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Longidorus hangzhouensis Zheng et al., 2001 (Nematoda: Longidoridae) from China, with a Description of First-stage Juvenile and a New Host Report

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Abstract: *Longidorus hangzhouensis* was redescribed from a population recovered from the rhizosphere of tea (*Camellia sinensis* L.) cultivated in Longjing Village, Hangzhou, Zhejiang Province, Eastern China, which is the type locality area of the species. It is characterized by a medium body size (3.2-3.9 mm), narrow lip region (8.1-10.5 µm), long odontostyle (106-119 µm) and short hemispherical tail (29-31µm). The main morphological characters correspond well with the original description of the type material collected from *Camellia japonica* in Fuyang, Zhejiang Province, except for the slightly longer tail. The revised codes for identifying *L. hangzhouensis* using the polytomous keys by Chen et al. (1997) and Loof & Chen (1999) are: A345-B1-C34-D1-E4-F1-G1-H1-I1. The phylogenetic relationships of the population were inferred by using D2-D3 expansion segments of 28S and 18S ribosomal DNA. *Longidorus hangzhouensis* clustered with other longidorids having East-Asiatic origin such as *L. camelliae* and *L. asiaticus* (being the closest species in D2-D3 28S and 18S rDNA phylogenetic reconstructions, respectively). The information included in this paper expands our knowledge on the occurrence and distribution of Longidoridae in China and provides additional data on host associations and morphology of *L. hangzhouensis*.

Key words: China, *Longidorus*, morphology, phylogeny, ribosomal DNA, tea

Introduction

The genus *Longidorus* Micoletzky, 1922 includes a number of species with a slender body ranging from 2 to 12 mm; they are economically important group causing severe damages to agricultural crops, also by transmitting plant viruses (Taylor & Brown 1997). Only 6% of 150 listed nominal *Longidorus* species have been reported as virus vectors (Decraemer & Robbins 2007). A list of 159 valid species was provided by Peneva et al. (2013) and up-to-date, the genus includes 168 species after the addition of nine recently described species: *L. perangustus* Roshan, Pourjam & Pedram, 2015, *L. persicus* Esmaeili, Heydari, Archidona-Yuste, Castillo & Palomares-Rius, 2017, *L. asiaticus* Trisciuzzi, Archidona-Yuste, Troccoli, Fanelli, De Luca, Vovlas & Castillo, 2015 and other six species described by Archidona-Juste et al. (2016). Hitherto, 15 species have been reported in mainland China (Zhang & Chen 1994, Wang et al. 1996, Zheng et al. 2002, Guo et al. 2011): *L. camelliae* Zheng, Peneva & Brown, 2000, *L. fangi* Xu & Cheng, 1991, *L. hangzhouensis* Zheng, Peng, Robbins & Brown, 2001, *L. intermedius* Kozlowska & Seinhorst, 1979, *L. fursti* Heyns, Coomans, Hutsebaut & Swart, 1987, *L. jiangsuensis* Xu & Hopper, 1990, *L. jonesi* Siddiqi, 1962, *L. litchii* Xu & Cheng, 1992, *L. macromucronatus* Siddiqi, 1962, *L. martini* Merny, 1966, *L. moniloides* Heyns, 1966, *L. pisi* Edward, Misra &

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Singh, 1964, *L. pawneensis* Luc & Coomans, 1988 and *L. henanus* Xu & Cheng, 1992.

Longidorus hangzhouensis was described from the rhizosphere of *Osmanthus* (Sweet-scented olive) and *Camellia japonica* L. (Japanese camellia) from Hangzhou and Fuyang, Zhejiang Province (Zheng et al. 2001). In this paper, morphological and molecular characterisation of a population of *L. hangzhouensis* recovered from the rhizosphere of Chinese tea grown in Longjing Village, Zhejiang Province, is provided.

Materials and Methods

Soil samples were collected in October 2015 from the rhizosphere of Chinese tea in Longjing Village, Hangzhou, Zhejiang, China, where is the type locality area of *L. hangzhouensis*. Soils and roots were taken from a depth of approximately 20-40 cm. A sub-sample of 500 g was processed for nematode extraction using sieving and decanting technique of Brown & Boag (1988). Residues were left overnight and nematode suspension was collected the next day. Suspension was examined under a stereomicroscope and target nematodes were handpicked and heat killed. Morphological examination and mounting of nematodes were derived from the method of Seinhorst (1959). Photographs of the specimens were taken using Leica DM5000B compound microscope with a digital camera. Measurements were made using LAS program and morphometric values were presented in micrometers (μm) except when noted otherwise.

DNA isolation and amplification

Genomic DNA was extracted from five individual nematodes as described by Ye et al. (2004). The DNA isolated from each individual nematode was directly amplified. The partial 18S region was amplified using the forward primer SSU_F_07 (AAA GAT TAA GCC ATG CAT G) and reverse primer SSU_R_81 (TGA TCC WKC YGC AGG TTC AC) (Trizciuzzi 2015) while D2-D3 expansion segment of 28S rDNA was amplified using D2A (5'-ACA AGT ACC GTG AGG GAA AGT TG-3') and D3B (5'-TCG GAA GGA ACC AGC TAC TA-3') primers (De Ley et al. 1999). PCR amplification were conducted in BIO-RAD S1000 thermal cycler with thermal cycling conditions of 95°C for 5 min pre-denaturation, 35 cycles at 94°C for 30 sec denaturation, 55°C for 45 sec annealing, 72°C for 2 min extension and 72°C for 10 min final extension (Oliveira et al. 2004). Aliquots of 2 μl PCR products were visualised in 1% Tris-acetate-EDTA (TAE) buffered agarose gel (100V, 400 mA, 30 min) stained with DuRed 10,000x dye (Cat#D009-500) and viewed under UV light. Amplified PCR products were purified following the instructions as described in Takara Minibest DNA fragment Purification kit Ver.4.0 (catalogue No. 9761) of the Takara ClonTech company in Shanghai, China. Purified DNA were cloned to pUCM-T vector and transformed in to DH 5-alpha competent cells. The transformants were screened on an ampicillin agar LB plates containing 400 μl IPTG, X-Gal and left at 37^0C overnight. Several clones of the nematode were isolated by white colonies and transferred to 5 ml LB containing 100 μl/μg ampicillin and incubated at 37^0C for 16 – 24 hs. Sequencing was done at SANGON Biotechnology Co., Ltd.

Sequence and phylogenetic analyses

The sequences of *L. hangzhouensis* were deposited in the GenBank with accession numbers KY945045 and KY945046 for 18S rRNA gene and D2-D3 expansion domains of 28S, respectively. The D2-D3 and 18S sequences were compared with those of other nematode species deposited in the GenBank database using BLASTn similarity search tool. The closest D2-D3 and 18S sequences to *L. hangzhouensis* were aligned using the GUIDANCE2 Server available at http://guidance.tau.ac.il/ with default parameters (Sela et al. 2015). Bayesian Inference (BI) algorithm implemented in MrBayes 3.2.5 was used for phylogenetic relationships reconstruction (Huelsenbeck & Ronquist 2001, Ronquist et al. 2012). For further details, see Lazarova et al. (2016).

Results

Longidorus hangzhouensis Zheng, Peng, Robbins & Brown, 2001

Morphological studies

(Table 1, 2; Figs. 1, 2)

Female: Body spiral to G-shaped when heat killed (Figs. 2 C-G). Cuticle smooth, consisting of two layers, 2 μm thick at the mid body and 9-11 μm at tail terminus. Lip region narrow and rounded anteriorly (Figs. 2 H-L), continuous with the body profile. Odontostyle relatively long, odontophore with weakly developed base. Pharyngeal bulb cylindrical measuring 83-94 x 14-18 μm (Fig. 2T), location of pharyngeal gland nuclei normal with dorsal gland nucleus at about anterior third of the bulb, nuclei of ventro-sub lateral gland at the middle. Cardia hemispherical. Reproductive system didelphic with equally developed branches extending 233 μm anteriorly

Table 1. Measurements (in µm and in the form, mean±standard deviation and range) of juvenile stages of Longidorus hangzhouensis recovered from Chinese tea in Longjing Village, Zhejiang, China.

Locality	**Longjing Village, Hangzhou, China**			
Host	***Camellia sinensis* (tea)**			
Stages	**J_1**	**J_2**	**J_3**	**J_4**
N	2	4	7	10
L (mm)	1.474, 1.45	1.81±3.4 (1.77-1.85)	2.06±1.8 (1.83-2.33)	2.5±3.8 (1.99-2.99)
Body diameter	30, 30	34.8±3.6 (32-40)	38.1±3.2 (33-42.5)	41.9±5.3 (36-49)
Pharynx	270, 273.5	278.1±5.5 (270-284)	285.5±10.2 (272-296)	304.8±32.9 (269-365)
Tail	36, 37	35.8±0.8 (35-37)	33.4±0.89 (32.0-34.2)	32±1.0 (32-34)
a	49.7, 47.9	52.4±5.6 (45.06-58.6)	54.1±3.7 (48.2-59.8)	59.8±3.3 (55.3-63.5)
b	5.5, 5.3	6.5±0.20 (6.2-6.7)	7.2±0.46 (6.6-7.9)	8.2±0.81 (7.4-10.3)
c	40.9. 40.3	50.4±1.4 (48.3-51.4)	61.7±6.6 (54.2-71.4)	88.02±9.6 (85.9-97.7)
c’	1.9, 1.7	1.3±0.11 (1.2-1.5)	1.1±0.1 (0.99-1.3)	0.99± 0.07 (0.89-1.1)
Odontostyle	75, 76	79.3±2.0 (78-82)	85.3±6.3 (81-92)	94.6±4.4 (90-102)
Odontophore	44, 39	53.7±1.4 (53-56)	55.7±3.3 (52-60)	59.2±4.4 (54-64.5)
Total stylet	119	133.1±2.6 (131-135.5)	142±6.7 (133-150)	153.8±7.2 (144.5-163)
Replacement odontostyle	84, 85	86.8±1.4 (79-88)	96.2±3.2 (92-100)	111.8±6.2 (99-117)
Guiding ring from oral aperture	27, 27	30.1±2.1 (27-31.5)	31.7±0.7 (31-33)	35.2±1.9 (32-38)
Width at lips	6, 6	6.9±0.5 (6-7.5)	7.5±0.4 (7-8)	8.2±1.04 (7-10)
Width at guiding ring	13, 16	18.8±1.5 (17-20.5)	19.8±1.1 (18-21)	22.8±1.4 (20-25)
Width at base of pharyngeal bulb	27, 26	32.2±2.9 (29-36)	38.2±3.1 (33-41)	42.9±5.3 (35.5-51)
Width at anus	19, 22	27.4±1.9 (25-29)	29.5±2.21 (26.5-32.3)	31.1 ±2.2 (28-34)

and 228 posteriorly (Fig. 2S), uteri narrow and 76-99 µm long. Tail short and symmetrical, terminus bluntly rounded to hemispherical (Figs. 2 M-Q). Based on the original description and the present study, the revision codes for identifying *L. hangzhouensis* using the polytomous key by Chen et al. (1997) are proposed A345-B1-C34-D1-E4-F12-G1-H1-I1.

Juveniles: Four developmental stages found, general morphology similar to adult, body habitus in all stages varies from open C to J-shaped. Tails of all juvenile stages appear conical but becoming hemispherical to bluntly conoid and *c’* value decreasing in successive stages. Juvenile stages were separated according to body length, position of replacement odontostyle and odontostyle length (Fig.1 A-D). In first stage juvenile (J_1), replacement odontostyle inserted in the posterior end of odontophore (Fig. 1A), while the three remaining juvenile stages can be separated accordingly to the length of functional and replacement odontostyle (Fig. 1C-D).

Molecular studies

The amplification products of D2-D3 expansion segments of 28S and 18S genes of *L. hangzhouensis* yielded fragments of 851 bp and 1770 bp, respectively. The D2-D3 and 18S sequences of *L. hangzhouensis* from China were obtained for the first time. For both 28S and 18S rDNA regions BLASTN results

Table 2. Morphometric comparisons of Longidorus hangzhouensis females from Longjing, Hangzhou, and reported specimens from other locality of Zhejiang, China. Measurements are in µm and in the form, mean ± standard deviation and range).

Origin Host	Longjing Village	Hangzhou	Fuyang	Ningbo
	Camellia sinensis (L.) (Chinese tea)	*Osmanthus delavayi* Franch. (Scented olive)	*Camellia japonica* L. (Japanese camellia)	*Metasequoia glyptostroboides* Hu & W.C. Cheng Metasequoia
N	8	8	12	22
L (mm)	3.37±2.4 (3.16-3.87)	4.24±0.55 (3.25-4.9)	3.49±0.28 (3.08-3.95)	3.50±0.53 (2.77-4.40)
a	62.9±3.3 (58.9-67)	62.4±3.5 (57-68)	62.6±3.5 (58-68)	61.48±5.26 (53.11-72.93)
b	9.8±1.0 (8.8-11.9)	12.4±3.4 (8.7-18.1)	9.9±1.2 (8.5-11.8)	8.45±0.79 (6.9-9.65)
c	134.2±11.3 (117.8-148.9)	138±15 (113-155)	133.4±12.2 (121-154)	118.79±19.54 (88.49-158.15)
c’	0.79±0.1 (0.66-0.88)	0.7±0.05 (0.7-0.8)	0.7±0.1 (0.7-0.8)	0.72±0.10 (0.55-0.95)
Tail	29.7±0.7 (29-31)	31.6±1.9 (30-34)	26.3±2.5 (23-30)	29.61±2.59 (24.86-35.07)
V	48.8±1.3 (47.2-51.1)	49.7±1.0 (48-51)	49.4±1.2 (47-50)	50.24±1.03 (49.71-52.24)
Odontostyle	111.3±4.4 (106-119)	118±9.8 (97-126)	111±3.6 (107-116)	125.92±5.85 (116.77-142.69)
Odontophore	62.1±2.7 (57-65)	65.2±6.3 (53-71)	57.1±2.1 (55-59)	69.76±6.96 (56.32-85.09)
Guiding ring from oral aperture	36.5±1.3 (35-39)	42.3±4.9 (39-47)	36.7±1.1 (35-38)	44.39±2.87 39.14-51.97)
Width at lips	8.9±0.9 (8-10.5)	10.6±0.9 (9-12)	8.9±0.6 (8-9.5)	12.25±0.84 (10.88-14)
Width at guiding ring	25.4±1.5 (23-28)	30.5±2.8 (27-35)	25.2±1.3 (23-27)	29.75±2.41 (25.71-34.27)
Width at base of pharyngeal bulb	52.6±5.1 (45.5-60)	55.8±5.8 (49-64)	47.9±3.5 (43-53)	49.85±7.99 (37.98-63.55)
Width at anus	37.9±4.4 (33-45)	44.1±4.4 (39-52)	35.7±1.9 (33-39)	41.75±6.07 (32.09-53.21)
Width at vulva	58.5±1.7 (56-61)	68.1±7.4 (57-81)	55.4±2.1 (53-58)	55.36±9.73 (42.25-72.43)

showed very high sequence similarity (99.8-99.9%) of current *L. hangzhouensis* population to several sequences (KJ741238-39; KJ755431-32) from Japan identified as *Longidorus* sp. JH-2014 (1-2 nt difference). We suppose that all *Longidorus* sp. JH-2014 sequences belong to the same species *L. hangzhouensis*, however no data on the morphology of this population have been published (Gu & He, unpublished).

The phylogenetic relationships of *L. hangzhouensis* with the closest species based on 28S and 18S rDNA sequence datasets are presented in Figs. 3-4. *Longidorus hangzhouensis* clusters with other *Longidorus* spp. having Asiatic origin in both phylogenetic reconstructions. In D2-D3 tree, *L. camelliae* (AY601585) is closely related to *L. hangzhouensis* and both species grouped in a well-supported clade with other longidorids from Asia: *L. asiaticus, Longidorus* sp. JH-2014 and *L.* cf. *hangzhouensis* (see Trisciuzzi et al. 2015, Gu & He, unpublished). In the 18S phylogenetic tree, *L. hangzhouensis* clustered with *L. asiaticus*, *L.* cf. *hangzhouensis* and *Longidorus* sp. JH-2014 with high support.

Discussion

The original description of *L. hangzhouensis* included only three juvenile stages (II - IV) and DNA sequences were not provided. *Longidorus hangzhouensis* sequences presented in GenBank and found in imported *Camellia* plants from Japan did not have morphological description (Gu & He unpublished). Hitherto, only few populations of this spe-

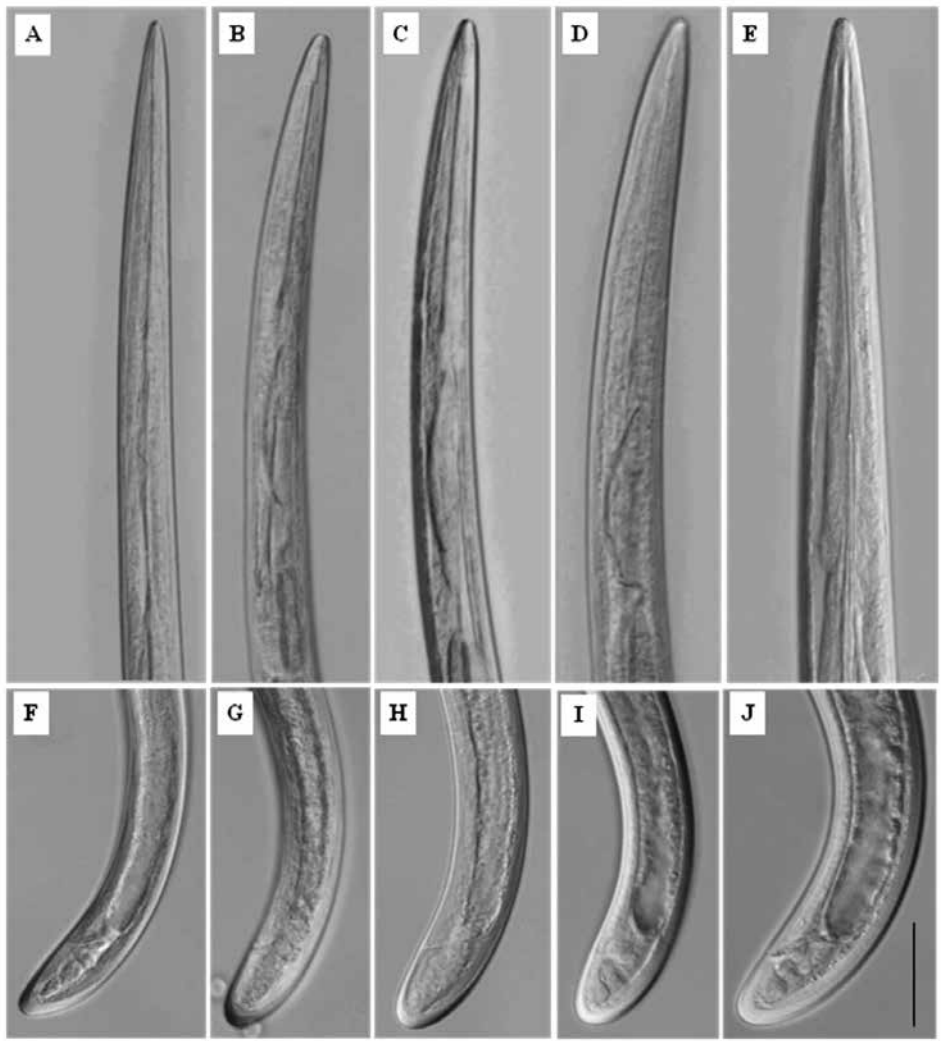

Fig. 1. *Longidorus hangzhouensis* Zheng, Peng, Robbins & Brown, 2001, juvenile stages to adult. A-D. Anterior region of J_1-J_4; E. Female head; F-I. Tails of J_1-J_4 ; J. Female tail.

cies have been reported and described from China (Guo et al. 2011) and no molecular data have been provided. In this study, *L. hangzhouensis* was recovered from the type locality area (Zheng et al. 2001). Morphologically, the population corresponds well to the specimens of *L. hangzhouensis* from Fuyang except for the slightly longer tail (*av*. 29.7 vs 26.3 µm), longer odontophore (*av*. 62.1 vs 51.1µm), greater anal and vulval body width (*av*. 37.9 vs 35.7 µm, *av.* 58.5 vs 55.4 µm). In comparison with the type population from Hangzhou, the two former populations have slightly narrower lip area (*av.* 8.9 and 8.9 vs 10.6 µm), slightly longer odontostyle (*av.* 111.3 and 111 vs 118 µm), shorter body (*av.* 3.37 and 3.49 vs 4.24 µm), somewhat more anterior position of guide ring (*av.* 36.5 and 36.7 vs 42.3 µm) and slightly shorter tail (*av.* 29.7 and 26.3 vs 31.6 µm). Guo et al. (2011) reported *L. hangzhouensis* in association with *Metasequoia glyptostroboides*; however, obvious dissimilarities in ranges of morphometrics are observed when compared to the type material, the specimens having longer odontostyle (117-143 vs 97-126 µm), wider lip region (11-14 vs 9-12 µm) and lower *b* and *c* values (*av.* b=8.45 vs. 12.4 µm; c=118.79 vs 138 µm). It is assumed that the common morphometric resemblance of *L. hangzhouensis* from Longjing and Fuyang are explained by relevant factors such as similar host association (*Camellia*) and the origin of locality (Zhejiang Province). Moreover, morphological variations were observed to those *L. hangzhouensis* populations recovered in different host, e.g. *Osmanthus* and *Metasequoia.*

The D2-D3 28S and 18S rDNA sequences of *L. hangzhouensis* described in the present study were almost identical to those deposited for *Longidorus* sp. JH-2014 by Gu & He. Even if no morphological data of the latter population have been provided, we assume that all *Longidorus* sp. JH-2014 sequences found in the imported *Camellia* plants from Japan belong to *L. hangzhouensis*. The same authors (Gu & He) have

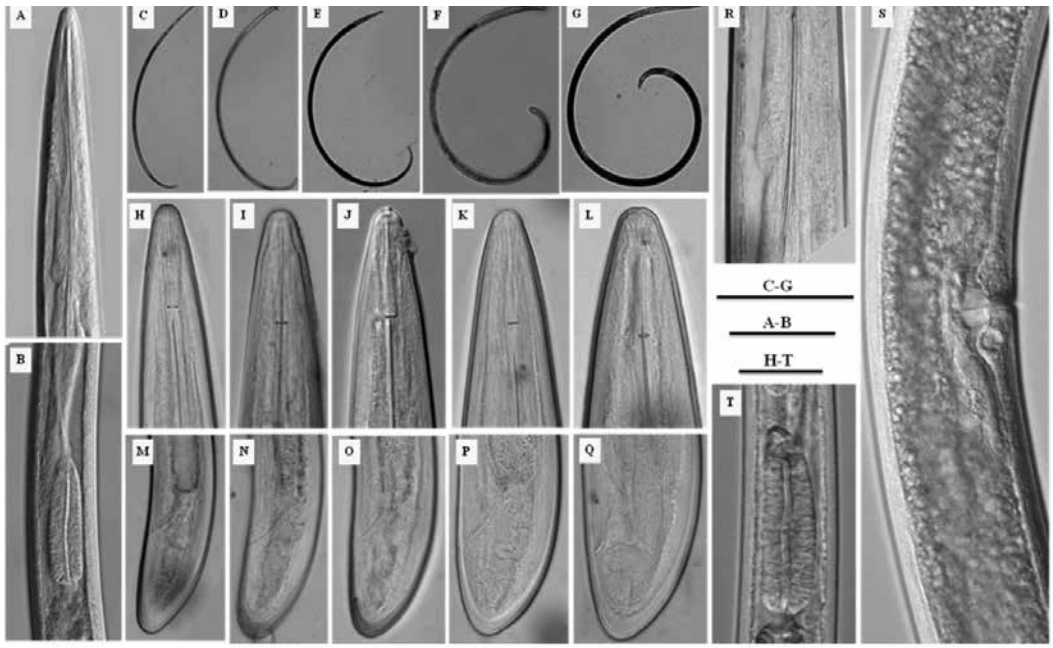

Fig. 2. *Longidorus hangzhouensis* Zheng, Peng, Robbins & Brown, 2001. A-B. Adult anterior region C-G. Habitus of juvenile stages to adult; H-L. Head region of J1-J4 and adult M-Q. Tail J1-J4 and adult R. Odontophore S. Female reproductive system; T. Pharyngeal bulb.

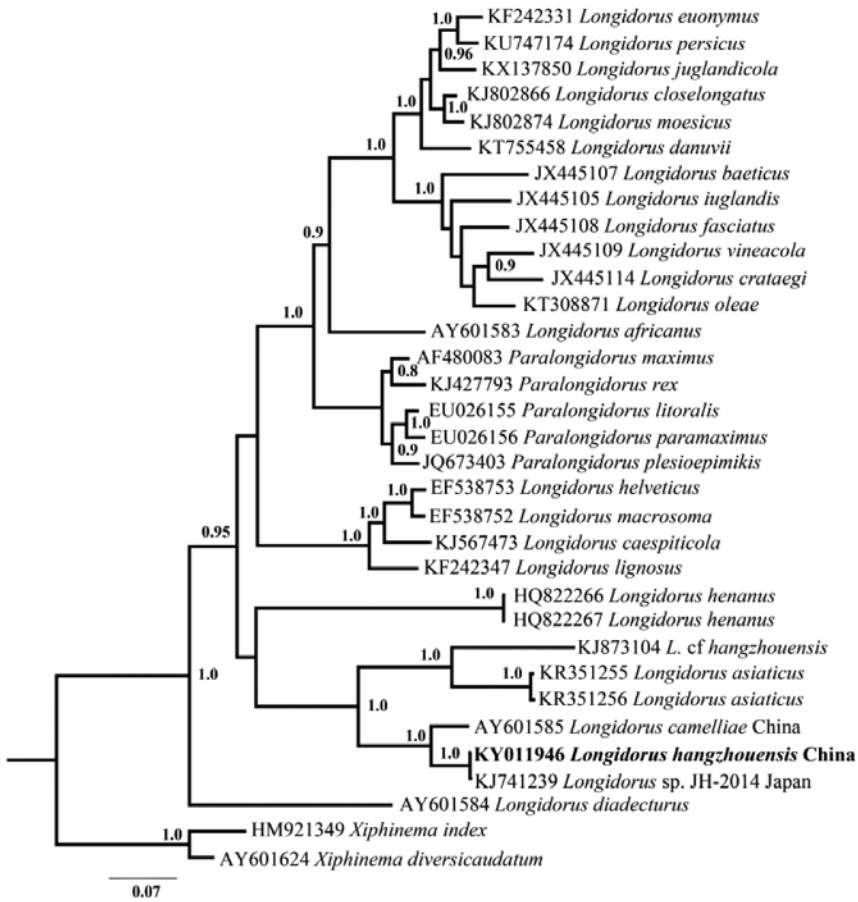

Fig. 3. Hypothesis of the phylogenetic relationships of *Longidorus hangzhouensis* based on D2-D3 28S rDNA inferred from a Bayesian analysis using GTR+G model.

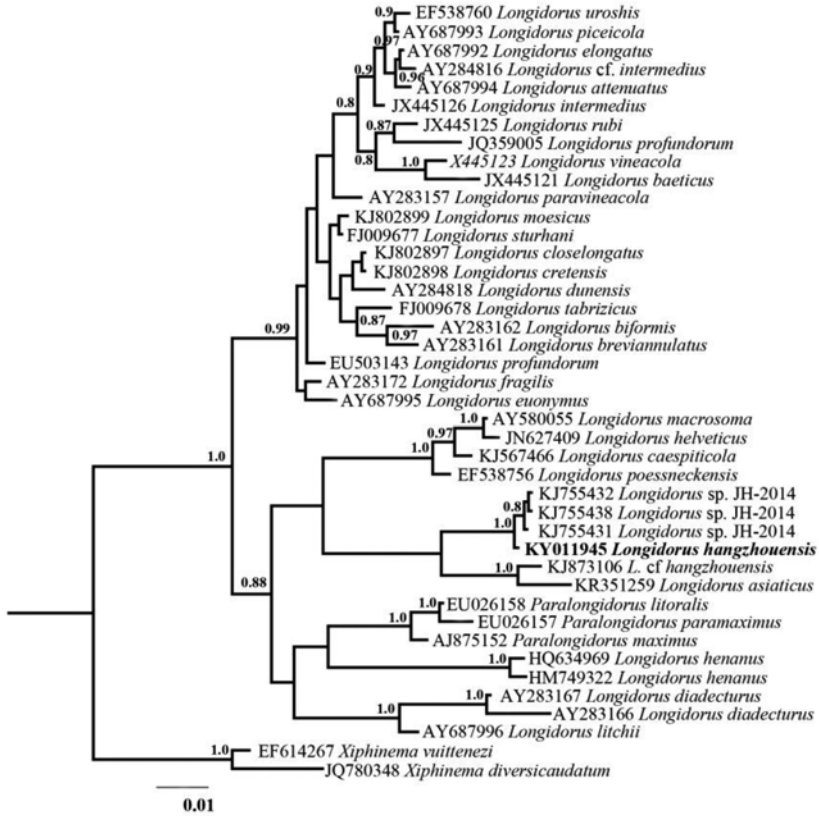

Fig. 4. Hypothesis of the phylogenetic relationships of *Longidorus hangzhouensis* based on 18S rDNA inferred from a Bayesian analysis using GTR+G model.

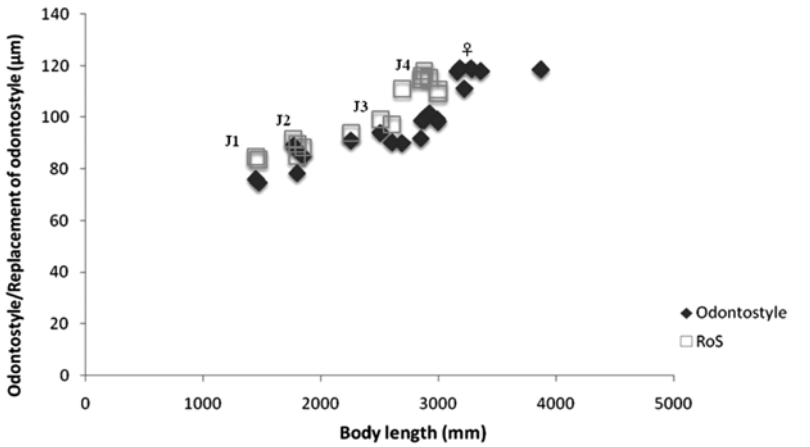

Fig. 5. Scatter plot of the relationships between functional odontostyle and replacement odontostyle (RoS) against body length of *Longidorus hangzhouensis* juveniles (J_1-J_4) and females.

deposited in GenBank another two sequences identified as *L. hangzhouensis* isolated from the same imported *Camellia* plants from Japan but these sequences were not related to *L. hangzhouensis* from China (Figs. 3, 4) collected from the type locality area. In fact, these sequences showed lower sequence similarity both for the D2-D3 domains and 18S rDNA gene of *L. hangzhouensis* determined in the present study (85 and 98%, respectively). Moreover, the relationships inferred in D2-D3 and 18S phylogenies showed closer relationship of the Japanese *L.* cf. *hangzhouensis* population with *L. asiaticus,* a species recently described by Trisciuzzi et al. (2015). We assume that both previously deposited sequences (KJ873104 and KJ873106) identified as *L. hangzhouensis* from Japan could be a result of misidentification; so far no publi-

cation with morphological description of this population is available (Gu & He unpublished).

Comparing the closest relationships of *L. hangzhouensis* population from Longjing to the sister species *L. camelliae* and *L. asiaticus*: *L. hangzhouensis* and *L. asiaticus* share almost similar range in body size (2.7-3.5 vs 3.2-3.8 µm), c' value (0.6-0.9 vs 0.66-0.88 µm), position of guiding ring (34.5-40.5 vs 35.3-39.2 µm) and having short conoid tail with rounded tip (23.5-33.5 vs 29-30.7 µm), while *L. camelliae* varies in having a higher *c'* value (1.1-1.4 µm) and elongated conical tail with broadly rounded terminus (32-43 µm). The only difference of the present population is the longer odontostyle (106-118.7 µm) as compared to *L. asiaticus* (78-92.5 µm) and *L. camelliae* (80-91 µm). Moreover, *L. hangzhouensis* and *L. asiaticus* were reported with four juvenile stages while *L. camelliae* has only three. The shape of first stage juvenile varied greatly from conoid-subdigitate with dorsal depression at hyaline region in *L. asiaticus*, bluntly conical terminus in *L. hangzhouensis* and slender conical in *L. camelliae*.

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