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Molecular Characterization of a *Xiphinema hunaniense* Population with Morphometric Data of all Four Juvenile Stages

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Abstract: A population of *Xiphinema hunaniense* Wang and Wu, 1992 with all four juvenile stages was found in the rhizosphere of *Pinus* sp. in Hangzhou, Zhejiang, China. Morphometrics of 18 females and 35 juveniles of this population are given herein. Detailed morphology and morphometrics of the four juvenile stages are provided. Further comparisons based on morphometrics of the population with previous studies of the females and the first-stage juveniles of *X. hunaniense* with *X. radicicola* are given, and morphological variation in *X. hunaniense* populations are discussed. A revised polytomous key code of Loof and Luc (1990) for *X. hunaniense* identification is provided, i.e., A1- B4- C4- D4/5- E1- F2(3)- G2- H2- I3- J4- K2- L1. In addition, the sequence of the D2 and D3 expansion region of the 28S rRNA gene was analyzed and compared with sequences of closely related species downloaded from the NCBI database. Cluster analysis of sequences confirmed and supported the species identifications.
Key words: China, juveniles, morphometrics, nematode, taxonomy, *Xiphinema hunaniense*.

Xiphinema hunaniense Wang & Wu, 1992 was first described from vineyard soils in Hunan province, China, and has been reported in the Chinese provinces of Hunan, Fujian, Shanghai, Zhejiang, Guangxi and Taiwan, from hosts including buntan (*Citrus grandis*), Japanese camellia (*Camellia japonica*), sago palm (*Cycas revolute*), grape (*Vitis vinifera*), Chinese hibiscus (*Hibiscus rosa-sinensis*), litchi (*Litchi chinensis*), longan (*Euphoria longana*), loquat (*Eriobotrya japonica*), mango (*Mangifera indica*), pear (*Pyrus pyrifolia* var. *yokoyama*), pine (*Pinus* sp.), sweet orange (*Citrus sinensis*) and some bonsai plants (*Camellia sasanpua*, *Ligustrum quihoui*). *Xiphinema hunaniense* has not been implicated as a virus vector.

Like most longidorid nematodes, *X. hunaniense* has four juvenile developmental stages and four molts before the adult stage. Only second, third, and fourth-stage juveniles of the species were reported by Wang and Wu (1992) and Zheng and Brown (1999). Chen et al. (2004) found four juvenile stages of the species, but only presented photographs with no detailed description or morphometrics of J1 specimens.

During an investigation of virus vector nematodes at the Institute of Biotechnology, Zhejiang University, Hangzhou, China, a population of *X. hunaniense* with all four juvenile stages was discovered. The presence of first-stage juveniles (J1) in the population provided an excellent opportunity to complete the morphometric data for this species. The present study presents the morphological and molecular variation among inter-species of *X. hunaniense*, including detailed morphological description and molecular characterization of the *X. hunaniense* population from Hangzhou, Zhejiang province, China, and a revised polytomous key code of Loof and Luc (1990) for identification of *X. hunaniense* is provided.

Materials and Methods

Morphological characterization: Nematodes were extracted from soil collected from the rhizosphere of *Pinus* sp. using the decanting and sieving method of Brown and Boag (1988). *Xiphinema hunaniense* specimens were handpicked from the samples, heat killed, and fixed in hot FG (formalin:glycerol 4:1) fixative for a minimum of 7 d. The fixed nematodes were processed to anhydrous glycerine by a modified Seinhorst (1959) method and mounted on slides. All observations of fixed nematodes were made with a light microscope, and photomicrographs and measurements were taken with the software Axiovision 3.1 (Zeiss, Germany).

PCR amplification and sequencing: Three samples were prepared for the population. For DNA extraction of each sample, one to four specimens of *X. hunaniense* were transferred into a 20-µl drop of double-distilled water on a clear glass slide and cut into fragments. The fragments, suspended in 10 µl water, were transferred into a 0.2 ml Eppendorf tube containing 8 µl Worm Lysis Buffer (125 mM KCI, 25 mM Tris-CI pH 8.3, 3.75 mM $MgC1_2$, 2.5 mM DTT, 1.125% Tween 20) and 2 µl of proteinase K (600 µg/ml). After storage at −70°C for 10 min, tubes were incubated at 65°C for 60 min, then at 95°C for 10 min. After centrifugation at 12,000 rpm for 2 min, 2 µl of the DNA suspension was added to the PCR reaction mixture containing 10 µl 10 X Taq incubation buffer, 20 µl 5 X Q solution, 200 µM of each dNTP (Taq PCR Core Kit, Qiagen, Germany), 1.5 µM of each the primers D2A (5′ ACA AGT ACC GTG AGG GAA AGT TG 3′) and D3B (5′ TCG GAA GGA ACC AGC TAC TA 3′) (synthesized by Sangon Technology & Services, Shanghai, China), 1 U Taq polymerase (Taq PCR Core Kit, Qiagen, Germany) and double-distilled water to a final volume of 25 µl. A fragment of the D2-D3 expansion region of the 28S rRNA gene was amplified using the following program: initial denaturation at 94°C for 3 min, 35 cycles at 94°C for 30 sec,

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54°C for 40 sec, and 72°C for 2 min followed by an extension at 72°C for 10 min. After DNA amplification, 3 μl of each PCR product was run on a 1% agarose gel (Zheng et al., 2003).

Purified PCR products were cloned into pUCM-T vector and transformed into DH5α high efficiency competent cells. Several clones of the nematode were isolated by blue/white selection and cycle-sequenced by Shanghai Sangon Biological Engineering Technology & Service Co., Ltd.; the DNA sequences were edited with the Chromas program (v1.3) (Technelysium Pty Ltd, Australia). Sequence of the D2-D3 expansion region of the 28S rRNA gene was deposited to GenBank (http://www.ncbi.nlm.nih.gov) (accession number EF026090).

RFLP and sequence analysis: The PCR products of the 28S region were purified and digested with six restriction enzymes according to the protocols of the company. Five microliters of each purified product was digested with each of the following restriction enzymes, Ava I, Bst EII, Hae III, Rsa I, Eco RI and Mbo I, for PCR products in the buffer stipulated by the manufacturer. The digested DNA was loaded on a 1.5% agarose gel, separated by electrophoresis (70V, 3 hr), stained with ethidium bromide, visualized on a TW-26 Macrovue UV transilluminator and photographed with a Kodak Digital Science 1-D system. Procedures for obtaining PCR-amplified products and endonuclease digestion of these products were repeated several times to verify the results.

For multiple sequence alignment analysis and NJ (neighbor joining method) tree construction, the sequences of the 28S rRNA gene of *X. hunaniense* in this study and those of *X. insigne*, *X. radicicola*, *X. chambersi* and *X. brasiliense* from Genbank were used. Multiple sequence alignments were made using ClustalX software with default options (Thompson et al., 1997), and the sequence distance percent identities were calculated with the ClustalW program of DNAStar software. NJ analysis of the aligned sequences utilized the MEGA2 tool. Bootstrap values based on 1,000 resamplings were determined, and the sequence of the D2-D3 expansion region of the 28S rRNA gene of *Longidorus elongatus* was used as an out-group taxon.

Results

Morphological characters and morphometrics: A total of 18 female and 35 juvenile specimens of *Xiphinema hunaniense* were examined. Morphometrics of females and juveniles and the key morphological characters are presented in Table 1 and Figure 1, respectively.

The female habitus was hook-shaped when heat-killed, identical with descriptions made by Wang and Wu (1992) and Zheng and Brown (1999), and none of the females bore eggs. Except for the genital tract, ju-

Table 1. Morphometrics of *X. hunaniense* population from *Pinus* sp., Hangzhou, Zhejiang Province, China.*

Stage	Female	J4	J3	J2	J1
n	18	11	14	4	6
L	2.16 ± 0.91 (2.04–2.40)	1.67 ± 0.09 (1.48–1.84)	1.26 ± 0.075 (1.13–1.36)	0.99 ± 0.55 (0.91–1.03)	0.80 ± 0.66 (0.75–0.91)
a	40.2 ± 1.7 (37.3–42.6)	36.2 ± 1.6 (33.8–38.8)	34.3 ± 1.8 (31.7–37.6)	32.7 ± 2.2 (30.1–35.1)	30.9 ± 1.7 (27.9–33.0)
b	5.6 ± 0.4 (5.1–6.6)	4.4 ± 0.4 (3.9–5.4)	3.8 ± 0.3 (3.4–4.2)	3.5 ± 0.2 (3.3–3.8)	3.4 ± 0.2 (3.1–3.6)
c	52.8 ± 6.6 (46.6–57.9)	29.7 ± 2.9 (24.1–33.9)	19.2 ± 0.8 (17.8–20.8)	13.1 ± 1.1 (11.5–13.9)	10.9 ± 0.5 (10.5–11.8)
c′	1.4 ± 0.1 (1.0–1.6)	2.1 ± 0.3 (1.6–2.5)	3.0 ± 0.2 (2.6–3.3)	4.2 ± 0.5 (3.7–4.8)	4.9 ± 0.3 (4.4–5.2)
V%	25.5 ± 0.9 (24.0–27.2)	—	—	—	—
Odontostyle	112.9 ± 2.1 (109.2–117.3)	93.5 ± 3.6 (88.8–99.1)	80.9 ± 1.9 (77.3–83.3)	65.4 ± 0.9 (64.3–66.6)	49.6 ± 1.1 (47.9–50.6)
Odontophore	70.5 ± 1.0 (68.3–71.6)	62.8 ± 1.5 (59.8–64.4)	53.1 ± 2.0 (50.3–56.7)	46.1 ± 2.1 (44.1–48.9)	37.5 ± 1.4 (36.3–39.4)
Total stylet	183.4 ± 2.4 (178.6–187.9)	155.4 ± 3.3 (152.1–162.4)	134.7 ± 3.6 (130.7–139)	111.5 ± 1.7 (109.8–113.7)	87.1 ± 2.1 (84.3–89.7)
Replacement of odontostyle no (of)	—	110.7 ± 1.8 (108.3–113.5)	95.7 ± 1.9 (93.5–98.9)	79.5 ± 2.5 (76.9–82.9)	67.6 ± 1.7 (66.1–70.5)
Tail length	41.5 ± 4.8 (31.1–46.5)	56.9 ± 5.8 (46.1–66.1)	66.0 ± 2.9 (61.3–69.9)	75.6 ± 2.7 (72.7–79.1)	72.7 ± 2.6 (70.8–77.4)
Body diam. at lip region	12.3 ± 0.3 (11.8–12.9)	10.6 ± 0.6 (9.9–11.6)	10.0 ± 0.2 (9.6–10.3)	8.6 ± 0.2 (8.4–8.9)	8.0 ± 0.2 (7.6–8.2)
Body diam. at base of esophagus	51.3 ± 2.1 (48.5–54.8)	46.2 ± 2.1 (43.7–49.1)	36.9 ± 2.0 (31.6–40.2)	30.4 ± 3.2 (26.9–34.3)	25.8 ± 3.2 (22.9–30.7)
Body diam. at vulva	53.9 ± 2.3 (50.9–56.2)	—	—	—	—
Body diam. at anus	30.0 ± 0.76 (28.9–31.3)	27.6 ± 1.4 (24.9–29.1)	22.2 ± 1.2 (19.9–23.8)	18.3 ± 1.7 (16.4–20.4)	15.0 ± 1.2 (13.7–16.9)

* All measurements in μm, except L in mm; Means ± SD, range in parentheses.

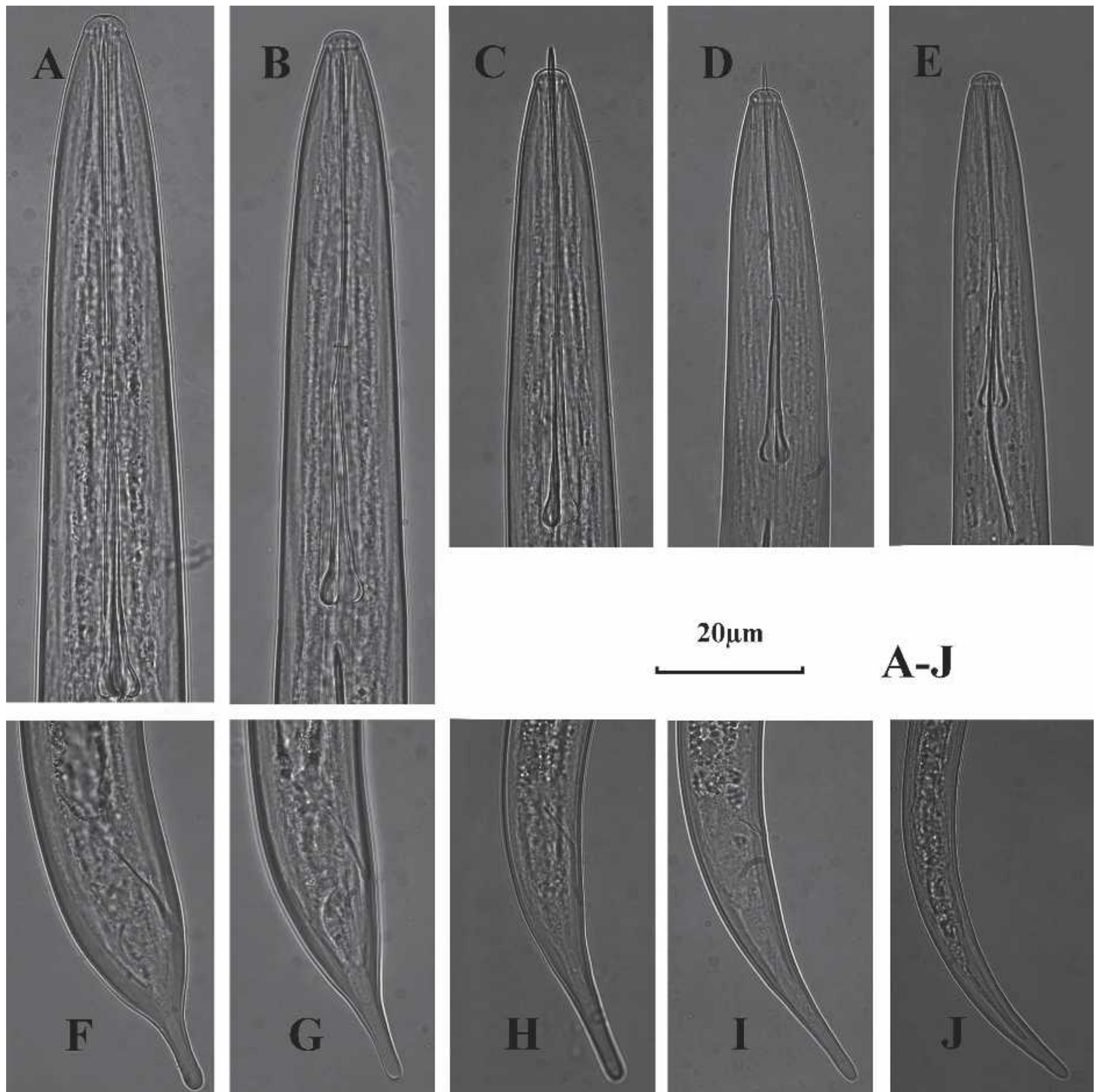

Fig. 1. Photomicrographs of *Xiphinema hunaniense*. A–E) Head regions of female, J4, J3, J2 and J1, respectively. F–J) Tail regions of female, J4, J3, J2 and J1, respectively.

veniles are generally similar to females. The *X. hunaniense* J1 is characterized by the position of the replacement odontostyle, which lies mostly within the odontophore, with the anterior tip near the base of the functional odontostyle (Fig. 1E). The body shape is curved, typical in J4, J3, posterior part of J2 and J1. Tails of J1 are long (c′ = 4.4–5.2), conical, uniformly tapering, without digitate terminus. With each juvenile molt, the tail to body length ratio (c) increases: J1 10.9, J2 13.1, J3 19.2, J4 29.7, and female 52.8. The average tail length increased slightly from 72.7 μm in J1 to 75.6 μm in J2, then decreased to 66 μm in J3, 56.9 μm in J4, and 41.5 μm in females (Table 1). The tail shape changed from conical, uniformly tapering in J1 to distinctly digitate in J4 and females (Fig. 1F-J). No males were found in this population.

After a comparison of several populations reported from China and the new Zhejaing population on *Pinus* sp. in the current study (Tables 1,2), ranges of morphometric variation are: length (1,580–2,500 μm), a (35–57), b (4.5–7.6), c (37–63), c′ (1.0–2.1), V (21.6–29.0), odontostyle (96–123 μm), odontophore (54–75 μm), total stylet (155–188 μm), tail length (31–48 μm), width at lip region (8.7–12.9 μm) and width at anus (21–31 μm). This *X. hunaniense* J1 data combined with the female data results in the following code for identification of *Xiphinema* species in the polytomous key of Loof and Luc (1990): A1-B4-C4-D4/5-E1-F2(3)-G2-H2-I3-J4-K2-L1.

TABLE 2. Morphometric comparisons of *X. hunaniense* and *X. radicicola* from different localities and hosts.*

Species	*X. hunaniense*				*X. radicicola*					
Origin and host	Hunan, grape (paratypes)[a]	Dashu, Litchi[b]	Fuyang, *Camellia japonica*	Zhangzhou, Plum[d]	Paralecotypes, orig.[c]	Ivory Coast, secondary forest[e]	Nigeria, *Bambusa vulgaris*[e]	Australia[e]	Malaysia, coconut[f]	Malaysia, Rambutan[f]
n	10	27	17	13	4	7	4	6	25	25
L	2290 (2070–2500)	1870 ± 120 (1580–2130)	1910 ± 150 (1733–2317)	2010 ± 130 (1740–2160)	2320 (2200–2430)	1780 (1600–1890)	1840 (1740–1930)	2600 (2500–2700)	2280 (1970–2690)	2030 ± 157 (1750–2320)
a	54 (51–57)	45.9 ± 3.64 (36.7–54.0)	45.0 ± 3.7 (36.9–47.7)	42 ± 5.3 (35–49)	53.5 (50.7–58.9)	41.2 (35.5–48.3)	41.7 (37.8–45.3)	64.4 (58–72)	55 (48–66)	52.3 ± 4.08 (45–61)
b	6.8 (5.9–7.6)	5.49 ± 0.74 (4.51–8.43)	5.6 ± 0.6 (4.6–6.4)	5.8 ± 0.7 (4.9–6.8)	6.2 (5.8–6.5)	5.5 (4.7–6.2)	4.3 (3.8–5.3)	6.5 (6.0–7.0)	6.2 (5.0–7.1)	5.65 ± 0.48 (4.3–6.7)
c	57 (53–63)	44.0 ± 3.68 (36.7–54.0)	47.5 ± 4.8 (39.2–58.5)	49 ± 6.3 (40–59)	37.8 (36.7–38.4)	35.6 (29.8–42.0)	31.0 (27.8–32.1)	48.4 (43–58)	45.9 (36.3–56.9)	36.2 ± 6.9 (27.4–49.1)
c′	1.5 (1.2–1.7)	1.66 ± 0.14 (1.30–2.00)	1.5 ± 0.2 (1.3–2.1)	1.6 ± 0.21 (1.2–1.9)	2.3 (2.2–2.4)	2.0 (1.7–2.4)	2.2 (2.1–2.4)	2.0 (1.7–2.4)	1.9 (1.6–2.3)	2.59 ± 0.44 (1.9–3.5)
V%	26 (24–27)	26.6 ± 0.84 (24.4–28.5)	26.0 ± 1.9 (21.6–28.7)	28 ± 1.4 (26–29)	28.4 (27.8–29.4)	29.6 (28.9–30.0)	31.6 (30.3–33.2)	27.6 (25–30)	26 (23–28)	26.4 ± 1.55 (23–29)
Odontostyle	112 (110–114)	111.8 ± 3.19 (105.0–115.0)	110.8 ± 5.0 (96.0–118.4)	120 ± 1.6 (118–123)	—	127 (125–130)	148–152	128 (124–131)	111 (103–123)	112.5 ± 7.28 (98–122)
Odontophore	71 (70–75)	64.6 ± 1.85 (61.7–68.3)	63.9 ± 6.0 (60.8–73.6)	57 ± 2.5 (54–61)	—	62 (59.5–64.5)	69–73	85 (70–84)	64 (60–66)	62.2 ± 2.28 (57–66)
Stylet	183 (180–187)	176.4 ± 3.38 (169.2–184.2)	174.9 ± 8.4 (155.2–184.0)	177 (172–184)	—	189 (186–194)	222 (219–223)	203 (194–210)	175 (167–188)	174.8 ± 8.48 (160–187)
Tail length	40 (37–45)	43.0 ± 3.0 (35.0–47.0)	39.7 ± 3.9 (36.7–45.2)	41 ± 4.5 (35–48)	60.5 (57.5–63.5)	50 (44–58)	59 (56–62)	59 (52–65)	50 (44–59)	57.9 ± 7.54 (46–74)
Width at lip region	12 (10–12)	—	9.8 ± 0.6 (8.7–10.9)	10 ± 0.5 (9–11)	—	—	—	—	11–12	10–12
Width at phryng./ intest. junction	23 (21–23)	—	40.8 ± 4.5 (35.5–52.2)	45 ± 4.3 (40–51)	—	—	—	—	—	—
Width at mid. body	42 (37–44)	—	42.7 ± 4.9 (37.4–55.5)	49 ± 5.1 (43–57)	—	—	—	—	42 (39–45)	38.8 ± 1.64 (34–41)
Width at anus	28 (27–31)	26.0 ± 1.0 (23.0–29.0)	26.3 ± 1.8 (21.3–28.4)	27 ± 1.3 (25–29)	—	—	—	—	26 (22–30)	23.5 ± 1.5 (20–26)

* All measurements in μm, except L in mm; Means ± SD, range in parentheses.
[a] From Wang and Wu, 1992.
[b] From Chen et al., 2004.
[c] From Zheng and Brown, 1999.
[d] From Pan et al., 2000.
[e] From Luc, 1981.
[f] From Razak and Loof, 1998.

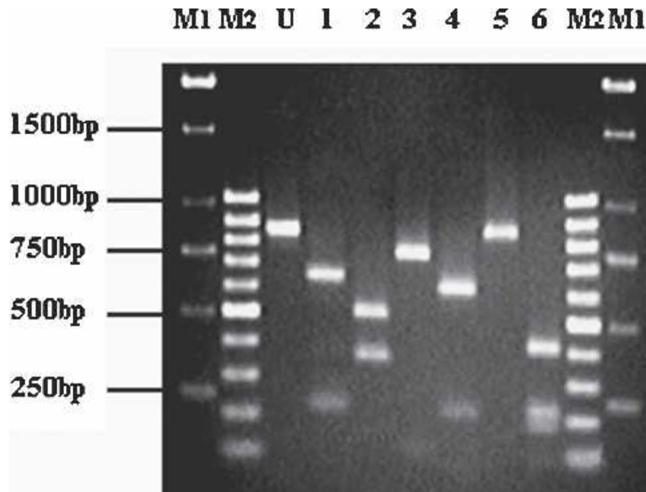

Fig. 2. Restriction fragments of amplified D2-D3 expansion region of the 28S rRNA gene of *X. hunaniense* from Hangzhou. M1: 1Kb marker; M2: 100 bp marker; U: undigested PCR product. 1: Ava I; 2: BstE II; 3: Hae III; 4: Rsa I; 5: Eco RI; 6: Mbo I.

Molecular characterization and relationship with other species: The sequence length of the D2-D3 expansion region of the 28S rRNA gene from *X. hunaniense* is 860 bp (including primers). No sequence variation was detected between the three sequenced samples through PCR-RFLP (Fig. 2 and Table 3) and sequence alignment techniques.

Based on the molecular phylogenetic tree (Fig. 3) generated from the 28S rDNA (D2-D3 expansion region of rRNA gene) sequence alignment, monophyly was implied for *X. insigne*, *X. brasiliense*, *X. chambersi*, *X. hunaniense* and *X. radicicola*, with *X. hunaniense* being closest to *X. insigne*. Similarity indices between 28S rDNA sequence of *X. hunaniense* and other species were 89.8% for *X. insigne*, 84.3% for *X. brasiliense*, 88.4% for *X. chambersi* and 80.5% for *X. radicicola*.

Discussion

The separation of the monodelphic *Xiphinema* species in the *X. radicicola* group, i.e., *X. radicicola*, *X. hunaniense*, *X. chambersi* and *X. brasiliense*, has proven difficult mainly because they all have a relatively short body length (L = 1.5–2.8 mm), possess an anteriorly situated vulva (V = 23–31%) and a simple posterior uterus lacking a Z organ or other ornamentation. Consequently, differentiation of the species in this group is often difficult (Cohn and Sher, 1972). The validity of *X. hunaniense* has been disputed. Loof et al. (1996) considered *X. hunaniense* a junior synonym of *X. radicicola* Goodey, 1936. The comparison of several paratype females of *X. radicicola* collected by Goodey and available in the Thorne component of the USDA nematode collection, Beltsville, MD, with paratype specimens of *X. hunaniense* resulted in Robbins and Wang (1998) re-establishing *X. hunaniense* a valid species. Based on observations of two *X. hunaniense* populations from different hosts in Fuyang, Hangzhou, to data by Cohn and Sher (1972) and by Luc (1981) for syntype and paralectotype specimens of *X. radicicola*, data from Wang and Wu (1992) and from Robbins and Wang (1998), led Zheng and Brown (1999) to concur with the results of Robbins and Wang (1998).

Both morphological and molecular data of the present study, especially the molecular characterization of the D2-D3 expansion region of the 28S rRNA gene, support *X. hunaniense* as a valid species. Compared to *X. radicicola*, *X. hunaniense* has a shorter tail (length ≤ 50 µm), smaller c′ ratio (usually about 1.5), and a shorter odontostyle length (average about 110 µm) (Tables 1,2).

The morphometrics of first-stage juveniles of *X. hunaniense* reported herein complete the morphological data and the code K2 of the species in Loof and Luc's (1990) polytomous key code. A comparison of the mean and range for J1 of *X. hunaniense* to the J1 of *X. radicicola* reported by Razak and Loof (1998) is as follows: odontostyle length 49.6 (47.9–50.6) vs. 49 (44–53) µm, replacement odontostyle length 67.6 (66.1–70.5) vs. 62 (54–70), tail length 72.7 (70.8–77.4) vs. 66 (61–74) and c′ 4.9 (4.4–5.2) vs. 5.3(4.6–6.7), respectively. Because the J1 morphometrics overlap, it is very difficult to differentiate the two species.

Although the polytomous keys aid in the identification of species of *Xiphinema* (Loof and Luc, 1990), those working on *Xiphinema radicicola* group taxonomy

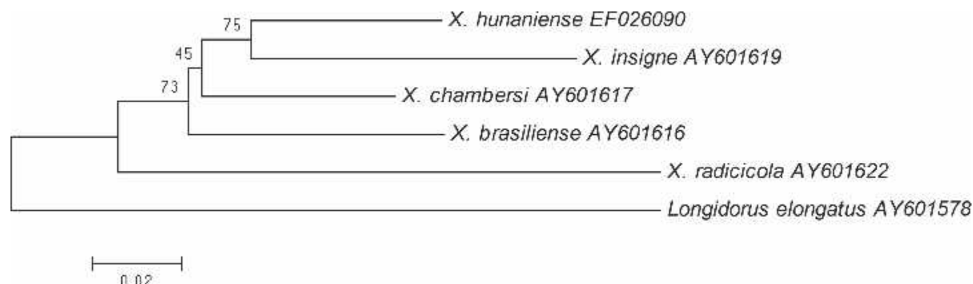

Fig. 3. Phylogenetic relationship of *X. hunaniense* populations to other *Xiphinema* species based upon sequences of D2-D3 expansion region of the 28S rRNA gene, rooted with *Longidorus elongatus*. The phylogenetic tree was constructed from rDNA sequences registered with GenBank, using MEGA2 with the Neighbor-Joining method. The scale represents a relative evolutionary distance, and the whole numbers are bootstrap values for 1,000 analyses.

Table 3. RFLP of D2–D3 expansion region of the 28S rRNA gene of *X. hunaniense* generated using DNAStar software (compared with Fig. 3).

PCR product (bp)	Restriction fragments (bp)					
	AvaI	BstEII	HaeIII	RsaI	EcoRI	MboI
860	643	501	765	611	—*	398
	217	359	95	206		217
				37		176
				6		65
						4

* "—" No cutting site in the fragment of PCR product.

should pay close attention to identification of these morphologically similar species when it is based only on morphological characters and morphometrics. It is more accurate to use molecular data combined with morphological characters to identify species within the *X. radicicola* group.

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