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Haplotype diversity of *Heterodera koreana* (Tylenchida: Heteroderidae), affecting bamboo in Korea

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Abstract In a survey of plant-parasitic nematodes in agricultural fields, cyst-forming nematodes were found in soil planted bamboo in Korea. The aim of this study was to identify the cyst nematodes based on morphological and molecular characteristics. As the results, the morphology and morphometrics of cysts and second-stage juveniles (J2s) were consistent with those of previous descriptions of *Heterodera koreana*. In phylogenetic analyses based on DNA sequences, these cyst nematodes were clustered together with clade of *H. koreana* in internal transcribed spacer (ITS) region, and large subunit D2-D3 segments (LSU D2-D3). These nematodes were clustered together with clade of *H. koreana* in cytochrome c oxidase subunit I (*COI*) gene, but a haplotype was different when compared with previous reported haplotypes (haplotype A-C) in Japan. This study showed these cyst nematodes were identified as *H. koreana*, and a new haplotype of *H. koreana* is distributed in Korea. We suggest that the new haplotype of *H. koreana* name as haplotype D.

Keywords *Afenestrata* group · Bamboo · *COI* · Haplotype · *Heterodera koreana*

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Introduction

Plant-parasitic nematodes (PPNs), which include over 4,100 species, interact with host crops (Decraemer & Hunt, 2006; Jones et al., 2013). Damage caused by PPNs has been estimated at 157 billion USD per year (Nicol et al., 2011). Among the PPNs, cyst nematodes are regarded as the most important PPNs worldwide, and contain eight genera as of 2021. Economic damage in many plants is caused by members of two genera, *Heterodera* and *Globodera*. The genus *Heterodera* contains some of the most important PPNs worldwide, and includes about 85 species including soybean cyst nematode (*Heterodera glycines*). The *Heterodera* species reported in Korea are as follows: soybean cyst nematode (*H. glycines*) on soybean in 1983 (Choi & Choi, 1983), Korean cyst nematode (*H. koreana*) on bamboo in 1992 (Vovlas et al., 1992), sugar beet cyst nematode (*H. schachtii*) on Kimchi-cabbage in 2011 (Kim et al., 2016), white soybean cyst nematode (*H. sojae*) on soybean in 2016 (Kang et al., 2016), clover cyst

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nematode (*H. trifolii*) on Kimchi-cabbage in 2017 (Mwamula et al., 2018), and rice cyst nematode (*H. oryzae*) on rice in 2020 (Mwesige et al., 2020).

H. koreana and *H. sojae* were first discovered in the Republic of Korea and were reported as novel cyst nematode species. Since then, the nematodes have been reported in other countries (Kang et al., 2016; Vovlas et al., 1992). *H. koreana* was described as *Afenestrata koreana* at first by Vovlas et al. (1992), but the genus *Afenestrata* was subsequently changed to *Heterodera* (Mundo-Ocampo et al., 2008). Because the genus *Afenestrata* was not classified as a specific clade and clustered together with *Heterodera* spp. in phylogenetic studies, synonymisation of *Afenestrata* with *Heterodera* was proposed (Mundo-Ocampo et al., 2008). Recently, bamboo has been cultivated as a crop for tea, and for seasoned vegetables in Korea, and damage by the Korean cyst nematode is expected. Since the Korean cyst nematode (*H. koreana*) was first reported, there have been no further findings or studies of the cyst nematode. A study of the cyst nematode based on its molecular characteristics is required because it is difficult to identify the *Heterodera* species using morphological and morphometrical characteristics.

In this study, we identified the cyst nematodes extracted from bamboo using morphological characteristics as well as molecular characteristics based on DNA barcoding genes such as LSU D2-D3 expansion segments, the ITS region, and the mitochondrial DNA *COI* gene. Furthermore, we identified the haplotype of the Korean cyst nematode based on the *COI* gene and named the new haplotype.

Materials and methods

Nematode isolation Soil samples were collected from the rhizosphere of bamboo in three fields in Sacheon city, Gyeongsangnam-do in Korea, from soil at a depth of approximately 15 cm using a soil sampler (diameter 2 cm). The samples were labeled as BC348, DR437, and DR1256, respectively (Table 1). Cysts were extracted by the sieving method using 850 µm and 250 µm mesh sieves (Kang et al., 2016). After extraction, the cysts and J2s were transferred into a watch glass containing tap water using forceps and pipette under a stereomicroscope (MZ205; Leica, Wetzlar, Germany) and were kept at 4 °C until further use.

Morphological analysis For light-microscopic observations, J2s were killed and fixed by addition of 80 °C FG 4:1 fixative (Southey, 1986). The nematodes were fixed for at least 24 h, then processed according to the Seinhorst method (Cid Del Prado Seinhorst, 1959; Vera & Subbotin, 2012). Specimens were mounted on Cobb slides and sealed with a paraffin ring and glycerin (Cobb, 1917). Vulval cones were cut under a stereomicroscope (M205; Leica, Wetzlar, Germany), and were transferred to glycerin on slide glasses. The nematodes were observed, measured, and photographed with the aid of a compound microscope (DM5000; Leica, Wetzlar, Germany) equipped with a microscope digital camera (DFC450; Leica, Wetzlar, Germany). The overall shape of the cysts, and the shape of the annule, vulval cone, head and lateral field were observed. The nematodes were identified morphologically based on the *Heterodera* species identification key authored by Subbotin et al. (2010).

Molecular analysis To extract genomic DNA, each single cyst of three different populations was transferred to a slide-glass on a small drop of distilled water, opened and its contents crushed using a filter paper chip (2 mm × 2 mm) and forceps. Using forceps, the chip with the crushed eggs and J2s was transferred into a PCR tube containing 30 µl lysis buffer (sterilized triple distilled water, 1 M Tris–HCl, 10% Triton-X 100, 100 µg/ml Proteinase K, 2 M KCl, 1 M $MgCl_2$) for extracting the nematode DNA

Table 1 Nematode samples used in this study

Population code	Collection date	Host plant	Coordinates	Accession No.		
				LSU D2-D3	ITS	*COI*
BC348	17th Dec. 2019	Bamboo	35.09973295964911, 128.0966177424182	MW642446	MW642449	MW642452
DR437	18th Dec. 2019	Bamboo	35.11422712938377, 128.1349239712546	MW642448	MW642450	MW642454
DR1256	17th Dec. 2019	Bamboo	35.10823722627927, 128.1225063270747	MW642447	MW642451	MW642453

(modified Iwahori et al., 2000). The tubes were incubated in a Thermal cycler (PTC-200, MJ Research, Alameda, CA, USA) at 60 °C for 1 h and 94 °C for 10 min.

Two ribosomal RNA fragments, i.e. the LSU D2-D3 segments, ITS regions, and *COI* gene of the mitochondrial genome were amplified. The primers for D2-D3 segment amplification were D2A (5'-ACAAGTACC GTGAGGGAAAGTTG-3') and D3B (5'-TCGGAA GGAACCAGCTACTA-3') (Subbotin et al., 2006). Primers for ITS amplification were TW81 (5'-GTT TCCGTAGGTGAACCTGC-3') and AB28 (5'-ATA TGCTTAAGTTCAGCGGGT-3') (Subbotin et al., 2000). A primer set of JB3 (5'-TTTTTTGGGCAT CCTGAGGTTTAT-3') and JB5 (5'-AGCACCTAA ACTTAAAACATAATGAAAATG-3') for *COI* gene was used in the PCR reaction (Derycke et al., 2005).

The PCR conditions were as follows: predenaturation stage; 94 °C for 5 min, cycling stage (n = 40); denaturation at 94 °C for 1 min, annealing at 56 °C (D2-D3 segments), 58 °C (ITS region), and 57 °C (*COI* gene) for 1 min, respectively, and extension at 72 °C for 2 min. The final extension was carried out at 72 °C for 10 min. In order to verify the PCR amplicon, electrophoresis was performed using 0.5 × TAE buffer on 1% agarose gel. The amplicon was subsequently purified using a commercial PCR Purification Kit (Qiagen, Valencia, CA). All strands of the PCR amplicons were cycle-sequenced with an ABI PRISM BigDye Terminator version 1.1 Cycle Sequencing Kit and electrophoresed in each direction on an ABI Prism ABI 377 Genetic Analyzer (PE Applied Biosystems, USA). The newly obtained sequences were submitted to the GenBank database (Table 1).

Phylogenetic analysis For phylogenetic study, the sequences of the three populations were compared with GenBank nematode sequences using the BLAST homology search program. The closest sequences were selected for phylogenetic analyses. Outgroup taxa for each dataset was chosen according to previous phylogenetic study for cyst-forming nematodes (Kang et al., 2016; Mwesige et al., 2020), *Cryphodera brinkmani* Karssen & van Aelst, 1999 and *Meloidodera sikhotealinien-sis* Eroshenko, 1978 (De Luca et al., 2013; Subbotin et al., 2000, 2017). The newly obtained and published sequences for each gene were aligned using Clustal W with default parameters (Thompson et al., 1994). Sequence alignments were manually edited using BioEdit (Hall 1999). The alignment quality was examined by eye, and optimized manually by adjusting the ambiguous nucleotide positions. Models of base substitution were evaluated using MODELTEST3.7 combined with PAUP4.0 (Huelsenbeck & Ronquist, 2001; Posada & Crandall, 1998; Swofford, 2003). The Akaike-supported model, the base frequency, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates in the AIC were then used in phylogenetic analyses. Bayesian analysis was performed to confirm the tree topology for each gene separately using MrBayes 3.1.2 running the chain for 1×10^6 generations and setting the 'burn-in' at 2500 (Huelsenbeck & Ronquist, 2001). The MCMC (Markov Chain Monte Carlo) method was used within a Bayesian framework to estimate posterior probabilities of the phylogenetic trees (Larget & Simon, 1999), and generate a 50% majority- rule consensus tree. The posterior probabilities are given on appropriate clades. Trees were visualized using TreeView (Page, 1996).

Data of *COI* gene analysis The sequences of the *COI* gene were assembled and aligned using MEGA version X (Kumer et al., 2018) with accession numbers LC202153-93, MW642452-4 and OL813218-9. DnaSP version 5.10.1 (Librado & Rozas, 2009) was used to carry out preliminary analyses of nucleotide polymorphism and haplotype variation, and to generate Arlequin haplotype files. We used PopART (Leigh & Bryant, 2015) to construct and examine median-joining haplotype networks (Bandelt et al., 1999), and Arlequin version 3.5.2.2 (Excoffier & Lischer, 2010) to estimate haplotype diversity and nucleotide diversity, and to test for genetic structure with an analysis of molecular variance (AMOVA).

Results

Morphological analysis Morphological characters and morphometric features of cysts, and vulval cone of cysts and J2s were examined and measured for species identification. Lemon-shaped cysts, variable

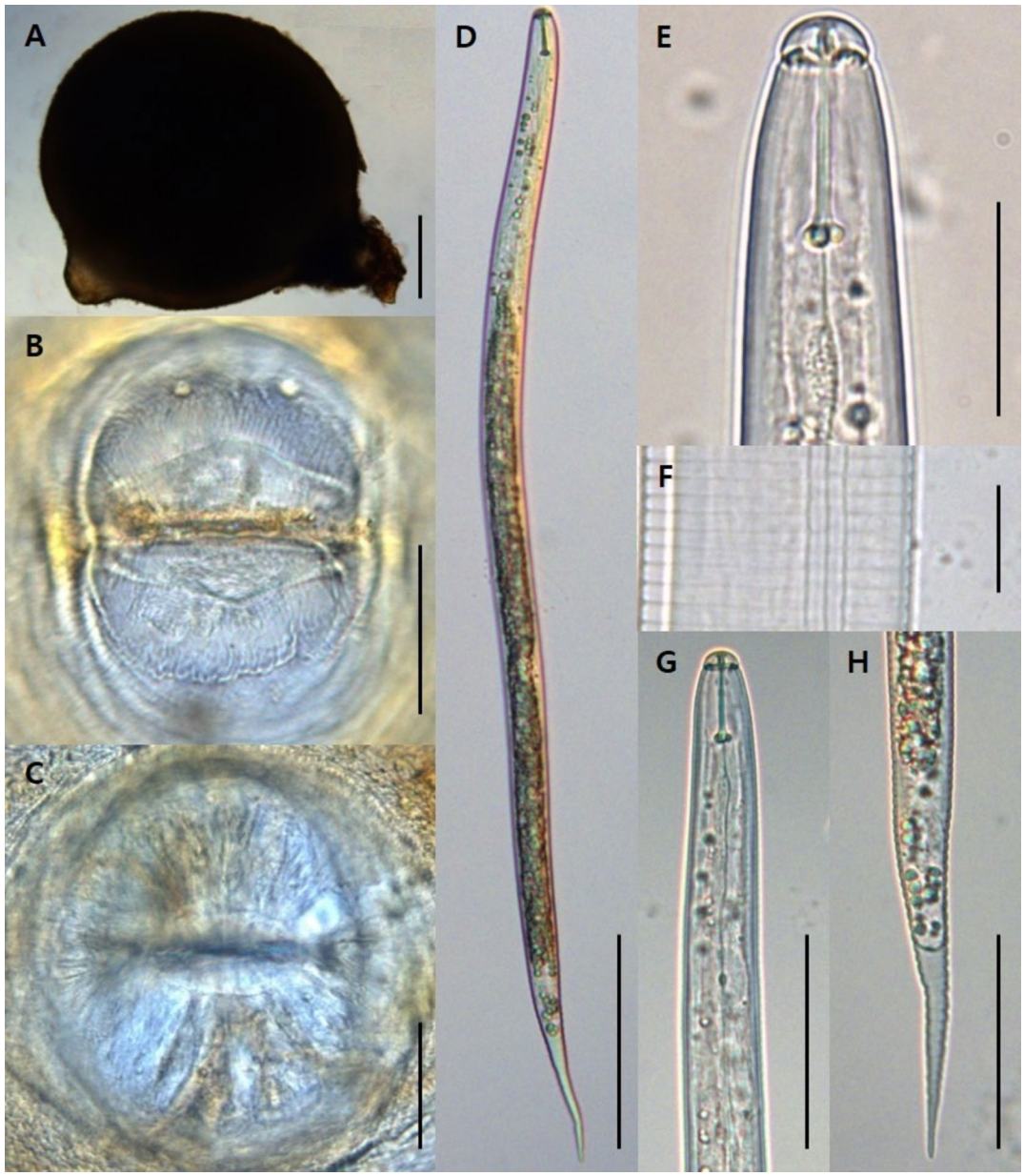

Fig. 1 The morphological characterization of cyst, vulval cone and second-stage juvenile of *Heterodera koreana*. **A**: Lemon-shaped cyst; **B**-**C**: Vulval cone; **D**: Entire body of second-stage juveniles; **E**: Head region of second-stage juveniles; **F**: Lateral field; **G**: Anterior of second-stage juveniles; **H**: Tail region. (Scale bars: A = 100 µm, B-C = 50 µm, D = 100 µm, E = 20 µm, F = 10 µm and G-H = 50 µm)

in size (377–903 µm) with distinct neck and vulval cones, were observed (Fig. 1A). The cuticle appeared light to dark brown in colour. The stylet and other pharyngeal structures were indistinct. A gelatinous egg sac was not observed and the cyst cuticle had an irregular zigzag pattern on mid-body. Vulval cone with lacking-fenestration was observed and covered with tuberculate pattern (Fig. 1B and C). J2s had a cylindrical body, tapering posteriorly, straight or slightly ventrally curved after fixation (Fig. 1D).

Table 2 Morphometric comparison of *Heterodera koreana* populations from Republic of Korea, Japan, China and Iran. All measurements are in µm and in the form: mean ± s.d. (range)

Stage	Character	Korea (this study)			Korea	Japan	China	Iran
		BC348	DR437	DR1256	Vovlas et al., 1992	Sekimoto et al., 2017	Wang et al., 2012	Maafi and Taheri, 2015
Cyst	n	7	10	7	20	10	5	21
	L (including neck)	647.4 ± 117.9 (509–903)	611.6 ± 81.5 (489–709)	592.1 ± 139.5 (377–844)	840 ± 149.0 (630–1174)	641 ± 65.8 (524–770)	748 ± 75.0 (700–860)	502 ± 70.0 (420–640)
	Body diam.	544.9 ± 100.9 (442.5–713.9)	521.4 ± 111.0 (326.6–620.1)	506.5 ± 102.3 (316.5–605.9)	520 ± 90.0 (400–653)	574 ± 60.7 (482–706)	680 ± 63.0 (620–760)	408 ± 60.0 (320–520)
	L/W ratio	1.2 ± 0.1 (0.9–1.4)	1.2 ± 0.2 (1.0–1.5)	1.2 ± 0.1 (1.0–1.4)	1.5 ± 0.4 (1.0–2.9)	1.1 ± 0.1 (1.0–1.2)	1.1 ± 0.1 (1.0–1.2)	1.2 ± 0.1 (1.1–1.5)
Vulval cone	Vulval slit length	48.6 ± 2.1 (46.0–51.6)	52.3 ± 2.7 (48.4–56.4)	48.2 ± 2.4 (44.0–51.9)	49.0 ± 4.3 (42.0–56.0)	49.9 ± 4.9 (40.5–59.8)	48.8 ± 5.1 (41.3–55.0)	51.9 ± 4.3 (46.0–59.0)
Second-stage Juvenile	n	10	10	10	20	20	20	14
	L	516.5 ± 17.7 (480–545)	487.9 ± 18.1 (454–514)	491.8 ± 10.6 (476–507)	446 ± 28.0 (390–509)	458 ± 17.3 (424–483)	513 ± 29.2 (448–553)	455 ± 11.3 (437–472)
	a	26.5 ± 1.1 (24.8–28.1)	25.3 ± 1.3 (23.2–27.1)	25.7 ± 0.5 (24.7–26.5)	28.0 ± 1.7 (25.0–32.0)	26.3 ± 0.8 (25.3–27.8)	29.9 ± 1.6 (26.0–32.2)	29.9 ± 0.9 (28.3–31.5)
	c	6.7 ± 0.2 (6.2–6.9)	6.6 ± 0.1 (6.4–6.8)	6.6 ± 0.1 (6.4–6.8)	6.7 ± 0.3 (6.1–7.3)	6.3 ± 0.2 (6.0–7.0)	6.7 ± 0.7 (6.2–8.1)	7.4 ± 0.9 (6.0–8.9)
	Stylet	21.0 ± 0.5 (20.5–21.8)	20.7 ± 0.4 (20.2–21.1)	20.7 ± 0.3 (20.3–21.0)	18.0 ± 1.5 (16.0–20.0)	17.5 ± 0.6 (16.4–18.9)	19.0 ± 0.9 (17.5–20.5)	18.1 ± 0.5 (17.0–19.0)
	Labial region height	4.7 ± 0.3 (4.4–5.2)	4.6 ± 0.5 (3.9–5.4)	4.7 ± 0.4 (4.0–5.2)		3.6 ± 0.2 (3.3–3.9)		3.0 (3.0)
	Labial region diam.	8.7 ± 0.3 (8.3–9.3)	8.5 ± 0.2 (8.3–8.8)	8.5 ± 0.2 (8.0–8.7)		8.1 ± 0.2 (7.7–8.4)		7.5 ± 0.5 (7.0–8.0)
	Anterior end to median bulb value	75.7 ± 1.2 (73.0–77.1)	71.8 ± 3.4 (63.8–75.6)	73.6 ± 2.0 (70.6–77.3)	72 ± 7.2 (65–98)	70 ± 3.0 (65–77)	72 ± 6.8 (60–80)	72 ± 1.7 (70–75)
	Anterior end to excretory pore	110.4 ± 2.9 (105.9–113.8)	102.9 ± 7.4 (84.3–110.3)	105.8 ± 5.0 (93.0–110.9)	95 ± 4.3 (88–103)	105 ± 3.0 (98–111)	111 ± 6.7 (97–118)	100 ± 2.5 (96–103)
	Body diam. at mid-body	19.5 ± 0.9 (18.0–21.1)	19.3 ± 1.0 (18.2–21.6)	19.2 ± 0.5 (18.4~20.0)	16.0 ± 1.0 (14.0–17.0)	17.4 ± 0.4 (16.5–18.1)	17.2 ± 0.5 (16.0–18.0)	15.2 ± 0.4 (15.0–16.0)
	Body diam. at anus	13.7 ± 0.6 (13.1–14.7)	13.1 ± 0.3 (12.4–13.4)	13.2 ± 0.6 (12.3–14.5)	–	12.2 ± 0.4 (11.5–12.7)	–	10.1 ± 1.0 (8.0–11.0)

Table 2 (continued)

Stage	Character	Korea (this study)			Korea	Japan	China	Iran
		BC348	DR437	DR1256	Vovlas et al., 1992	Sekimoto et al., 2017	Wang et al., 2012	Maafi and Taheri, 2015
	Tail length	76.7 ± 4.4 (70.6–85.0)	74.0 ± 3.3 (68.2–78.9)	74.6 ± 1.4 (72.0–76.7)	66 ± 4.4 (59–74)	72 ± 4.4 (63–79)	77 ± 8.9 (62–88)	62 ± 6.9 (51–74)
	Hyaline region	52.4 ± 4.3 (48.1–60.1)	51.0 ± 1.8 (48.8–53.5)	49.3 ± 3.0 (45.5–55.0)	40 ± 2.5 (35–46)	41 ± 3.2 (33–45)	49 ± 5.2 (39–56)	44 ± 1.8 (40–47)

The stylet had a length of 20–21 µm and was well developed, stylet-knobs were oviform (Fig. 1E and G), and the body length ranged from 454 to 545 µm (Table 2). The J2s had three incisures in the lateral field (Fig. 1F). Anus and hyaline part of tail were distinct, and the hyaline terminal section averaged 50.9 (45.5–55.0) µm long (Fig. 1H).

Molecular and phylogenetic analysis The LSU D2-D3 segments, ITS region, and *CO*I gene of the mtDNA were amplified as indicated in the methodology section. The sequenced LSU D2-D3 segments, ITS region, and *CO*I are 751–755, 902–908, and 424–425 bp, respectively. A BLASTn search on the LSU D2-D3 segments and ITS region revealed similarities with the Afenestrata group of *Heterodera* species such as *H. Koreana* and *H. hainanensis*. The highest match of the LSU D2-D3 segment sequences was *H. koreana* (LC202092), with 100% identities and no gaps. The ITS region results also revealed that the most similar species was *H. koreana* (KX640828), with 99.89% identities (901/902) and no insertions/deletions. In addition, a BLASTn search of *H. koreana* on the *CO*I revealed high-scoring matches with *H. koreana* (LC202153), which is the species isolated from *Phyllostachys nigra* var. *henonis* in Iwate in Japan. The identities between the Korean population (this study) and *H. koreana* (LC202153) were 98.21% (385/392), with no insertions/deletions.

The molecular phylogenetic relationships of Korean populations of *H. koreana* were shown in Figs. 2, 3, and 4. The phylogenetic tree of the LSU D2-D3 segments of *Heterodera* species is shown in Fig. 2. The average nucleotide composition was as follows: 19.33% A, 21.47% C, 33.92 G and 25.28% T. Using *Cryphodera brinkmani* and *Meloidodera sikhotealiniensis* as the outgroup taxa, the molecular phylogeny strongly supported monophyly of *Heterodera* species. Phylogenetic tree inferred from the ITS region dataset is shown in Fig. 3. The average nucleotide composition was as follows: 19.17% A, 22.44% C, 29.04% G and 29.35% T. Three Korean populations of *Heterodera* species were close to *H. koreana* when *C. brinkmani* and *M. sikhotealiniensis* were used as the outgroup taxa. The results showed that the BC348, DR437, and DR1256 populations belong to the '*Afenestrata*' group clade.

The phylogenetic tree based on the *CO*I gene of mtDNA is shown in Fig. 4. The average nucleotide

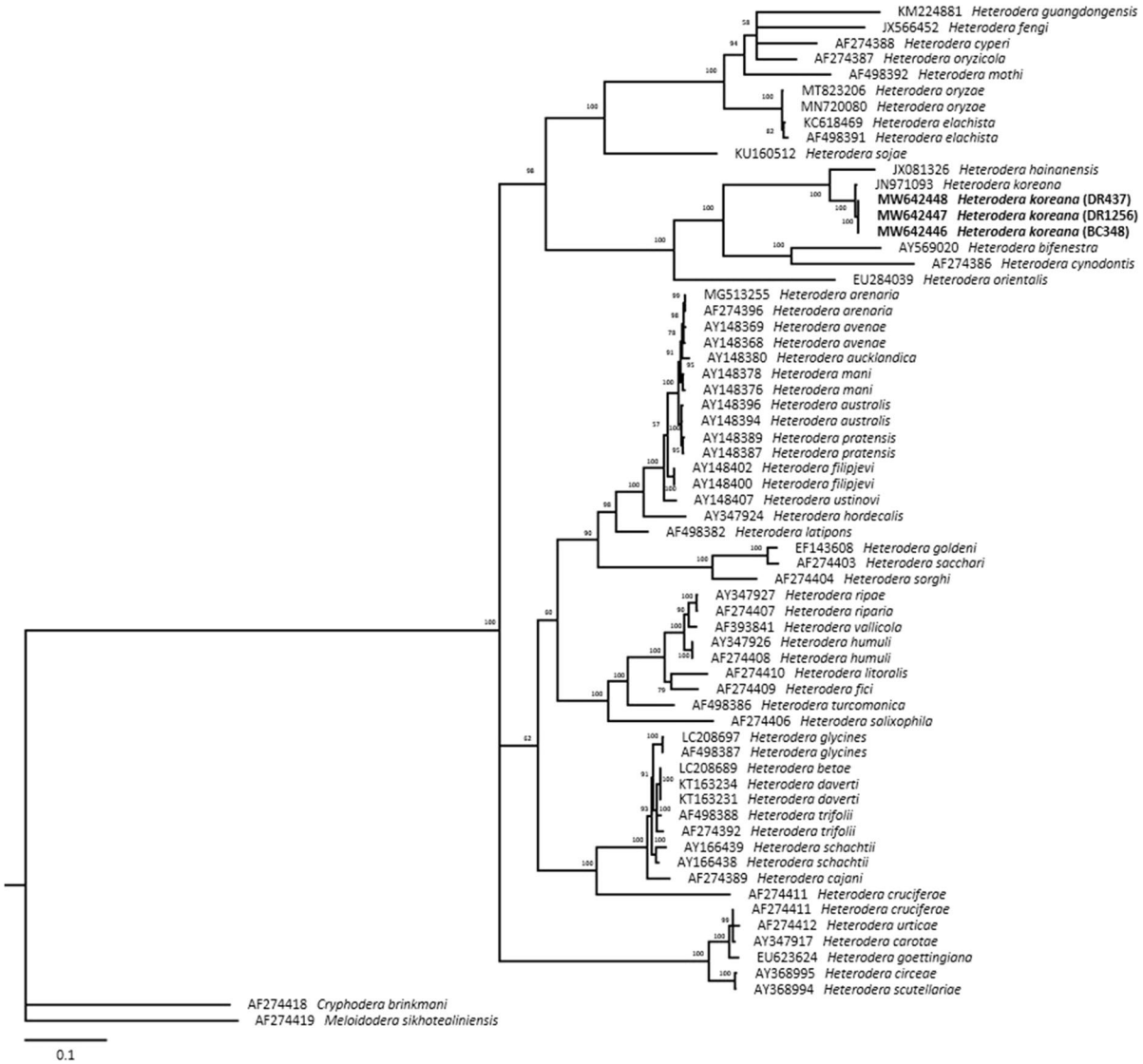

Fig. 2 Phylogenetic relationships within population and species of *Heterodera*. Bayesian 50% majority rule consensus tree from two runs as inferred from the analysis of the D2-D3 of 28S rDNA gene sequences under the GTR + I + G model. Posterior probability values more than 50% are given in appropriate clades. Newly sequenced samples are indicated by bold font

composition was as follows: 24.50% A, 8.68% C, 14.18 G and 52.64% T. Using *Rotylenchus eximius* and *R. urmiaensis* as the outgroup taxa, the molecular phylogenetic relationship of the dataset, which contains three Korean populations and previously registered data in NCBI, was closest to *H. koreana*. However, the three Korean populations were not clustered together with the Japanese haplotype of *H. koreana*, haplotype A-C, and were classified to a new clade of *H. koreana*.

Data of *CO*I gene analysis We identified four haplotypes (Haplotypes A–C, and Korean haplotype) in the 46 *CO*I sequences (357 bp), and the dataset included 21 polymorphisms (Table 3). The network analysis showed a radial-shaped haplotype network with the most common Haplotypes A (48%) and B (43%) occupying a central position with the rest of the haplotypes differing by up to 13 substitutions (Fig. 5). Haplotypes A and C were found in Japan. Haplotype

B was found in both Japan and the USA. The Korean haplotype was newly found in this study in Korea. We analyzed haplotype diversity and nucleotide diversity by regional populations. In the Japan populations, the haplotype diversity was 0.5317 ± 0.0319 and the nucleotide diversity was 0.0090 ± 0.0053. The Tajima's D statistic was negative for the Japan populations (-0.68579). The Korean and USA populations had no diversity in the haplotype and the nucleotide, because there was no variation in the regional populations.

Pairwise F_{ST}, which is a fixation index between regional populations, was calculated to estimate the genetic differentiation that can be caused by the genetic structure. The Korean population had high fixation index values of 1.000 with the USA populations and 0.680 with the Japanese populations, indicating a differentiated genetic structure to the Korean population. On the other hand, the Japanese and USA populations had low differentiation, with a fixation index of 0.177. The AMOVA showed that variation by region was responsible for 60.46% of the total variation. The remaining 39.54% of the variance was explained by the variation among populations within region (7.76%) and variation within populations (31.78%) (Table 4).

Discussion

During a PPNs survey in 2020, three populations of *Heterodera* species were isolated from the

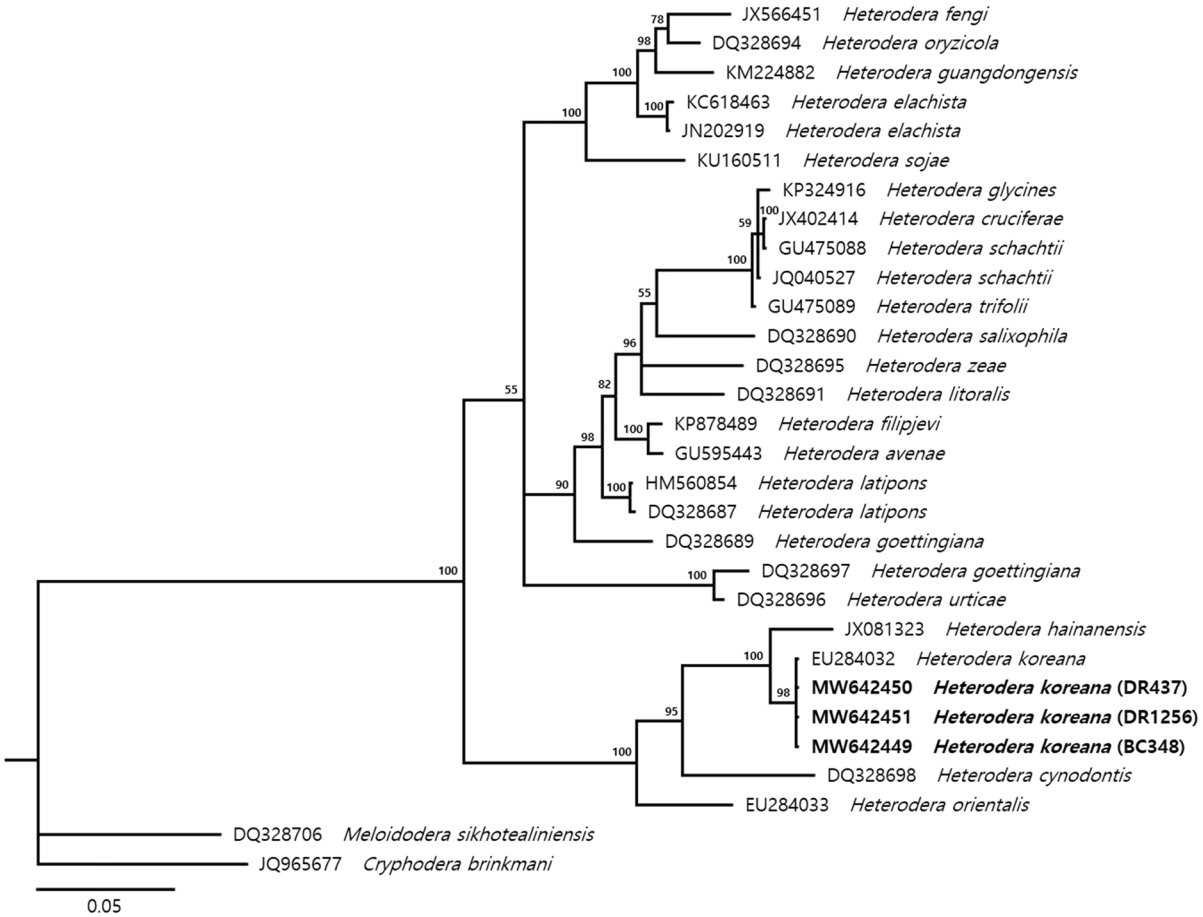

Fig. 3 Phylogenetic relationships within population and species of *Heterodera*. Bayesian 50% majority rule consensus tree from two runs as inferred from the analysis of the ITS rRNA gene sequences under the TVM+I+G model. Posterior probability values more than 50% are given in appropriate clades. Newly sequenced samples are indicated by bold font

rhizosphere of bamboo in the Republic of Korea. Three cyst-forming nematodes were identified as *H. koreana* using a morphological identification key for the *Afenestrata* sensu stricto group together with phylogenetic analysis (Table 2, Figs. 1 and 2). The genus *Heterodera* contains seven sensu stricto groups, which are the *Afenestrata*, *Avenae*, *Cyperi*, *Goettingiana*, *Humuli*, *Sacchari* and *Schachtii* groups (Subbotin et al., 2010). Bayesian trees inferred from LSU D2-D3 segments and the ITS region showed that *H. koreana* is related to the *Afenestrata* group (Figs. 2 and 3). The *Afenestrata* group includes the following seven species, which are *H. africana*, *H. axonopi*, *H. bamboosi*, *H. hainanensis*, *H. koreana*, *H. orientalis* and *H. saccharophila* (Mundo-Ocampo et al., 2008; Zhuo et al., 2013). Of these, three *Heterodera* species, which are *H. bamboosi*, *H. koreana* and *H. hainanensis*, have been recorded from bamboo in the world (Kaushal & Swarup, 1988; Vovlas et al., 1992; Zhuo et al., 2013), but the sequences of *H. bamboosi* were absent in GenBank. Nevertheless, *H. koreana* could be distinguish from *H. bamboosi* by the shorter body length in J2 (446 vs. 472 µm), and the vulval cone present in females (Subbotin et al., 2010).

Morphology and morphometrics of the cysts and the J2s of the three Korean populations were

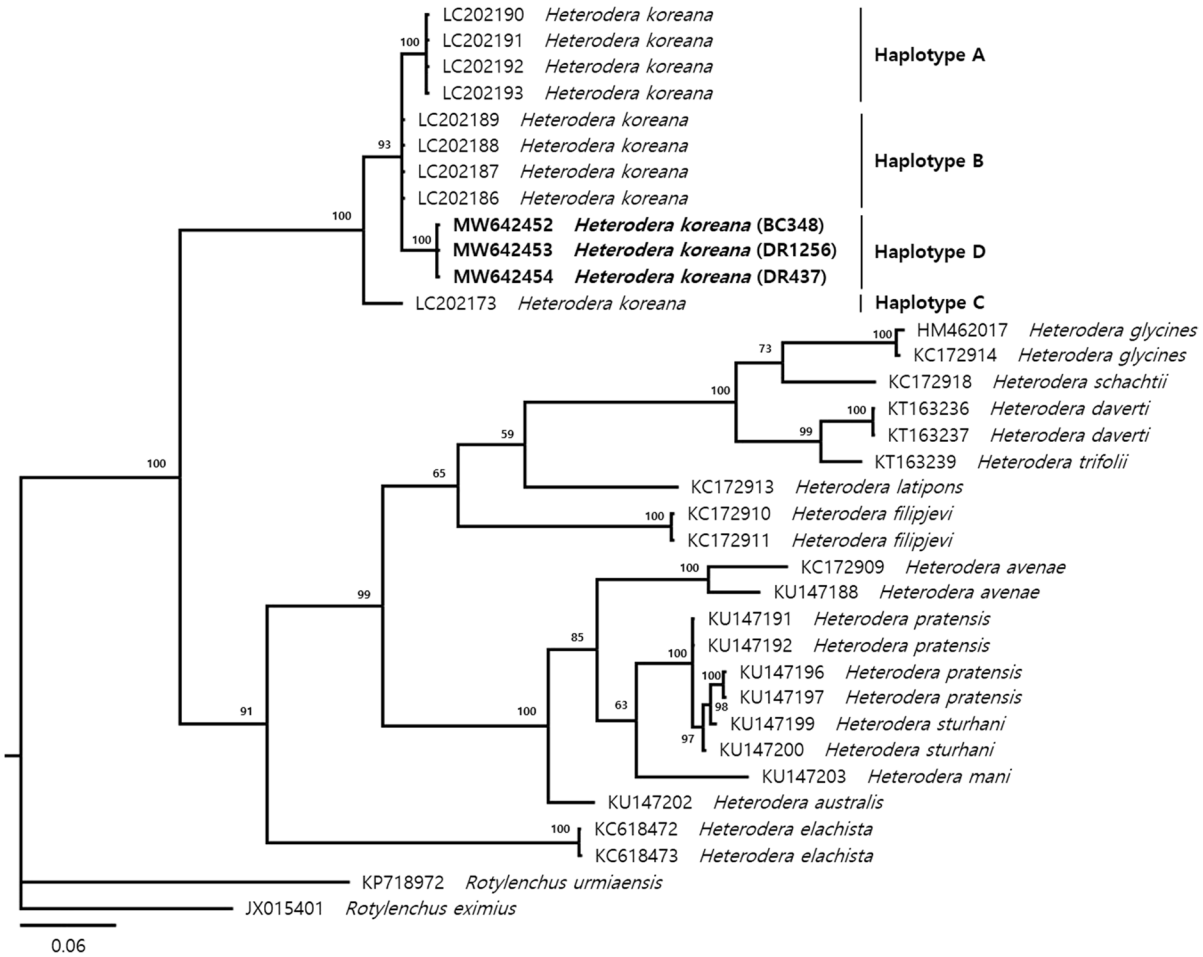

Fig. 4 Phylogenetic relationships between *Heterodera* species. Bayesian 50% majority rule consensus tree as inferred from the analysis of the *COI* gene sequence alignment under the TVM+I+G model. Posterior probabilities over 50% are given for appropriate clades. Newly obtained sequences are indicated by bold font

consistent with the described *H. koreana* in China (Wang et al., 2012). However, these populations differed from the original descriptions in the Republic of Korea and Japanese populations by the shorter cyst body length, the longer J2 body length, tail length, and the length of the hyaline region (Sekimoto et al., 2017; Vovlas et al., 1992). The 'c' value and 'tail length' in the Korean populations were inconsistent with the original description of the Iranian population (Maafi and Taheri, 2015). These differences in morphology could be explained as a result of intraspecific variation (Wang et al., 2012).

Recently, molecular and phylogenetic analysis based on DNA barcoding genes such as the LSU D2-D3 segments, ITS region, and the mtDNA *COI* gene have become very important to speed up and simplify the identification of animals including plant-parasitic nematodes. The *COI* gene is a very powerful DNA barcoding marker, and has been used in barcoding *Heterodera* species since 2005 (Blok & Powers, 2009; Derycke et al., 2005; Subbotin et al., 2015; Vovlas et al., 2015). However, our phylogenetic study based on the *COI* showed the haplotype of the Korean populations did not cluster together with Japanese populations (Fig. 4).

In a previous study, three *COI* haplotypes of *H. koreana* were found in Japan, and named as haplotypes A, B and C (Table 3). Haplotype A is dominant in Japan (Sekimoto et al., 2017). However, our study showed that a new haplotype of *H. koreana* was present in Korea, because the haplotype of the Korean populations was distinguished from the Japanese haplotype, and the AMOVA analysis also showed substantial regional differences (60.46%) between the Korean populations and Japanese populations, which was greater than that between the total populations (31.78%) (Table 4). Thus, we suggest that a new haplotype of *H. koreana* is present in the Republic of Korea and name it as haplotype D (Table 3 and Fig. 5).

To investigate associations between genetic haplotype and phenotype in the nematodes, we performed morphological comparisons between the Korean haplotype and the haplotypes reported in China and Japan. Our study showed that the whole length and hyaline portion in J2s in the Korean populations were similar to that of the Chinese population (Wang et al., 2012) (Table 2). However, our results showed that the Korean population had a longer J2 body

Table 3 Variable position in the four *COI* haplotypes of *Heterodera koreana* populations

Haplotype	Accession No.	Variable position (bp)																			
		33	45	57	72	75	81	87	132	159	165	171	186	189	192	199	213	228	257	267	288	327
A	LC202164-7	A	G	A	C	T	A	A	G	G	A	T	A	A	T	G	A	C	G	T	A	G
	LC202169-83	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	LC202190-3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B	LC202153-63	A	G	A	T	G	A	A	A	G	A	T	A	A	T	G	T	T	G	T	A	G
	LC202168	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	LC202184-9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	OL813218-9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C	LC202173	A	A	T	T	T	G	A	A	A	A	A	G	T	C	A	T	T	A	T	G	A
D	MW642452-4	G	G	A	T	A	A	G	G	A	G	T	A	A	T	G	T	T	G	C	A	G

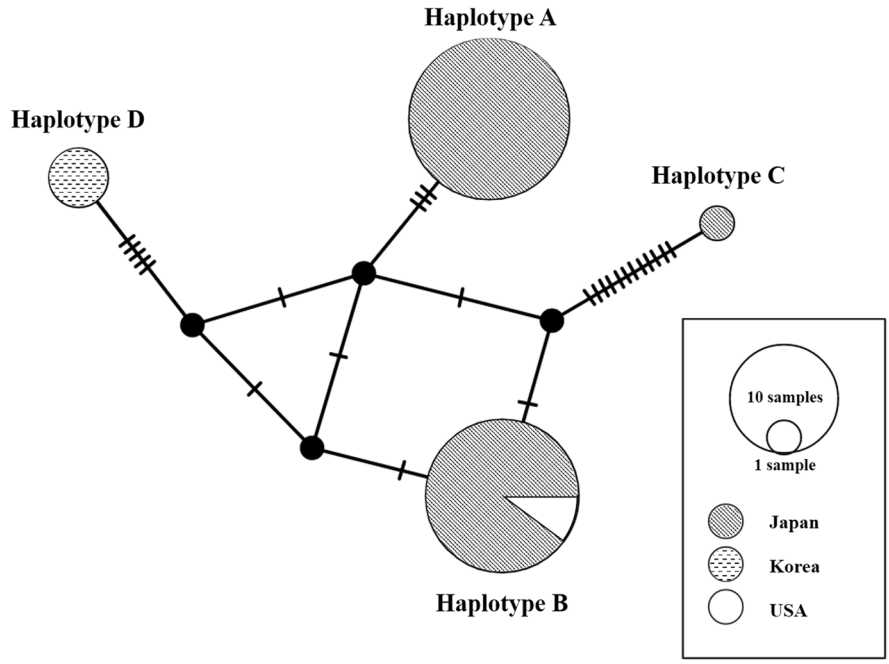

Fig. 5 Median-joining haplotype network. Haplotypes mentioned in text are indicated. Black circle represents an un-sampled haplotype. Mutations are indicated with hatch-marks

Table 4 Analysis of molecular variance of three populations of *Heterodera koreana*. Group refers to the pooling of Japan and USA together and Korea alone

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation indices	p-value[a]
Among group	1	18.887	2.76860	60.46	$F_{CT}=0.60460$	0.00089
Among populations within groups	1	2.810	0.35515	7.76	$F_{SC}=0.68216$	0.03317
Within populations	43	62.585	1.45547	31.78	$F_{ST}=0.19615$	0.00000
Total	45	84.283	4.57922			

[a] Significance tests were performed with 10,100 permutations

length (499 ± 15.5 vs. 458 ± 17.3 µm) and a longer hyaline length of the J2 tail (51 ± 3.0 vs. 41 ± 3.2 µm) than those of the Japanese population (Sekimoto et al., 2017) (Table 2). Thus, we suggest that the differences in the haplotype of the *COI* gene sequence may affect the morphological differences within the species *H. koreana*. The studies on the association between the genotype of LSU D2-D3, ITS regions, *COI*, and morphological characteristics were conducted in marine nematodes. The results showed that highly divergent genotype clusters were accompanied by morphological differences (Derycke et al., 2008), and in the *COI* gene and ITS region particularly (Fonseca et al., 2008). Because the sequence of the Chinese haplotype is absent in NCBI Genbank, further study of the association between genetic haplotype and phenotype is required. In addition, the various haplotypes could be distributed in Republic of Korea due to the similar morphology between the original description of *H. koreana* in Republic of Korea and those of the Japanese population (Sekimoto et al., 2017; Vovlas et al., 1992). The haplotype may be associated with phenotypes of the nematodes like ecotype, and pathotype. Therefore, further study of correlations between the haplotypes of the nematodes (Haplotype A, B, C and, D) and phenotypes in bamboo, including a pathogenicity, is required.

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Declarations

Ethics approval All the authors certify that the work carried out in this research followed the principles of ethical and professional conduct have been followed.

Conflicts of interest No potential conflict of interest relevant to this article was reported. Additionally, the authors certify that soil samplings did not involve any species endangered or protected in Republic of Korea.

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