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Cryphodera sinensis n. sp. (Nematoda: Heteroderidae), a non-cyst-forming parasitic nematode from the root of ramie *Boehmeria nivea* in China

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Abstract

Cryphodera sinensis n. sp. is described from ramie (*Boehmeria nivea*) based on the morphology and molecular analyses of rRNA small subunit (SSU), D2D3 expansion domains of large subunit (LSU D2D3) and internal transcribed spacer (ITS). This new species is characterized by oval females with a distinct subcrystalline layer and pronounced and protruding vulval lip, distinctly concave vulva–anus profile and a vulva–anus distance of 29.5–35.8 μm. Males possess two annuli in the lip region, a stylet 27–32.5 μm in length with round knobs sloping slightly posteriorly, lateral fields with three lines, spicules 20–28 μm long and the presence of a short cloacal tube. Second-stage juveniles possess three lip annuli, a stylet 28–31 μm in length with well-developed knobs projected anteriorly and three lines along the lateral field. The pointed tail, 52–65 μm long, possesses a mucro-like tip and a hyaline region, 24.5–35 μm long. Large phasmids with a lens-like structure are located 2–6 annuli posterior to the anus. Phylogenetic analysis shows that the species has unique SSU, LSU D2D3 and ITS rRNA sequences. Phylogenetic relationships of the three rDNA sequences of *C. sinensis* n. sp. and other cystoid/cyst nematodes are analysed together with a comparison of other species within the genus *Cryphodera*.

Introduction

The family Heteroderidae of plant-parasitic nematodes contains cyst-forming (or cyst) genera and non-cyst-forming (or cystoid) genera. In China, cyst-forming genera, including *Heterodera* Schmidt, 1871, *Globodera* Skarbilovich, 1959 and *Cactodera* Krall & Krall, 1978, have been reported (Chen *et al.*, 1994; Pan *et al.*, 1997; Zhuo *et al.*, 2013), but not non-cyst-forming genera. *Cryphodera* Colbran, 1966, a non-cyst-forming genus, was established in 1966. This genus currently comprises six species (Karssen & Van Aelst, 1999). During a survey of cyst nematodes in China's tropical and subtropical regions in May 2012, a *Cryphodera* species was recovered from ramie (*Boehmeria nivea* (Linn.) Gaudich) in Yiyang,

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a city in Hunan Province in the subtropics. Comparative morphological, morphometric and molecular studies of the nematode revealed that it differed from all other nominal species in the genus and it is herein described as a new species, *Cryphodera sinensis* n. sp. Phylogenetic analyses on small subunit (SSU), D2D3 expansion domains of large subunit (LSU D2D3) and internal transcribed spacer (ITS) rRNA sequences were performed to investigate the relationship of *C. sinensis* n. sp. with other available DNA sequences of cystoid and cyst nematodes.

Materials and methods

Collection and examination of nematodes

Cryphodera sinensis n. sp. was extracted from ramie in Yiyang City, Hunan Province, China. Females were isolated from soil samples around ramie roots by the sieving technique (Subbotin *et al.*, 2010a) or dissected from roots. Males and second-stage juveniles (J2) were extracted from soil samples by the Baermann funnel method. J2s were also released from crushed females.

Females were transferred to 4% formalin and processed according to the method by Bajaj *et al.* (1989). Perineal patterns of mature females were prepared by the procedures for *Meloidogyne* (Hartman & Sasser, 1985). Males and J2s were relaxed by gentle heat, fixed in 4% formalin and processed by the glycerin-ethanol method (Feng, 2001). Nematodes were observed, illustrated, measured and photographed with the aid of a Nikon ECLIPSE Ni microscope equipped with a Nikon Digital Sight Camera and exclusive NIS-Elements BR software (Nikon, Japan).

Molecular analysis

For molecular analysis, the DNA samples of single J2s and males were prepared according to the method by Mundo-Ocampo *et al.* (2008). Three rRNA fragments, i.e. SSU, LSU D2D3 and ITS, were amplified. SSU was amplified as two partially overlapping fragments using two pairs of primers. The first primer pair was 988F (5′-CTCAAAGATTAAGCCATGC-3′) and 1912R (5′-TTTACGGTCAGAACTAGGG-3′) and, the second primer pair was 1813F (5′-CTGCGTGAGAGGTGAAAT-3′) and 2646R (5′-GCTACCTTGTTACGACTTTT-3′) (Holterman *et al.*, 2009). Detailed protocols of polymerase chain reaction (PCR) amplification were as described by Holterman *et al.* (2009). Two partial SSU fragments were integrated as a near-full-length SSU sequence using DNAStar software (Burland, 2000). Primers for LSU D2D3 amplification were D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) (Subbotin *et al.*, 2006). Primers for ITS amplification were TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) (Subbotin *et al.*, 2000). Detailed protocols of PCR amplification were as described by Tanha Maafi *et al.* (2003). The sequencing program was the same as described in Zhuo *et al.* (2010). The newly obtained sequences were deposited in the GenBank database.

The sequences of *C. sinensis* n. sp. were compared with GenBank accessions from other nematode species using the BLAST homology search program (http://blast.ncbi.nlm.nih.gov/). The closest sequences were selected in phylogenetic analyses. Outgroup taxa for each dataset were chosen according to previous molecular phylogenetic analysis for cyst nematodes (Subbotin *et al.*, 2001; Mundo-Ocampo *et al.*, 2008; Bernard *et al.*, 2010). DNA sequences were aligned by ClustalW (http://workbench.sdsc.edu, Bioinformatics and Computational Biology group, Department of Bioengineering, UC San Diego, California, USA), using default parameters. The alignment quality was examined by eye and optimized. The model of base substitution was evaluated using MODELTEST (Posada & Crandall, 1998; Huelsenbeck & Ronquist, 2001). The Akaike-supported model, the base frequencies, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates were used in phylogenetic analyses. Bayesian analysis was performed to confirm the tree topology for each gene separately using MrBayes 3.1.0 (Huelsenbeck & Ronquist, 2001) running the chain for 1×10^6 generations and setting the 'burn-in' at 1000. The MCMC (Markov Chain Monte Carlo) method was used within a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees (Larget & Simon, 1999) using the 50% majority-rule.

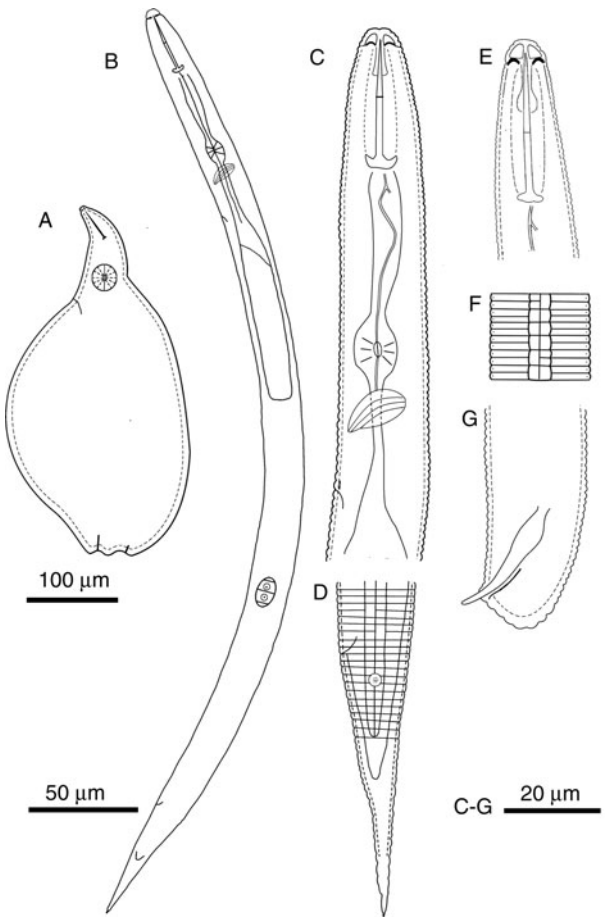

Fig. 1. *Cryphodera sinensis* n. sp.: (A) female; (B) second-stage juvenile (J2); (C) anterior region of J2; (D) lateral field and tail of J2; (E) head region of male; (F) lateral field of male; (G) tail of male.

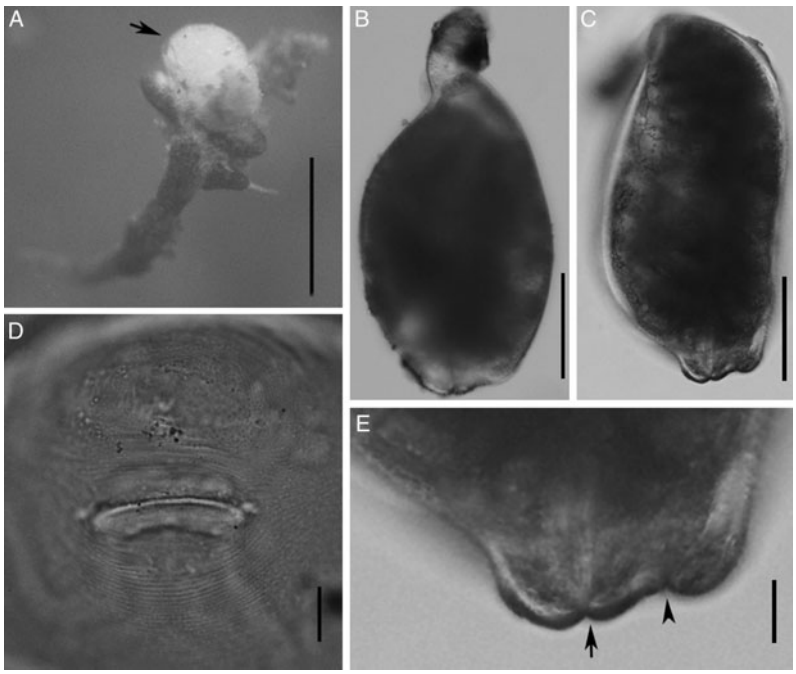

Fig. 2. Females of *Cryphodera sinensis* n. sp. under the light microscrope: (A) a female (arrow) parasitizing a root of ramie; (B, C) entire body; (D) perineal pattern; (E) vulva–anus region (arrow = vulva; arrowhead = anus). Scale bars: 1000 μm (A); 100 μm (B, C); 20 μm (D, E).

Results

Description of Cryphodera sinensis *n. sp*

In the morphometrics of females, males and second-stage juveniles (figs 1–4, tables 1 and 2), abbreviations follow those of Siddiqi (2000).

Female

Body oval without vulval cone, completely annulated, surrounded by a distinct subcrystalline layer. Neck distinct, usually bent laterally. Labial region not offset, labial disc distinct. Except for the stylet and median bulb, other internal structures difficult to observe. Stylet long, with rounded knobs sloping posteriorly. Median bulb nearly rounded to oval, with distinct valve plates situated centrally. Excretory pore located posterior to median bulb. Vulva terminal, lips pronounced and protruding. Anus subterminal, dorsal, near the vulva. Area between anus and vulva distinctly concave. Perineal pattern ovoid with fine, smooth and continuous striae. Striae surround anus and vulva respectively. Phasmids indistinct. Eggs and juveniles (55 ± 19 (30–75)) retained inside the body. No cyst formed.

Male

Body slender, ventrally curved when heat-relaxed. Lateral fields with three incisures, areolated. Annulus width at mid-body 1.7–2.1 μm. Labial region slightly offset, with a prominent labial disc and two post-labial annuli, basal annulus broader and longitudinal striations present. Stylet long, with rounded knobs sloping slightly posteriorly. Median bulb oval, occupying *c.* 50% of corresponding body diameter, with 3–4 μm long

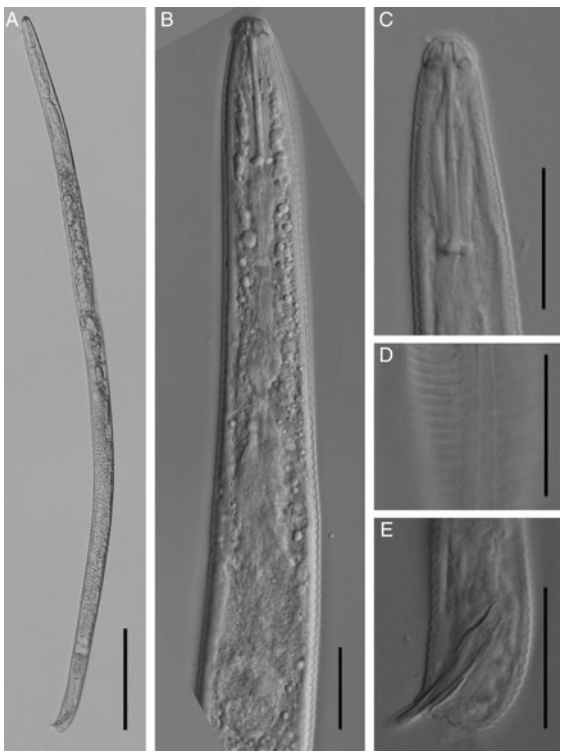

Fig. 3. Males of *Cryphodera sinensis* n. sp. under the light microscope: (A) entire body; (B) pharyngeal region; (C) anterior region; (D) lateral lines in the middle body; (E) tail. Scale bars: 100 μm (A); 20 μm (B–E).

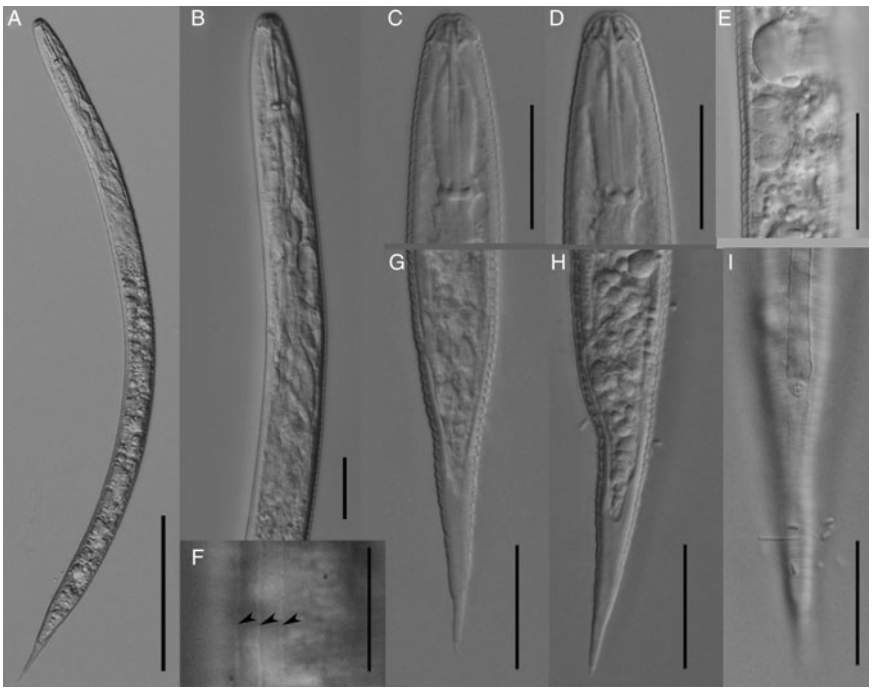

Fig. 4. Second-stage juveniles of *Cryphodera sinensis* n. sp. under the light microscope: (A) entire body; (B) pharyngeal region; (C, D) anterior region; (E) genital promordium; (F) lateral lines (arrowed); (G, H) tail; (I) phasmids. Scale bars: 100 μm (A); 20 μm (B–E, G–I); 10 μm (F).

valve plates situated centrally. Pharyngeal glands developed, ventrally overlapping the intestine. Hemizonid distinct, posterior to median bulb, *c.* 4 μm long, 3–6 annuli anterior to excretory pore. Testis single, anteriorly outstretched. Spicules slender, arcuate with blunt rounded tip. Gubernaculum thin and almost straight. Tail very short, hemispherical, anal lips forming a short tube. Phasmids indistinct.

Second-stage juveniles (J2)

Body slender, tapering posteriorly, ventrally curved when heat-relaxed. Labial region slightly offset, hemispherical, with one weakly offset labial disc and three distinct post-labial annuli. Stylet long, cone straight, shaft cylindrical. Stylet knobs well developed with distinct anterior projection. Median bulb oval, occupying *c.* 50% of corresponding body diameter, with 3–4 μm long valve plates situated centrally. Pharyngeal glands filling body cavity. Hemizonid distinct, immediately anterior to excretory pore. Lateral fields with three incisures, areolated. Annulus width at mid-body 1.7–2 μm. Genital primordium oval, 12.5 (10–14) μm long, 7.9 (6–10) μm wide, 159 (145–174) μm anterior to tail tip. Tail conoid, gradually tapering to a point terminus with mucro-like tip, hyaline region relatively long, occupying 44.8–54.7% of tail length, posterior half not annulated. Phasmids large, lens-like, 2–6 annuli posterior to anus.

Table 1. Morphometrics in μm of females of *Cryphodera sinensis* n. sp.

		Paratypes		
Character	Holotype	*n*	Mean ± SD	Range
Body length (L)	447.5	12	460.9 ± 53.1	362.9–526.8
Neck length	107.5	12	107.1 ± 19.4	79.3–141.7
Body diameter	187.5	12	240.1 ± 51.6	172.4–314.0
Stylet length	35.4	6	32.1 ± 2.6	28.0–35.4
Length of medium bulb	25.3	7	27.4 ± 3.4	21.9–32.3
Width of medium bulb	22.8	7	24.9 ± 4.4	17.1–31.4
Excretory pore from anterior end	118.4	4	124.4 ± 7.7	115.0–132.1
Vulva–anus distance	32.5	10	31.4 ± 2.0	29.5–35.8
Median bulb valve from anterior end	72.5	7	73.8 ± 8.9	67.5–80.0
a = L/max. width	2.4	12	2.0 ± 0.4	1.6–3.0
Vulval slit length	–	5	51.3 ± 6.8	42.8–58.3

n, number of specimens observed; SD, standard deviation.

Table 2. Morphometrics in μm of males and second-stage juveniles of *Cryphodera sinensis* n. sp.

Character	Male Paratypes ($n = 11$)		Second-stage juvenile Paratypes ($n = 30$)	
	Mean ± SD	Range	Mean ± SD	Range
Body length (L)	670.0 ± 165	447.8–915.9	435.2 ± 25.4	388.8–474.5
a = L/ max. width	31.6 ± 3.0	28.1–36.2	22.6 ± 1.5	19.4–25.9
b = L/oesophageal length	–	–	3.9 ± 0.2	3.7–4.4
b′ = L/ distance from head end to posterior end of oesophageal glands	4.5 ± 0.7	3.4–5.7	2.7 ± 0.2	2.4–3.2
c = L/ tail length	197.2 ± 49.1	137.2–271.3	7.8 ± 0.5	6.4–8.6
c′ = tail length/anal body width	–	–	4.1 ± 0.3	3.6–4.7
T = (distance between cloaca and anterior-most part of testis /L) × 100	50.9 ± 6.2	40.3–58.6	–	–
Height of lip region	4.5 ± 0.4	4.0–5.0	4.3 ± 0.3	4.0–5.0
Diameter of lip region	8.4 ± 0.6	8.0–9.5	9.3 ± 0.3	9.0–10.0
Stylet length	30.1 ± 1.9	27.0–32.5	29.1 ± 0.8	28.0–31.0
Length of stylet cone	–	–	14.2 ± 0.7	13.0–16.0
Length of stylet shaft and knobs	14.7 ± 1	13.0–16.0	14.9 ± 0.5	14.0–15.6
Height of stylet knobs	–	–	2.3 ± 0.2	2.0–2.9
Width of stylet knobs	–	–	5.9 ± 0.4	5.0–6.5
DGO = distance of dorsal oesophageal gland orifice to base of stylet	3.4 ± 0.3	3.0–3.8	5.4 ± 0.7	4.5–6.5
Length of medium bulb	11.7 ± 1.0	11.0–14.0	13.4 ± 1.1	11.0–15.0
Width of medium bulb	7.8 ± 1.0	6.5–10.0	9.8 ± 1.0	7.5–12.0
Median bulb valve from anterior end	75.9 ± 6.3	67.0–84.8	75.3 ± 4.8	66.0–88.6
Hemizoid from anterior end	110.6 ± 12	96–127.8	102.4 ± 5.9	90.0–111.3
Excretory pore from anterior end	121.8 ± 12.7	106.0–140.4	104.2 ± 5.9	91.7–113.2
Pharyngeal gland from anterior end	149.3 ± 21.8	124.0–187.2	164.2 ± 9.8	147.5–181.3
Body diameter at mid-body	21 ± 3.4	15.7–25.3	19.3 ± 0.6	18.0–20.0
Body diameter at anus	12.9 ± 1.9	10.0–16.0	13.8 ± 0.7	12.5–15.2
Tail length	3.8 ± 1.7	2.2–6.1	56.0 ± 3.2	52.0–65.0
Tail-hyaline portion	–	–	28.3 ± 2.3	24.5–35.0
Phasmid to tail tip	–	–	48.5 ± 3.2	43.5–56
Tail-hyaline portion/Stylet length	–	–	0.97 ± 0.07	0.86–1.13
L/Median bulb valve from anterior end	–	–	5.8 ± 0.3	5.1–6.3
Tail length/Tail-Hyaline portion	–	–	2 ± 0.09	1.8–2.2
Testis length	349.3 ± 111.9	218.0–525.0	–	–
Spicule length	24.3 ± 2.8	20.0–28.0	–	–
Gubernaculum length	6.6 ± 0.4	6.0–7.0	–	–

n, number of specimens observed; SD, standard deviation.

Type host, material and etymology

Type host. Roots and rhizosphere of ramie (*Boehmeria nivea*) from Yiyang City (latitude 28°55.977′N, longitude 112°25.818′E), Hunan Province, China. Material collected in May 2011. The soil type is clay and the climate is subtropical.

Deposition of type specimens. Holotype female, two paratype males and six paratype J2s are deposited on slides in the USDA Nematode Collection, Beltsville, Massachusetts, USA; one paratype female, two paratype males and five paratype J2s deposited on slides in the Department of Nematology, University of California, Riverside, California, USA. Other voucher specimens are available at the Plant Nematode Research Laboratory, College of Natural Resources and Environment, South China Agricultural University, Guangzhou, China.

Etymology. Specific epithet refers to the geographical location (adj. sinensis = Chinese, from China).

Diagnosis and relationships

Cryphodera sinensis n. sp. has the general characteristics of the genus *Cryphodera*. Diagnostic characters of the new species and all species comparisons in the genus *Cryphodera* are compiled in tables 3 and 4, and a key to all species within *Cryphodera* is also provided. The new species, on the basis of pronounced and protruding vulval lips, concave anus–vulva profile, three lateral lines in males and J2s, three post-labial annuli and anteriorly concave stylet knobs in J2s, appears morphologically most similar to *C. eucalypti* Colbran, 1966; *C. kalesari* Bajaj, Walia, Dabur & Bhatti, 1989 and *C. brinkmani* Karssen & Van Aelst, 1999.

Cryphodera sinensis n. sp. differs from *C. eucalypti* by the shorter vulva–anus distance (29.5–35.8 v. 40–70 μm), the presence of cloacal tubus, the tail terminus shape of the J2 (point with mucro-like tip v. bluntly rounded), the shorter J2 stylet (28–31 v. 31–35 μm), the stylet knob shape of J2 (distinctly anteriorly projected v. anterior faces flat or slightly pointed anteriorly) and the longer J2 tail (56.0 ± 3.2 v. 51.6 ± 1.3 μm). It differs from *C. kalesari* by the larger female (362.9–526.8 v. 272–353 μm in length; 172.4–314.0 v. 112–288 μm in width), the shorter female stylet (28.0–35.4 v. 37–42 μm), the shorter vulva–anus distance (29.5–35.8 v. 48–72 μm), the longer J2 stylet (28.0–31.0 v. 24–29 μm), the tail terminus shape of

Table 3. Comparative morphometrics (in μm) of females and males in species of *Cryphodera* with ranges in brackets.

	C. sinensis n. sp.	*C. eucalypti*		*C. kalesari*	*C. brinkmani*	*C. podocarpi*	*C. nothophagi*	*C. coxi*
	Present study	Colbran, 1966	Wouts, 1973a	Bajaj *et al.*, 1989	Karssen & Van Aelst, 1999	Wouts, 1973a	Wouts, 1973a	Wouts, 1973a
FEMALE								
Body length (L)	460.9 ± 53.1 (362.9–526.8)	400–636	353 (286–428)	311 ± 21 (272–353)	450 ± 52 (384–544)	393 (300–473)	341 (261–423)	331 (218–455)
Body diameter	240.1 ± 51.6 (172.4–314.0)	201–274	210 (147–290)	205.5 ± 51 (112–288)	238 ± 35 (160–339)	276 (217–335)	226 (148–305)	184 (116–369)
Number of lip annulus	–	–	1	2–3	–	2	2 more or less	2 more or less
Vulval slit length	51.3 ± 6.8 (42.8–58.3)	45 (44–49)	–	35	51 ± 3.8 (44–60)	–	–	–
Vulva–anus distance	31.4 ± 2.0 (29.5–35.8)	47 (45–70)	45 (40–51)	57 ± 7.5 (48–72)	49 ± 2.9 (44–54)	65 (58–70)	46.4 (41–57)	52.5 (38–70)
Vulval lip profile	Pronounced and protruding	–	Distinctly protruding	Protruding	Slightly protruding to protruding	Slightly protruding	Slightly protruding	Slightly protruding
Anus-vulva profile	Distinctly concave	Flat to concave	Concave	Distinctly concave	Flat to concave	Flat	Flat	Flat
MALE								
Body length	670.0 ± 165 (447.8–915.9)	596–894	801 (700–900)	–	832 ± 88 (704–899)	884 (764–973)	715 (577–841)	710 (546–883)
a = L/max. width	31.6 ± 3.0 (28.1–36.2)	27.1–42.4	38.8 (32–43)	–	34.6 ± 1.9 (31.9–36.4)	35.7 (33–38)	37.5 (36–39)	36.0 (31–42)
Number of lip annuli	2	3	2	–	2	3–4	2–3	2
Stylet length	30.1 ± 1.9 (27.0–32.5)	29.4–37.1	35.4 (33–37.5)	–	37.5 ± 1.8 (35.4–39.2)	37.0 (32–39)	34.8 (31–38)	32.3 (28–35)
Stylet knobs	Rounded, sloping slightly posteriorly	Directly posteriorly	Rounded with more or less flattened anterior faces	–	Rounded, pointing slightly anteriorly and set off	Anterior face flat or somewhat pointed	Anterior face flat or somewhat pointed	Anterior face flat or somewhat pointed
DGO	3.4 ± 0.3 (3.0–3.8)	5.0–7.8	–	–	5.4 ± 0.8 (4.4–6.3)	–	–	–
Number of lateral lines	3	3	3	–	3	4	4	4
Spicule length	24.3 ± 2.8 (20.0–28.0)	21.2–28	24	–	28.4 ± 2.2 (26.5–31.6)	24, 27	25.3 (22–29)	24.9 (24–25.5)
Gubernaculum length	6.6 ± 0.4 (6.0–7.0)	6.4–9	7.5	–	9.2 ± 0.4 (8.9–9.5)	7, 9	7 (6–8)	5.5 (5–6)
Cloacal tubus	Present	Absent	Absent	–	Absent	Absent	Absent	Absent

DGO, distance of dorsal oesophageal gland orifice to base of stylet.
Data referred to Colbran (1966), Wouts (1973a), Bajaj *et al.* (1989), Karssen & Van Aelst (1999), Siddiqi (2000) and Sturhan (2010). –, not available.

Table 4. Comparative morphometrics (in μm) of second-stage juveniles (J2) in species of *Cryphodera* with ranges in brackets.

	C. sinensis n. sp.	*C. eucalypti*		*C. kalesari*	*C. brinkmani*	*C. podocarpi*	*C. nothophagi*	*C. coxi*
	Present study	Colbran, 1966	Wouts, 1973a	Bajaj *et al.*, 1989	Karssen & Van Aelst, 1999	Wouts, 1973a	Wouts, 1973a	Wouts, 1973a
Body length (L)	435.2 ± 25.4 (388.8–474.5)	379–461	428 ± 6	383 ± 19 (353–424)	393 ± 23 (450–541)	525 ± 5	450 ± 4	457 ± 4
a = L/ max. width	22.6 ± 1.5 (19.4–25.9)	22.0–26.0	24.8 ± 0.6	22 ± 1.5 (18–26)	23.2 ± 1.3 (20.3–24.6)	27.2 ± 0.5	24.5 ± 0.3	25.3 ± 0.4
b = L/oesophageal length	3.9 ± 0.2 (3.7–4.4)	3.5–4.8	3.7 ± 0.06	3.84 ± 0.38 (3.2–5.0)	–	3.8 ± 0.06	3.7 ± 0.05	3.7 ± 0.04
c = L/tail length	7.8 ± 0.5 (6.4–8.6)	7.9–9.8	8.3 ± 0.15	8.4 ± 1.2 (7–13)	7.1 ± 0.4 (6.4–7.8)	7.7 ± 0.07	8.2 ± 0.08	8.5 ± 0.08
Number of lip annuli	3	3	3	3	4	5	4	4
Stylet length	29.1 ± 0.8 (28.0–31.0)	–†	32.9 ± 0.3 (31–35)	26.5 ± 1 (24–29)	33.3 ± 1.0 (31.6–35.4)	38.7 ± 0.4	33.9 ± 0.2	34.4 ± 0.2
Stylet knobs	Project distinctly anteriorly	Concave	Flat or pointing slightly anteriorly	Flat to concave	Concave	Pointing distinctly anteriorly	Flat anteriorly	Pointing slightly anteriorly
DGO	5.4 ± 0.7 (4.5–6.5)	4.3–5.0	5.5 ± 0.17	3–4	5.8 ± 0.6 (4.4–7.0)	6.0 ± 0.26	5.6 ± 0.17	6.1 ± 0.28
Number of lateral lines	3	3	3	3	3	3	3 (occasionally 4)‡	3
Tail length	56.0 ± 3.2 (52.0–65.0)	–	51.6 ± 1.3	46.5 ± 6 (27–54)	69.7 ± 4.4 (61.3–76.5)	68.9 ± 0.1	55.3 ± 0.8	53.7 ± 0.8
Hyaline portion of tail	28.3 ± 2.3 (24.5–35.0)	–	27.6 ± 1.2	21.5 ± 2.5 (18–26)	37.2 ± 3.6 (30.3–42.3)	36.0 ± 0.1	30.1 ± 0.7	30.6 ± 0.5
Tail terminus	Point with mucro-like tip	Bluntly rounded	Narrow rounded	Narrow rounded	Point with mucro-like tip	Narrow rounded	Narrow rounded	Narrow rounded

DGO, distance of dorsal oesophageal gland orifice to base of stylet.
Data referred to Colbran (1966), Wouts (1973a), Bajaj *et al.* (1989), Karssen & Van Aelst (1999), Siddiqi (2000) and Sturhan (2010). –, not available.
† Colbran (1966) reported stylet length for J2 of *C. eucalypti* was 26–35.9 μm. Wouts (1973a) mentioned that the minimum 26 μm belonged to J2 of *Heterodera*. Bajaj *et al.* (1989) and Karssen & Van Aelst (1999) accepted the point of Wouts (1973a). Thus, the value of *C. eucalypti* described by Colbran (1966) is omitted here.
‡ Wouts (1973a) described four incisures in the field of *C. nothophagi* J2. Sturhan (2010) re-examined paratype J2 of *C. nothophagi*, which revealed three incisures in the field, with the inner incisure occasionally diverging into two. Thus, the point of Sturhan (2010) is accepted here.

the J2 (point with mucro-like tip v. narrow rounded), the longer J2 tail and hyaline portion of tail (52.0–65.0 v. 27–54 μm; 24.5–35 v. 18–26 μm) and the presence of males in *C. sinensis* n. sp. but no males found in *C. kalesari*. From *C. brinkmani*, the new species differs by the shorter female stylet (28.0–35.4 v. 39.2–42.3 μm), shorter vulva–anus distance (29.5–35.8 v. 44–54 μm), shorter male stylet (27.0–32.5 v. 35.4–39.2 μm), fewer J2 lip annuli (3 v. 4), shorter J2 stylet (28.0–31.0 v. 31.6–35.4 μm) and shorter J2 tail (52.0–65.0 v. 61.3–76.5 μm).

In addition, *C. sinensis* n. sp. can be easily distinguished from the remaining three species of *Cryphodera*, namely *C. podocarpi* (Wouts, 1973) Luc, Taylor & Cadet, 1978, *C. nothophagi* (Wouts, 1973) Luc, Taylor & Cadet, 1978 and *C. coxi* (Wouts, 1973) Luc, Taylor & Cadet, 1978, by the shape of the vulval lips (pronounced and protruding v. protruding slightly out of body contour), anus–vulva profile (concave v. flat), the shorter vulva–anus distance (29.5–35.8 v. at least 38 μm), fewer incisures in the male lateral field (3 v. 4) and fewer lip annuli in J2 (3 v. 4 in *C. nothophagi* and *C. coxi*, and 5 in *C. podocarpi*).

Molecular profiles and phylogenetic relationships

The 1740-bp near-full-length SSU sequence was based on DNA template of a single J2. The GenBank accession number of the sequence is JX566453. A BLASTN search of *C. sinensis* n. sp. revealed a high match with the only sequenced *Cryphodera* species in this gene, i.e. *C. brinkmani* (GenBank accession number JQ965679). The identity of the two sequences was 99.6% (1651/1657) without a gap.

The rDNA sequences of 787-bp LSU D2D3 from a single male (body length up to 900 μm), 788-bp LSU D2D3 from another single male (body length only 500 μm, the same size as a J2) and the same J2 as mentioned above were sequenced, respectively. GenBank accession numbers of the three sequences are JX566454, JX566455 and JX566456. The identities of the two male sequences were 99.5% (783/788) with one insertion/deletion (0.1%). The identities of the juvenile sequence and the two male sequences were 99.4% (782/788) with one insertion/deletion (0.1%) and 99.6% (785/788) without a gap, respectively. Intraspecific D2D3 sequence variation for *C. sinensis* n. sp. was 3–7 nucleotides (0.4–0.6%). A BLASTN search of *C. sinensis* n. sp. on the LSU D2D3 revealed a high match with the only sequenced *Cryphodera* species in this gene, i.e. *C. brinkmani* (GenBank accession number DQ328705). The identities of the sequence from *C. brinkmani* and the three sequences from the new species were 93.8% (621/661) with one insertion/deletion (0.1%), 94% (622/661) without a gap, and 93.8% (621/661) without a gap. The interspecific D2D3 sequence variation was 39–40 nucleotides (6%).

The 1037-bp, 1040-bp and 1042-bp ITS-rDNA sequences were sequenced, respectively, based on the same DNA template of males and J2. GenBank accession numbers of the three sequences are JX566457, JX566458 and JX566459. The identities of the two male sequences were 98.8% (1026/1040) with three insertions/deletions (0.2%). The identities of the juvenile sequence and the two male sequences were 98.5% (1022/1042) with five insertions/deletions (0.4%) and 98.8% (1027/1043) with four insertions/deletions (0.3%), respectively. Intraspecific ITS sequence variation for *C. sinensis* n. sp. was 17–25 nucleotides (1.1–1.5%). A BLASTN search of *C. sinensis* n. sp. on the ITS revealed high matches with four *Cryphodera* isolates, including three unidentified isolates and *C. brinkmani*, the highest being with an unidentified isolate, namely *Cryphodera* sp. A (GenBank accession number JF894388–JF894396). The identities of the sequences from the new species and *Cryphodera* sp. A were 97.1–98.9% (631–640/647, 627–640/648 or 632/649) with 0–3 gaps. Comparing with *C. brinkmani* (AF274418), the three sequences of *C. sinensis* n. sp. had 212, 219 and 224 variable sites with 82.2, 81.9 and 81.1% identities (777/989, 772/996 and 778/997) and 78, 91 and 90 insertions/deletions (7.9, 9.1 and 9.0%), respectively. The identities of *C. sinensis* n. sp. (JX566457–JX566459) and *Cryphodera* sp. B (JF894400–JF894402) ranged from 77.3 to 78.7%, and *C. sinensis* n. sp. (JX566457–JX566459) and *Cryphodera* sp. C (JF894397–JF894399), 83.0–83.7%. The interspecific ITS sequence variation within *Cryphodera* ranged from 15.1 to 22.8%.

The molecular phylogenetic relationships of the new species are shown in figs 5–7. A phylogenetic tree based on SSU from a multiple alignment of 1753 total characters was constructed (fig. 5). This dataset has 1551 constant characters (88.5%). The average nucleotide composition is as follows: 23.95% A, 23.93% C, 27.49% G and 24.63% T. When using *Helicotylenchus dihystera* and *Scutellonema bradys* within the family Hoplolaimidae as outgroup taxa, the molecular phylogeny strongly supported a monophyly of Heteroderidae with 99%. *Cryphodera sinensis* n. sp. is in a 100%-supported monophyletic clade with *C. brinkmani*.

A phylogenetic tree based on LSU D2D3 was from a multiple alignment of 887 total characters (fig. 6). This dataset has 576 constant characters (64.9%). The average nucleotide composition is as follows: 19.76% A, 22.62% C, 33.24% G and 24.38% T. When using *Rotylenchus vitis* and *Rotylenchulus reniformis* within the family Hoplolaimidae as outgroup taxa, the molecular phylogeny barely supported a monophyly of Heteroderidae with 65% support. *Cryphodera sinensis* n. sp. is positioned in the cluster of the family Heteroderidae and is in a 100%-supported monophyletic clade with *C. brinkmani*. This clade is sister to *Meloidodera alni*, another cystoid nematode with 100% support.

A phylogenetic tree based on ITS-rRNA was from a multiple alignment of 932 total characters (fig. 7). This dataset has 586 constant characters (62.9%). The average nucleotide composition is as follows: 21.17% A, 21.86% C, 27.89% G and 29.08% T. Using *Punctodera punctata* as outgroup taxon, all *Cryphodera* sequences reside within a well-supported monophyletic clade with 100% support. The three sequences of *C. sinensis* n. sp. are positioned within a clade with *Cryphodera* sp. A with 100% support.

Discussion

Since the genus *Cryphodera* and the type species *C. eucalypti* were proposed in 1966 (Colbran, 1966), five other species, namely *C. podocarpi*, *C. nothophagi*, *C. coxi*, *C. kalesari* and *C. brinkmani*, have been recognized in this

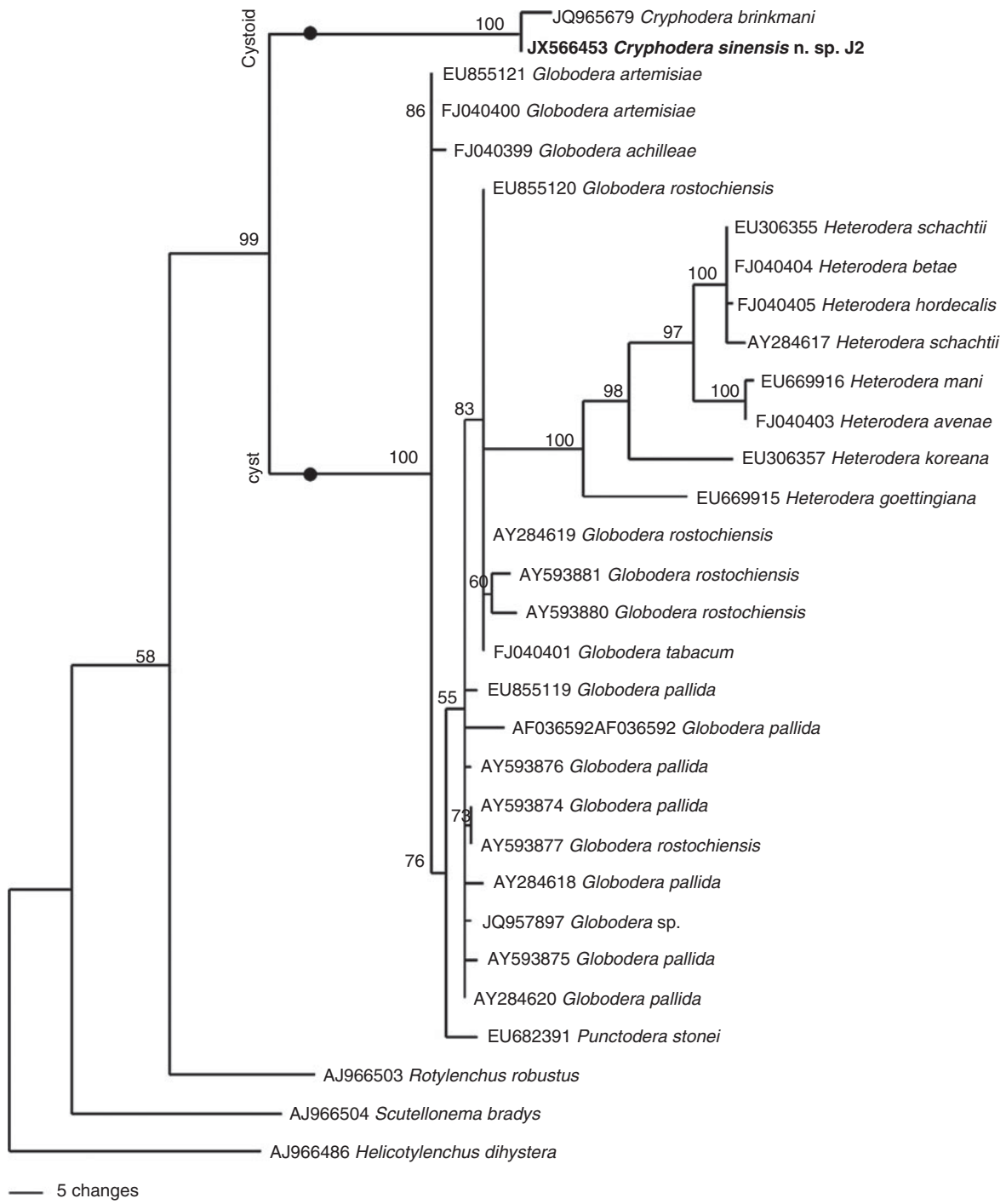

Fig. 5. The 10,001st Bayesian tree inferred from SSU under TrN + I + G model (−lnL = 4228.6084; freqA = 0.2395; freqC = 0.2393; freqG = 0.2749; freqT = 0.2463; R(a) = 1; R(b) = 3.3399; R(c) = 1; R(d) = 1; R(e) = 8.6593; R(f) = 1; Pinva = 0.7202; Shape = 0.7907). Posterior probability values exceeding 50% are given on appropriate clades.

genus (Karssen & Van Aelst, 1999; Siddiqi, 2000). All these species were found in Oceania and Asia, e.g. *C. eucalypti* in Australia (Colbran, 1966), *C. podocarpi*, *C. nothophagi* and *C. coxi* in New Zealand (Wouts, 1973a), *C. kalesari* in India (Bajaj *et al.*, 1989) and *C. brinkmani* from Japan (Karssen & Van Aelst, 1999). In China, non-cyst-forming genera have never been reported to date. This is the first record of the non-cyst-forming genus *Cryphodera* in China.

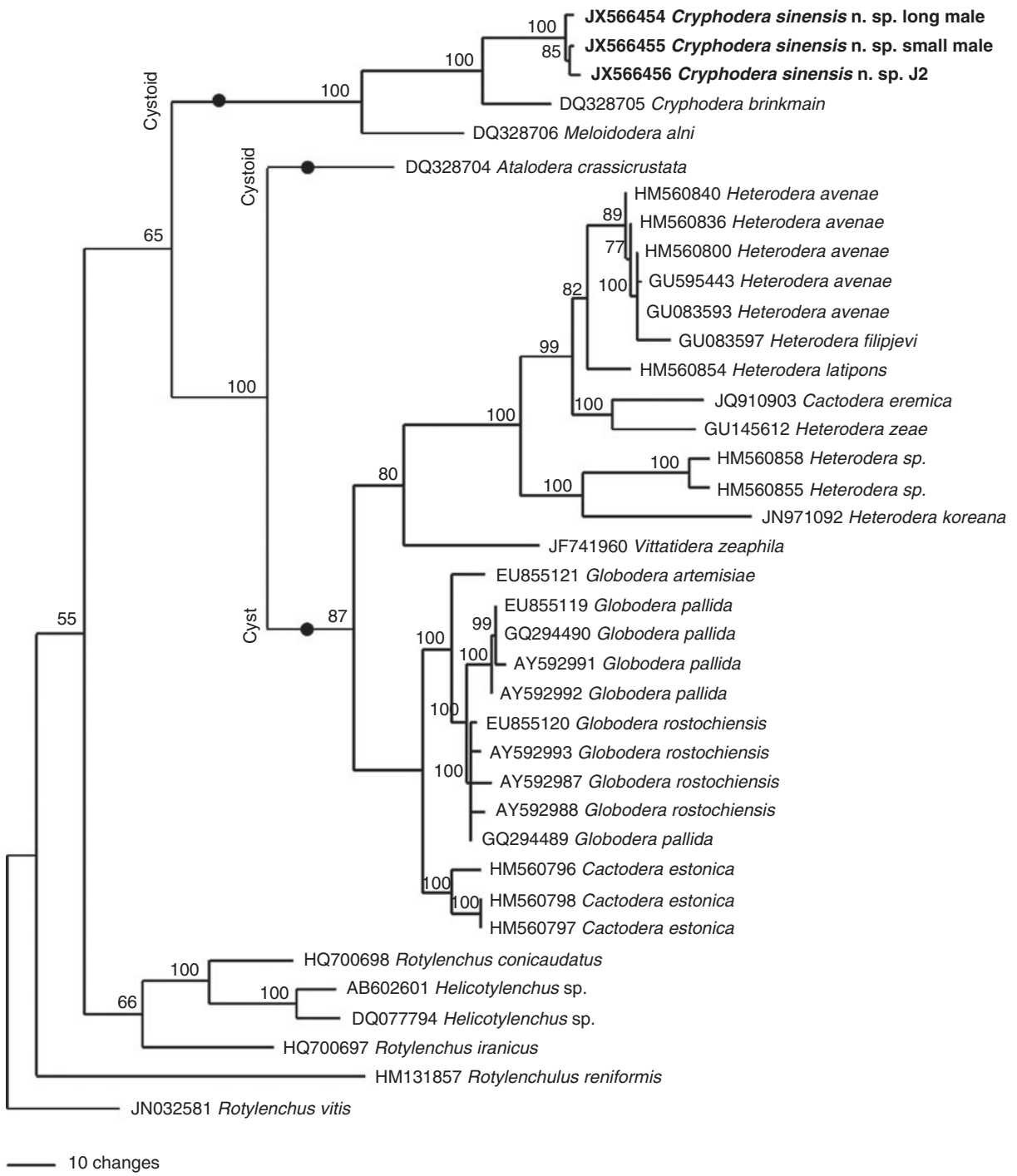

Fig. 6. The 10,001st Bayesian tree inferred from LSU D2D3 under TVMef + I + G model (−lnL = 4735.6504; freqA = 0.1976; freqC = 0.2262; freqG = 0.3324; freqT = 0.2438; R(a) = 0.5072; R(b) = 4.9033; R(c) = 1.9702; R(d) = 0.5097; R(e) = 9.0255; R(f) = 1; Pinva = 0.4639; Shape = 0.9702). Posterior probability values exceeding 50% are given on appropriate clades.

In this study, *C. sinensis* n. sp., a non-cyst-forming nematode, was obtained from *Boehmeria nivea* in Hunan Province, China. It's worthy to note that two obviously different sizes of males were present in the same population of *C. sinensis* n. sp. The longest male is about twice the length of the J2 and the smallest male is shorter than J2, but no morphological differences are observed. In order to clarify if these two very different sized males and juveniles are the same, DNA from a single longer or smaller male and J2 were sequenced separately, and LSU

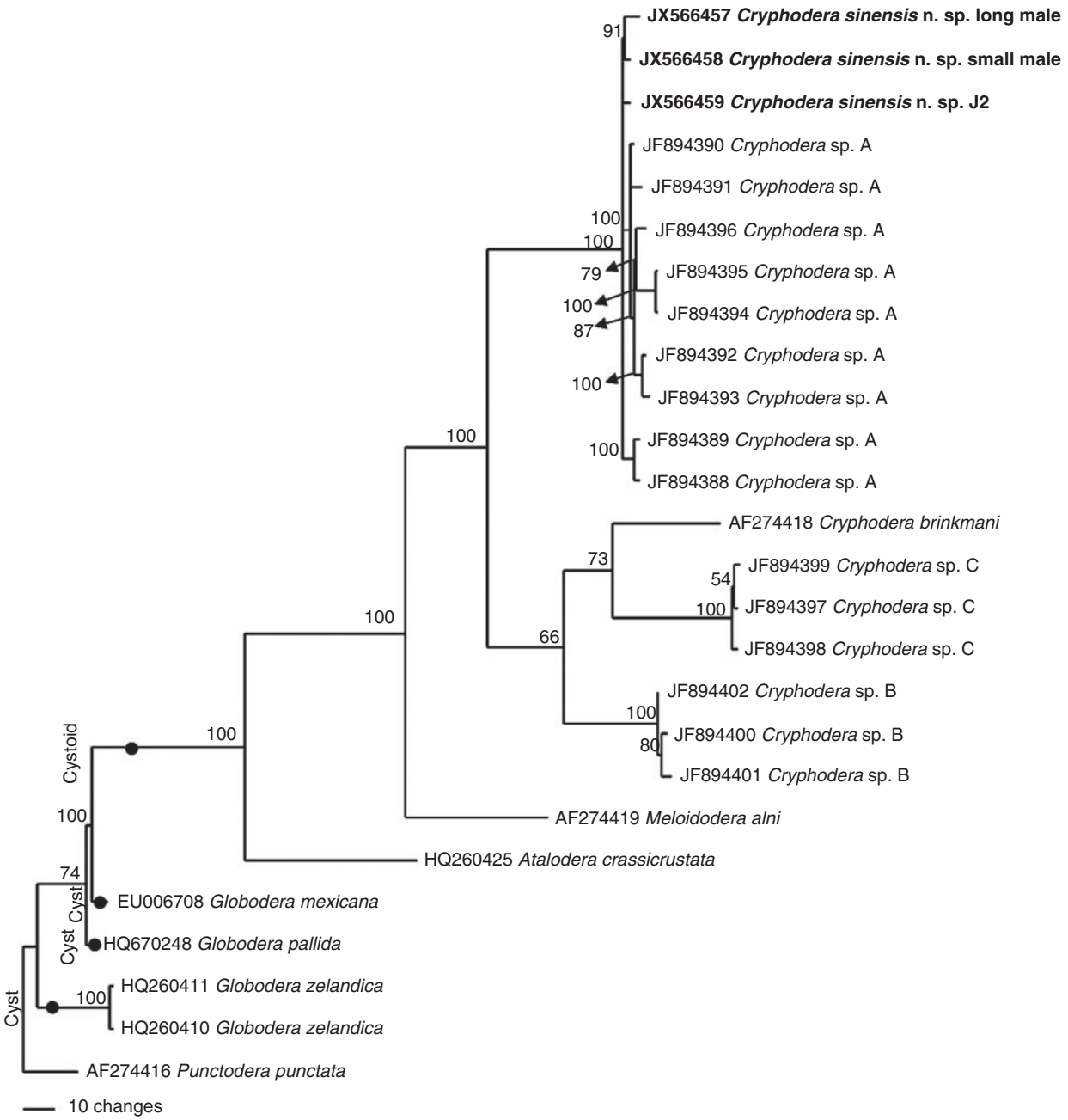

Fig. 7. The 10,001st Bayesian tree inferred from ITS under GTR + I + G model (−lnL = 3759.562; freqA = 0.2117; freqC = 0.2186; freqG = 0.2789; freqT = 0.2908; R(a) = 1.0389; R(b) = 6.3099; R(c) = 1.5176; R(d) = 0.1822; R(e) = 6.3099; R(f) = 1; Pinva = 0; Shape = 0.6136). Posterior probability values exceeding 50% are given on appropriate clades.

D2D3 (JX566454–JX566456) and ITS (JX566457–JX566459) were obtained. The sequence comparison results revealed that D2D3 and ITS sequence variations were 3–7 nucleotides (0.4–0.6%) and 17–25 nucleotides (1.1–1.5%) respectively. In *Heterodera orientalis*, ITS sequence variation was up to 1.4% (Mundo-Ocampo *et al.*, 2008). Intraspecific ITS sequence variation for *Cryphodera* sp. A was 2.5% (Nguyen *et al.*, 2011). *Cryphodera* sp. A was found in a survey for Heteroderidae from natural forests in Vietnam. Three *Cryphodera* species including *Cryphodera* sp. A, *Cryphodera* sp. B and *Cryphodera* sp. C could be distinguished by molecular data (Nguyen *et al.*, 2011). Interestingly, the identities of the ITS sequences from *C. sinensis* n. sp. and *Cryphodera* sp. A are 97–99% and the phylogenetic tree based on ITS-rRNA showed that *C. sinensis* n. sp. is in the same clade with *Cryphodera* sp. A with 100% support and short branch differences. Based on these data and morphological observations, molecular

variation among the males of different sizes and juveniles is intraspecific, suggesting that *C. sinensis* n. sp. and *Cryphodera* sp. A are the same species.

Diagnostic morphological characters of *Cryphodera* were proposed by Siddiqi (2000). Except for the shorter vulva–anus distance and the presence of cloacal tubus, other morphological characters of *C. sinensis* n. sp. conform to the diagnostic characters of *Cryphodera*. In the new species, the range of vulva–anus distance is from 29.5 to 35.8 μm, but the minimum vulva–anus distance is 38 μm in other *Cryphodera* species. Moreover, males of *C. sinensis* n. sp. have cloacal tubus v. no cloacal tubus in other *Cryphodera* species. A similar difference was also reported in *Heterodera*. Males of only some species of *Heterodera* have cloacal tubus (Subbotin *et al.*, 2010b). The phylogenetic analysis placed *C. sinensis* n. sp. in a 100%-supported monophyletic clade with *C. brinkmani* based on SSU and LSU D2D3 and in a 100%-supported clade of *Cryphodera* including *Cryphodera* sp. A, *Cryphodera* sp. B, *Cryphodera* sp. C and *C. brinkmani* based on ITS. Therefore, the presence of a cloacal tube is variation at the species level. In the genus *Cryphodera*, the perineal pattern was only previously examined for *C. brinkmani* (Karssen & Van Aelst, 1999). Striae between the vulva and anus in *C. sinensis* n. sp. are less than those in *C. brinkmani*. The perineal pattern in the genus *Meloidogyne* is an important diagnostic character to distinguish some species. Unfortunately, this feature has not been examined for other *Cryphodera* species and needs further study. Through this study, *C. sinensis* n. sp. represents the seventh *Cryphodera* species. Due to this addition, two morphological characters of the genus *Cryphodera* need to be updated, i.e. range of the vulva–anus distance could be 29.5 to 72 μm and a cloacal tube could be absent or present.

About nine genera within Heteroderidae are non-cyst-forming genera according to Siddiqi (2000) including *Cryphodera*, *Meloidodera* and *Atalodera*. Different classifications among these three genera were proposed. For instance, Wouts (1972, 1973a, b) placed *Cryphodera* and *Meloidodera* in the subfamily Meloidoderinae and *Atalodera* in Ataloderinae; however, Siddiqi (2000) proposed *Cryphodera* and *Atalodera* in the subfamily Ataloderinae and *Meloidodera* in the subfamily Meloidoderinae. In our phylogenetic trees based on LSU D2D3 and ITS, *Cryphodera* is more closely related to *Meloidodera* than *Atalodera*, which is similar to the tree inferred from ITS1 rRNA gene reported by Vovlas *et al.* (2013). Unfortunately, few DNA sequence data are available for non-cyst-forming genera. As more sequence data become available, the classification of non-cyst-forming nematodes in the Heteroderidae should be re-evaluated.

Key to species within the genus *Cryphodera*

1 – J2 with three lip annuli 2
– J2 with four to five lip annuli 4
2 – Vulva–anus distance <40 μm; male present, with cloacal tubus; tail of J2 with point, mucro-like tip . . . . . *C. sinensis* n. sp.
– Vulva-anus distance ≥ 40 μm; male absent, if present, lacking cloacal tubus; tail of J2 with rounded terminus 3
3 – Female with one lip annulus; stylet length of J2 ≥ 31 μm . . . . . *C. eucalypti*
– Female with two to three lip annuli; stylet length of J2 <30 μm . . . . . *C. kalesari*
4 – Male with three incisures in lateral field; tail of J2 with point, mucro-like tip. . . . . *C. brinkmani*
– Male with four incisures in lateral field; tail of J2 with narrow rounded terminus 5
5 – J2 with five lip annuli . . . . . *C. podocarpi*
– J2 with four lip annuli 6
6 – J2 with conical lip region and distinct flat labial disc; stylet knobs of J2 flat anteriorly. . . . . *C. nothophagi*
– J2 with hemispherical lip region and inconspicuous labial disc; stylet knobs of J2 slightly pointing anteriorly . . . . . *C. coxi*

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