Argentina	Chaves and Mondino (2013)
X. krugi	*Zea mays*	corn	Sadaillo, Argentina	Chaves and Mondino (2013)
X. krugi	*Fragaria* sp.	strawberry	Coronda, Sta Fe, Argentina	Chaves and Mondino (2013)
X. krugi	*Solanum melongena*	eggplant	Helvecia Sta Fe, Argentina	Medera (2013)
X. krugi	*Saccharum officinarum*	sugarcane	Cartago, Costa Rica	Peraza-Padilla *et al*. (2016)
X. krugi	*Cynodon* sp.	star grass	Alajuela, Costa Rica	Peraza-Padilla *et al*. (2016)
X. krugi	*Hevea brasiliensis*	rubber	Puntarena, Costa Rica	Peraza-Padilla *et al.* (2016)
X. krugi	*Citrus* sp.	citrus	Surinam	Loof and Maas (1972)
X. krugi	*Citrus* sp.	citrus	Senegal	Luc and Hunt (1978)
X. krugi	-	hardwood	Florida, USA	Ye and Robbins (2010)
X. krugi	*Lilium longiflorum*	easter lily	Sri Lanka	Southey and Luc (1973)
X. krugi	*Ulmus parviflora*	elm	Hongkong	Shen *et al.* (1999)
X. krugi	-	unknown	Antioquia, Colombia	Volcy, 1990
X. krugi	*Ceiba speciosa*	silk floss	China	In present study

segments of 28S and ITS1 rDNA regions yielded a fragment length of approximately 840 bp and 1227 bp, respectively. Blast homology of *X. krugi* (KY011942) using D2-D3 segments showed 95% similarity to closely related *Xiphinema* spp. available in GenBank. Meanwhile *X. krugi* (KY011940) showed 99% similarity in ITS1 region to *X. krugi* populations reported by Oliveira *et al.* (2006).

In phylogenetic analyses of ITS1 region using Maximum Likelihood, from the Argentinean intercepted *X. krugi* by Quarantine authorities in China (KY011940) was clustered in a (PP = 0.98) clade of *X. krugi* populations classified as genotype profile B (DQ017149-DQ017151) reported by Oliveira *et al.* (2006) (Fig. 3). The phylogenetic analysis using 28S region of *X. krugi* (KY011942) showed PP=0.83 of similarity value to the sequences of *X. krugi* from Costa Rica deposited in GenBank (KX931060) (Fig. 4).

Discussion

Morphometric heterogeneity of *X. krugi* tail varies in shape and tip structure, which is comparably and could be used as an important diagnostic character for separating populations. Luc and Hunt (1978) classified 6 populations *X. krugi* using tail characters. Tail terminus could vary from specimens having fairly long peg to specimens without peg (Coomans *et al.,* 2001) and variation on tail shape also occurs from sub-conoid with slight bulge extremity to conoid rounded with distinct peg (Luc and Hunt, 1978). Additionally, studies carried out by Oliveira *et*

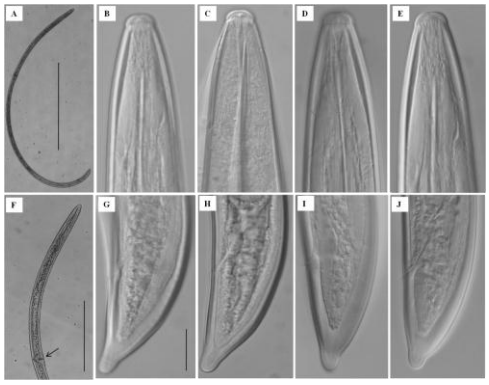

Fig. 1: *Xiphinema krugi*: A-C. Habitus; B-C. Head of *X. krugi* intercepted from Argentina D-E. Head of *X. krugi* from USA; F. Position of vulva from anterior; G-H. Tail of *X. krugi* intercepted from Argentina; I-J. Tail of *X. krugi* USA (Scale bars: A=100 µm; F=50 µm; B-E, G-J= 10 µm)

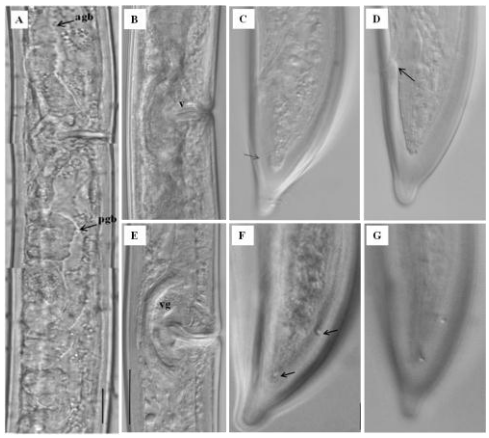

Fig. 2: *Xiphinema krugi:* A. Reproductive system of *X. krugi* intercept from Argentina showing reduced anterior genital branch (agb) and normal development of posterior genital branch (pgb); B, E. vulva and vaginal orientation of *X. krugi* intercept from Argentina; C. faint oblique lines in tail terminus; D. anus position; F. Position of tail papillae in *X. krugi* intercept from Argentina; G. Tail papillae in *X. krugi* USA (Scale bar: A=5 µm; B-G=10 µm)

al. (2006) clearly separated *X. krugi* populations into four distinct groups or morphospecies based on principal component analysis (PCA) of morphometric characters and four distinct genotype profiles using the ITS1 region (Type A-D).

Morphometric characters in our study agreed with the morphometric characters previously reported by Chaves and Mondino (2013) for *X. krugi* populations from Argentina, and from Brazilian studies of *X. krugi* population reported by Oliveira *et al.* (2006). Our studies showed very slight variations in morphometric values of c' ratio (1.1 vs. 1.3 vs. 1.2 µm), odontostyle length (114 vs. 112 vs. 116 µm), ratio of anterior genital branch as expressed to diameter of vulva

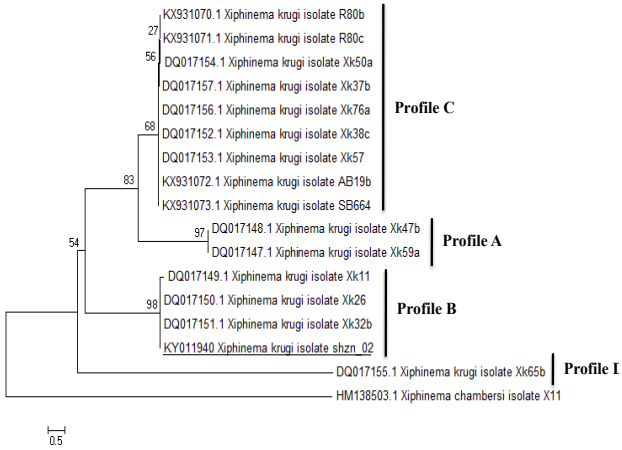

Fig. 3: Maximum likelihood tree showing relationships of *X. krugi* population based on ITS1 region. Sequence data sets of *X. krugi* populations reported by Oliveira *et al.* (2006) were used to construct the tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap tests (1000 replicates) and is shown above the branches

(1.8 vs. 1.6 vs. 2.2 µm).

Based from Luc and Hunt (1978) classification of *X. krugi* tail character, the *X. krugi* intercepted in China is most similar to category 12 (Florida tail), a tail of conoid shape with rounded terminus and a distinct ventral peg at tail extremity. Additionally, the morphometric of intercepted population agreed well on the morphometric characters of *X. krugi* population PX32b from Brazil which falls in the category of morphospecies type B as classified by Oliveira *et al.* (2006).

Molecular analysis of *X. krugi* intercepted population showed homologies to putative populations of *X. krugi* Brazil and *X. krugi* Costa Rica but phylogenetic relationship only revealed a well-supported clade (PP=0.98) to *X. krugi* genotypes profile B (DQ017149-DQ017151) of Brazilian putative species, while comparatively different to *X. krugi* Costa Rica (KX931072-KX931073) from which the latter was clustered to profile C (Fig. 3). The genotypic difference between the *X. krugi* intercept in China and recently described *X. krugi* from Costa Rica is explained from dissimilarity of tail shape of Costa Rican population having a sub-conoid tail with a slight bulge at the extremity but never considered a peg. In contrast, further interpretation and comparison of phylogenetic relationship of 28S region cannot be clearly interpreted due to very few *X. krugi* 28S sequence deposited in the GenBank.

Delineation of *X. krugi* using morphology and sequence data were addressed in this study to consider studying intra-population variability in the future. Oliveira *et al.* (2006) and Peraza-Padilla *et al.* (2016) agreed that *X. krugi* is a possible complex species because of diversity of tail characters between populations. The wide difference of

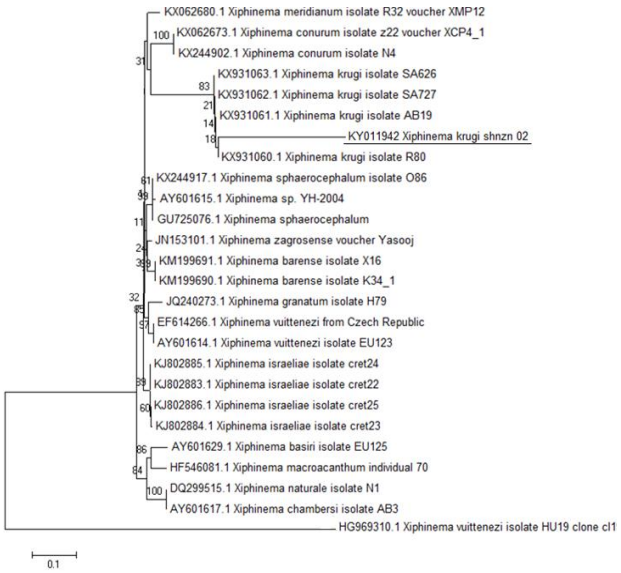

Fig. 4: Phylogenetic relationships of *X. krugi* population as inferred from partial D2-D3 expansion segments with comparison of *Xiphinema* spp. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above the branches

tail shape may be due to geographical intraspecific variability and may possibly comprise cryptic species with distance genotypes. Recent description of *X. krugi* from Costa Rica and *X. krugi* intercepted in China is one of a conclusive evidence of intra-species variability among *X. krugi* populations.

In conclusion, a study of different populations is essential to clarify this species complex. It is also necessary to consider in future studies providing a mitochondrial DNA sequence (*COI*) which may offer a more accurate confirmation of *X. krugi* status. This is the first interception report of *X. krugi* from Argentina in China associated with a possible host *Ceiba speciosa.*

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