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Molecular and Morphological Identification of *Xiphinema hunaniense* on the *Juniperus chinensis* Imported from Thailand

Long H[1,2,3], Ling XY[1,3], Li FR[1,2,3], Li YN[1,2,3] and Zheng Y[1,2,3]

[1]Shenzhen Entry-exit Inspection and Quarantine Bureau, Shenzen, 518045 Guangdong P.R., China
[2]State Key Quarantine Laboratory of Legume Pest & Plant Pathogenic Fungi of AQSIQ, Shenzhen, 518045 Guangdong P.R., China
[3]Shenzhen Key Laboratory of Inspection Research & Development of Alien Pests, Shenzhen, 518045 Guangdong P.R., China

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Abstract

A population of alien nematode was collected from the roots of Chinese juniper (*Juniperus chinensis*). The morphology and morphometrical traits of the collected females were in agreement with these of *Xiphinema hunaniense* described in the original references, except for a few differences, such as the tail length and "c " ratio. The sequences searching and alignment and phylogenetic analysis, which were based on the DNA sequence of D2-D3 expansion regions of 28S rDNA gene, further suggested that the species of this isolated nematode is *X. hunaniense*. In China, it is the first time that *X. hunaniense* was intercepted from this new host imported from Tailand.

Key words – Alien nematode– *Xiphinema hunaniense*– Chinese juniper– morphology– phylogenetic analysis

Introduction

Xiphinema hunaniense Wang & Wu, 1992 was first described from vineyard soils in Hunan province, China (Wang & Wu, 1992). This species is morphologically close to *X. radicicola* Goodey, 1936 and *X. brasiliense* Lordello, 1951. *X. hunaniense* was once considered as a junior synonym of *X. radicicola* (Loof et al., 1996). But this view was denied by Robbins & Wang (1998). Instead they re-established *X. hunaniense* a valid species. Zheng & Brown (1999) further established the validity of the taxonomic status of *X. hunaniense* based on comparing two *X. hunaniense* populations from different hosts in Hangzhou, China with syntype and paralectotype specimens of *X. Radicicola* (Cohn & Sher, 1972; Luc, 1981).

X. hunaniense has been reported in some Chinese provinces (Wu et al., 2007). The reported hosts included grape (*Vitis vinifera*), buntan (*Citrus grandis*), Chinese hibiscus (*Hibiscus rosasinensis*), Japanese camellia (*Camellia japonica*), litchi (*Litchi chinensis*), longan (*Euphoria longana*), loquat (*Eriobotrya japonica*), mango (*Mangifera indica*), pear (*Pyrus pyrifolia* var. *yokoyama*), pine (*Pinus* sp.), sago palm (*Cycas revolute*), sweet orange (*Citrus sinensis*) and some bonsai plants (*Camellia sasanpua*, *Ligustrum quihoui*) (Wu et al., 2007). Chen et al. (2004) reported eight populations of *X. hunaniense* colleted from different host plants in Taiwan, China, and discussed the variations of different *X. hunaniense* populations. Wu et al. (2007) described a *X.*

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Corresponding Author: Long Hai– e-mail – longsi1979@163.com

hunaniense population found in Zhejiang, China, and released the molecular data (Wu et al., 2007).

In this paper, a population of *X. hunaniense* was firstly intercepted by Shenzhen Entry-Exit Inspection and Quarantine Bureau on Chinese juniper (*Juniperus chinensis*) that imported from Tailand. And the morphological characterization and molecular data of the female *X. hunaniense* population on this new host were presented and compared with the ones from China.

Materials & Methods

Morphological characterization

Specimens of *X. hunaniense* used in this study were isolated and collected from the infested soil and root of *Juniperus chinensis* that were imported from Tailand with the decanting and sieving method of Brown & Boag (1988). Nematodes were handpicked from the samples, killed by gentle heat, fixed in a solution of 4% of formaldehyde + 1% glycerol, and treated with glycerol according to reported method (Hooper, 1986). All observations of fixed nematodes were under a Zeiss Image.M1 light microscope, whereas photographs and measurements were taken with the software Axiovision 4.7 (Zeiss, Germany). Abbreviations used in Table 1 are defined in Siddiqi (2000).

Molecular identification

Nematode DNA was extracted from single individuals. One nematode was moved to a clean glass slide, suspended in a 12-µl drop of lysis buffer (1X*Ex Taq* buffer, 100 µg/ml proteinase K, 2 mM $MgC1_2$) and cut into fragments. The fragments together with the lysis buffer were then transferred into a 0.2 ml sterile eppendorf tube. After freezing at −70°C for 10 min, tubes were incubated at 60°C for 60 min, then at 95°C for 15 min.The treatment solution above were then served as DNA sample for PCR. For PCR reaction, 10 µl of the DNA sample was added to the PCR reaction mixture containing 1X *Ex Taq* buffer, 0.2 mM of each dNTPs, 2.0 mM $MgCl_2$, 1 U *Ex Taq* polymerase (*TaKaRa Ex Taq*®, TaKaRa, Dalian, China), 0.4 µM of each the primers D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAG CTACTA-3') (synthesized by Sangon Technology & Services, Shanghai, China) and double-distilled water was added to a final volume of 50 µl. PCR amplification was followed the program: initial denaturation at 94°C for 4 min, 40 cycles at 94°C for 30 sec, 55°C for 40 sec, and 72°C for 1 min followed by an extension at 72°C for 10 min. After DNA amplification, PCR product was checked by running on a 1.5% agarose gel with 5 µl of each PCR product. The other PCR products of five samples were sequenced by Shanghai Sangon Biological Engineering Technology & Service Co., Ltd..The DNA sequences were edited with the BioEdit (v7.0) (Hall, 1999) and submitted to the GenBank database (www.ncbi.nlm.nih.gov) under accession number KF290564.

Phylogenetic analysis

28S rDNA gene sequences of *X. hunaniense* in this study and those of related species from GenBank database (www.ncbi.nlm.nih.gov) were used for phylogenetic re-construction, and *Longidorus elongatus* (AY601578) as outgroup taxa. The newly obtained and published sequences for each gene were aligned using Clustal W (Thompson et al., 1994) with default parameters. Sequence alignments were manually edited using BioEdit (Hall, 1999). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura et al., 2011).

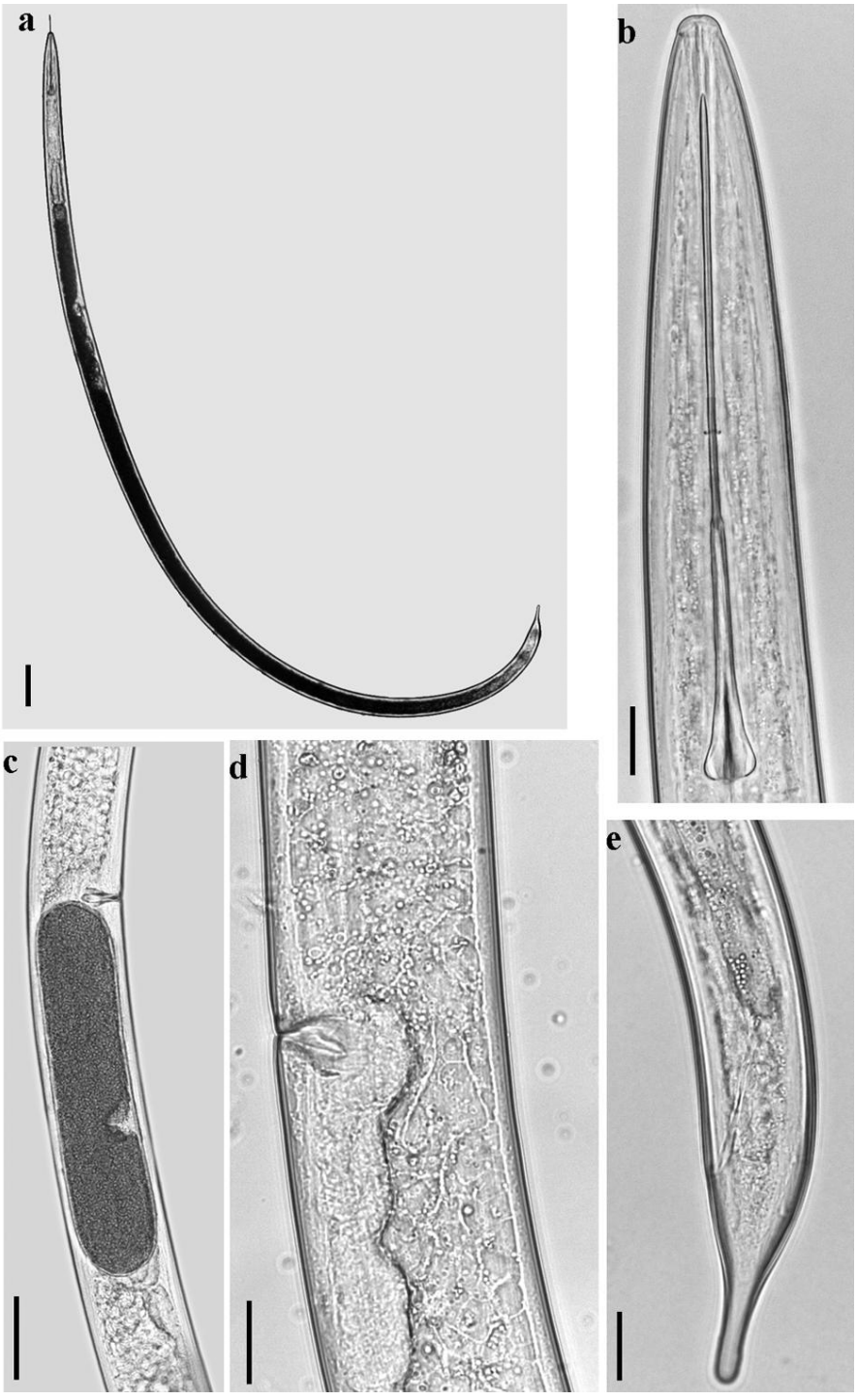
Results

Morphological characters and morphometrics

A total of 12 female specimens were examined. Morphology and morphometrics of them fit closely to those of the original descriptions of the species that Wang and Wu reported (Wang & Wu, 1992). As presented in Figs. 1, the female body is hook-shaped (Figs. 1a). It has one long mucro on the middle of tail terminal (Figs. 1e). And the posterior genital tract has only one ovary (Figs. 1c) comparing to the descriptions in the original literature that it divided into two ovarian branches

(Wang & Wu, 1992). Additionally, a few females whose vagina slope backwards was observed (Figs. 1d). Males were not found in this population.

The new Thailand population of *X. hunaniense* on *Juniperus chinensis* in the current study and several other populations reported from China was compared in Table 1. The ranges of morphometric variation are: L (1.73–2.50), a (35–57), b (4.6–8.2), c (29.2–63), c'(1.0–2.9), V% (21.6–2.9), odontostyle (96–123.3), odontophore (54–75.8), stylet (155.2–195.0), tail (31.1–77.4), width at lip region (8.7–13.5), width at mid. body (37–63.8), width at anus (21.3–35.6).



Figs 1 –Light microscopy micrographs of *Xiphinema hunaniense* female detected from Thailand. a Entire female. b Head regions of female. c-d Vulva of female; e Tail regions of female. (Scale bars: a, =100 μm;c=50 μm; all others=20 μm).

Molecular characterization and phylogenetic relationships of *Xiphinema hunaniense*

The amplification of D2-D3 expansion segments of 28S rDNA yielded a single fragment of approximately 800 bp based on gel electrophoresis. No sequence variation was detected among the five sequenced samples. DNA sequences searching and alignment using BLAST showed that D2-D3 expansion segments of 28S rDNA of *X. hunaniense* (KF290564) from Thailand matched well (99% similarity) with those of the *X. hunaniense* deposited in GenBank (EF188840, EF026090, EF188841 and EF188839), with only one nucleotide (817/818 identities), eight nucleotides (810/818 identities), nine nucleotides (809/818 identities) and ten nucleotides (808/818 identities) differences respectively, and presenting zero gap (0/818), and one gap (1/818), respectively. The next close species was *X. italiae* (FJ713153) differing by 87 nucleotides (89% similarity, 738/825 identities), and presenting 22 gaps (2%, 22/825). Surprisingly, the 28S rDNA sequence of *X. hunaniense* from Thailand was only 87% similar to *X. brasiliense* (AY601616) with 696/800 identities and 40 gaps (5%, 40/800); and 81% similar to *X. radicicola* (AY601622) with 651/799 identities and 27 gaps (3%, 27/799).

Table 1 Morphometrics comparison of *X. hunaniense* female populations from Thailand and China.*

Origin and host	Thailand, Juniper	China, grape (paratypes)[a]	China, Camellia[b]	China, Plum[c]	China, Loquat[d]	China, *Pinus* sp.[e]
n	12	10	17	13	18	18
L	2.27 ± 0.13 (2.04–2.49)	2.29 (2.07–2.50)	1.91 ± 0.15 (1.73–2.23)	2.01 ± 0.13 (1.74–2.16)	2.10 ± 0.09 (1.95–2.27)	2.16 ± 0.91 (2.04–2.40)
a	40.5 ± 3.08 (36.2–45.5)	54 (51–57)	45.0 ± 3.7 (36.9–47.7)	42 ± 5.3 (35–49)	46.7 ± 3.32 (41.0–53.0)	40.2 ± 1.7 (37.3–42.6)
b	5.88 ± 0.82 (4.9–8.2)	6.8 (5.9–7.6)	5.6 ± 0.6 (4.6–6.4)	5.8 ± 0.7 (4.9–6.8)	5.66 ± 0.54 (4.88–7.09)	5.6 ± 0.4 (5.1–6.6)
c	35.35 ± 4.24 (29.2–46.1)	57 (53–63)	47.5 ± 4.8 (39.2–58.5)	49 ± 6.3 (40–59)	40.4 ± 4.01 (32.3–47.3)	52.8 ± 6.6 (46.6–57.9)
c’	2.24 ± 0.31 (1.8–2.9)	1.5 (1.2–1.7)	1.5 ± 0.2 (1.3–2.1)	1.6 ± 0.21 (1.2–1.9)	1.86 ± 0.20 (1.50–2.18）	1.4 ± 0.1 (1.0–1.6)
V%	26.6 ± 1.12 (24.5–28.8)	26 (24–27)	26.0 ± 1.9 (21.6–28.7)	28 ± 1.4 (26–29)	26.1 ± 0.67 (25.1–27.4)	25.5 ± 0.9 (24.0–27.2)
Odontostyle	114.53 ± 2.97 (109.8–119.7)	112 (110–114)	110.8 ± 5.0 (96.0–118.4)	120 ± 1.6 (118–123)	118.9 ± 2.99 (114.1–123.3)	112.9 ± 2.1 (109.2–117.3)
Odontophore	71.5 ± 1.86 (67.4–75.0)	71 (70–75)	63.9 ± 6.0 (60.8–73.6)	57 ± 2.5 (54–61)	71.6 ± 2.55 (66.7–75.8)	70.5 ± 1.0 (68.3–71.6)
Stylet	186.03 ± 2.82 (181.0–191.7)	183 (180–187)	174.9 ± 8.4 (155.2–184.0)	177 (172–184)	190.6 ± 3.52 (183.3–195.0)	183.4 ± 2.4 (178.6–187.9)
Tail	65.31 ± 8.16 (48.3–77.4)	40 (37–45)	39.7 ± 3.9 (36.7–45.2)	41 ± 4.5 (35–48)	53.0 ± 5.0 (46.0–64.0)	41.5 ± 4.8 (31.1–46.5)
Width at lip region	12.13 ± 0.69 (10.7–13.5)	12 (10–12)	9.8 ± 0.6 (8.7–10.9)	10 ± 0.5 (9–11)	–	12.3 ± 0.3 (11.8–12.9)
Width at mid. body	58.27 ± 3.94 (49.2–63.8)	42 (37–44)	42.7 ± 4.9 (37.4–55.5)	49 ± 5.1 (43–57)	–	–
Width at anus	29.48 ± 3.60 (22.3–35.6)	28 (27–31)	26.3 ± 1.8 (21.3–28.4)	27 ± 1.3 (25–29)	28.0 ± 2.0 (25.0–32.0)	30.0 ± 0.76 (28.9–31.3)

* All measurements in μm, except L in mm; Means ± SD (min.–max.), “–”=No data.

[a] From Wang & Wu, 1992.

[b] From Zheng & Brown, 1999.

[c] From Pan et al., 2000.

[d] From Chen et al., 2004.

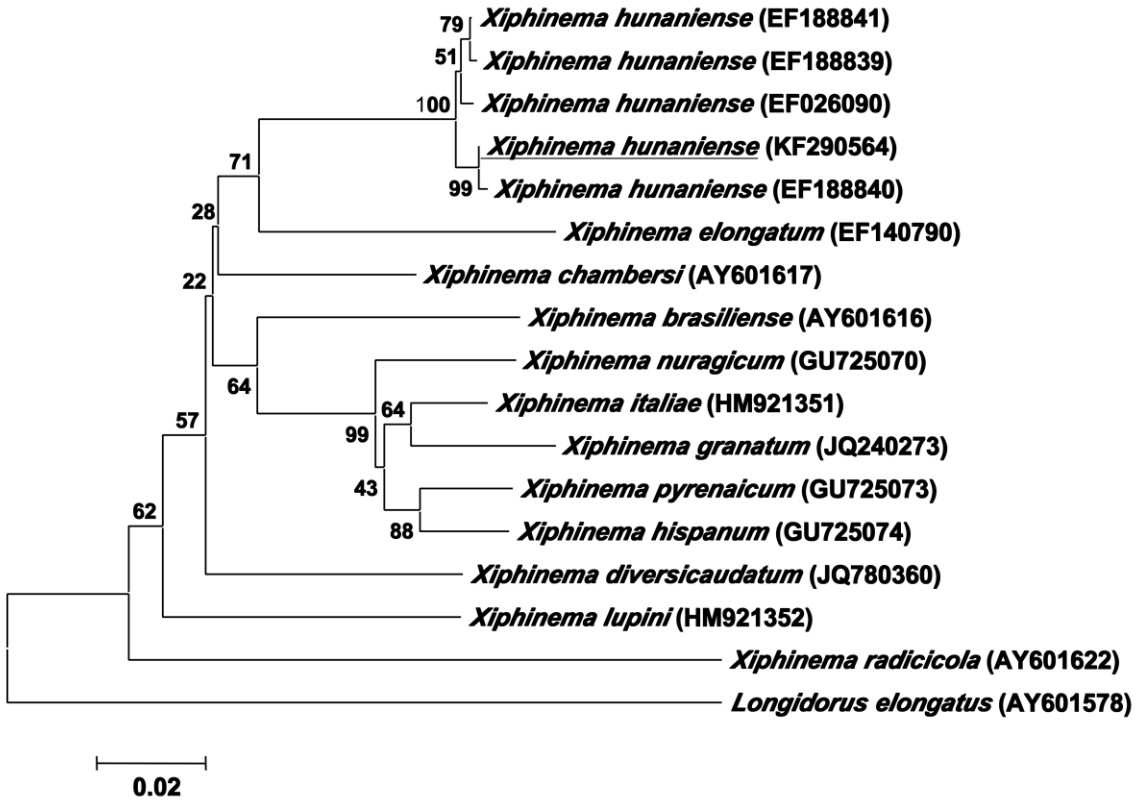
[e] From Wu et al., 2007.

Phylogenetic relationships among *Xiphinema* nematodes were inferred from analyses of 28S rRNA gene of a multiple edited alignment including 17 sequences using MEGA5.0, and NJ tree was constructed (Figs 2). Phylogenetic analysis of 28S rDNA of this study demonstrated that Thailand *X. hunaniense* clustered together with *X. hunaniense* populations from China.

Discussion

The morphology of *X. hunaniense*, *X. radicicola,* and *X. brasiliense* is similar. Loof et al. (1996) considered *X. hunaniense* as a junior synonym of *X. radicicola.* However, later studies (Robbins & Wang, 1998; Zheng & Brown, 1999) re-established the validity of the taxonomic status of *X. hunaniense.*

A comparison of the mean and range of the morphometrics value of the female *X. hunaniense* from Thailand with from China populations (Wang & Wu, 1992; Zheng & Brown, 1999; Pan et al., 2000; Chen et al., 2004; Wu et al., 2007) was made. It showed that the measurement rangs of the *X. hunaniense* populations are partial overlapping. Comparing to the population fom China, the ones from Thailand has smaller c value, higher c' value and higher values in tail length and body width. The a and b values are smaller than those of paratypes (Wang & Wu, 1992), but they are close to those of the rest populations. The odontophore and stylet are higher than those of Camellia and Plum populations, which are close to those of the remaining populations. The rest measurement values are similar to other *X. hunaniense* populations. The same situations happened to the studies of Chen et al. (Chen et al., 2007). According to the report, the Xhun8 *X. hunaniense* population has smaller c value, higher c'value and bigger tail than those of Xhun1-7 populations. Unlike the original descriptions (Wang & Wu,1992), the Thailand female has a posterior genital tract with only single ovary.



Figs 2 –Phylogenetic relationships within *Xiphinema* species based upon sequences of D2-D3 expansion region of the 28S rRNA gene, rooted with *Longidorus elongatus*. The phylogenetic tree was constructed from rDNA sequences registered with GenBank, using MEGA5 with the Neighbor-Joining method. Branch lengths are proportional to the amount of sequence change, which are indicated by the scale bar below the tree, and the whole numbers are bootstrap values for 1,000 analyses.Newly obtained sequence in this study is underlined.

Furthermore, the other studies did not report that the posterior genital tract of the female divided into two branches (Zheng & Brown, 1999; Pan et al., 2000; Chen et al., 2004; Wu et al., 2007) . The values of L [2.1 (2.0-2.2)], V% [27 (26-28)] and stylet [187 (181-196)] of the *X. brasiliense* from Ceylon (Corn & Sher, 1972) are close to those of Thailand *X. hunaniense*. And a comparison of the means and ranges of *X. hunaniense* (Table 1) with those of *X. radicicola* (Luc, 1981; Razak & Loof, 1998) was also made. The measurement ranges of the two species are also partial overlapping. The means analysis with one-way anova demonstrated that the c, c', V%, tail length, width at anus values have a certain influence on differentiating the two species, and the rest measurement values have no significant influence. Perhaps this is one reason that the two species are often confused (Loof et al., 1996). Whether variable morphology and morphometrics within and between populations of *X. hunaniense* or similar morphology and morphometrics among *X. hunaniense*, *X. brasiliense* and *X. radicicola* indeed affect the identification results only based on the morphology and morphometrics. The molecular data of rDNA sequences is more convincing than the morphometrics data, because it is stable within and between populations of *X. hunaniense* (Chen et al., 2004; Wu et al., 2007). Therefore, *X. hunaniense* identification based on sequencing of 28S rDNA combining with morphological and morphometrical data is more rational.

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