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Morphological and Molecular Characterization of *Heterodera schachtii* and the Newly Recorded Cyst Nematode, *H. trifolii* Associated with Chinese Cabbage in Korea

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The sugar beet cyst nematode, *Heterodera schachtii* is a well known pathogen on Chinese cabbage in the highland fields of Korea. However, a race of cyst forming nematode with close morphological resemblance to *H. trifolii* was recently isolated from the same Chinese cabbage fields. Morphological species differentiation between the two cyst nematodes is challenging, with only minor differences between them. Thus, this study described the newly intercepted *H. trifolii* population, and reviewed morphological and molecular characteristics conceivably essential in differentiating the two nematode species. A comparison of morphometric measurements of both infective juveniles and vulval cones of cysts showed significant differences between the two cyst nematodes. When total RNA and genomic DNA were extracted from a mixed field population, *CO*I genes and ITS regions were clearly amplified with primers of the two *Heterodera* species, suggesting that *Heterodera* population collected from the Chinese cabbage field consisted of a mixture of two species. *CO*I and ITS of *H. trifolii* were predominantly amplified from nucleotides prepared from *H. trifolii* monoxenic population whereas those of *H. schachtii* were strongly detected in *H. schachtii* monoxenic cultures. Thus, this study confirms the coexistence of the two species in some Chinese cabbage fields; and the presence of *H. trifolii* in Korea is reported here for the first time.

Keywords **:** infective juvenile, morphometrics, vulval cone

Handling Associate Editor **:** Lee, Yong Hoon

The clover cyst nematode (CCN), *Heterodera trifolii* Goffart, 1932 and the sugar beet cyst nematode (SBCN), *H. schachtii* Schmidt, 1871 constitute the *H. schachtii sensu stricto* group, along with *H. ciceri*, *H. betae*, *H. galeopsidis*, *H. daverti*, *H. lespedezae*, *H. glycines*, *H. medicaginis* and *H. rosii* (Subbotin et al., 2000). *H. schachtii* is a predominant threat in sugar beet production, and attacks over 200 plant species within 23 different plant families; most notably, the Chenopodiaceae and Cruciferae families (Amiri et al., 2002; Steele, 1965). The species has a wide distribution in several Asian countries, Europe, Australia, North America, Africa and South America (Baldwin and Mundo-Ocampo, 1991; Evans and Rowe, 1998). By contrast, *H. trifolii* is known to attack over 86 plant species within 9 families (Goodey et al., 1965; Mercer and Woodfield, 1986). The species is economically important on clover and sugar beet (Ambrogioni et al., 1999; Amiri et al., 2002), and has a wide distribution, mostly in European countries like Netherlands, Sweden, Germany and Italy (Ambrogioni et al., 1999). It is also recorded in New Zealand, UK, USA, Russia, Canada and recently Japan (Inserra et al., 1993; Kimpinski et al., 1993; Sekimoto et al., 2016,

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2017).

H. schachtii and *H. trifolii* have similarities in host range and thus, coexistence as mixed populations on the same host is possible (Steele and Whitehand, 1984). Distinguishing the two species from each other especially at field level may not be feasible since both exhibit similar morphological body characteristics and symptoms of damage. However, there exist some minor morphological and morphometrical differences useful in distinguishing all species of the *H. schachtii sensu stricto* group from each other at laboratory level (Amiri et al., 2002).

The SBCN has been identified as one of the serious pests of economic importance on Chinese cabbage production in Korea (Lee et al., 2013). Reports indicate increasing dissemination and infestation rates within the highland Chinese cabbage growing areas of Gangwon-do province (from 11.2 ha in 2011 to 70 ha in 2015) (Kwon et al., 2016). However, a race of cyst nematode with close morphological resemblance to *H. trifolii* was recently isolated from the same Chinese cabbage fields manifesting symptoms of damage similar to *H. schachtii*. Chinese cabbage, *Brassica rapa chinensis* L. (Bae-chu) is a highly valued economic crop and the leading vegetable in both production and consumption in Korea (Chang et al., 2008; Kim et al., 2014). Thus, pest and disease management is very crucial, and efforts to combat cyst nematodes are underway (Kim et al., 2016). However, correct species identification of plant parasitic nematodes is a vital milestone preceding successful nematode control and management strategies. Therefore, this study aimed at providing a detailed morphological, morphometric and molecular description of the newly intercepted *H. trifolii* population in Korea and review characteristics conceivably essential in distinguishing the two nematode species.

Materials and Methods

Cyst nematode populations. Samples of *H. trifolii* (YS503) and *H. schachtii* (GC147) populations were taken from Jeongseon cabbage fields (Table 1), as part of a survey to determine the dispersal rate of cyst nematodes in the infested cabbage fields based on field symptoms of stunted growth, yellowing and wilting of Chinese cabbage plants. YS503 and GC147 were subsequently cultured and maintained as monoxenic cultures on Chinese cabbage in pots at National Academy of Agricultural Science laboratories, Wanju, Korea. Mixed populations (TB372) were sampled from Chinese cabbage fields of Taebaek. Cysts were extracted from infested field soil samples and Chinese cabbage roots using modified Cobb's decanting and sieving method (EPPO, 2013). Cysts were recovered from a 250 µm sieve and filtered through Whatman® 150 mm filter paper (GE Healthcare Life Sciences, Marlborough, USA) before handpicking using fine forceps under a stereomicroscope Nikon SMZ 1000 (Nikon, Tokyo, Japan). Infective second stage juveniles (J2s) were extracted from the soil using a modified Baermann funnel method (EPPO, 2013).

Morphological identification. Twenty J2 specimens of each species emerging from monoxenic cultured cysts (*H. trifolii* YS503 and *H. schachtii* GC147 populations) were heat-killed on temporary slides and directly observed or first fixed and mounted in pure glycerin (Seinhorst, 1959). Ten vulval cones were prepared from undamaged cysts using a method described in Van Bezooijen (2006). Undamaged cysts were soaked in water before vulval cones were excised and transferred into a drop of glycerin-gelatin mixture. Measurements and pictures were made using Zeiss imager Z2 microscope (Carl Zeiss, Gottingen, Germany), fitted with a software AxioVision; Material Science Software for Research and Engineering (Carl Zeiss, Gottingen, Germany), and identification was made based on the key provided by Wouts and Baldwin (1998). All measurements were recorded in µm. Standard deviation and measurement range were calculated and recorded for each arithmetic mean.

Molecular characterization. For molecular characterization, two procedures were performed using species specific and universal primers, respectively. Sequences of *CO*I, ITS and beta-tubulin of the two *Heterodera* species were compared and species-specific primers were designed for

Table 1. The nematode populations used in this study

Population	Date	Host plant	Coordinates
GC147	27th Jul 2016	Chinese cabbage	37.3330700, 128.8464880
YS503	16th Aug 2017	Chinese cabbage	37.4397300, 128.8565550
TB372	9th Oct 2017	Chinese cabbage	37.2558180, 128.9830310

Coordinates for GC147 and YS503 represent individual cabbage fields where samples were taken in Jeongseon, respectively while Coordinates for TB372 represent cabbage field where mixed populations were sampled in Taebaek.

*CO*I and ITS, while conserved regions of beta-tubulin were selected for positive control amplification (Supplementary Table 1, Supplementary Fig. 1). Total RNA and genomic DNA were extracted from cysts with TRI Reagent® (MRC, Cincinnati, OH, USA) according to the manufacturer's protocol with some modifications. Six cysts (from TB372 population), or individual cyst (from YS503 and GC147 populations) were transferred into an eppendorf tube and completely homogenized with 200 µl TRI Reagent® (MRC) using a bullet blender (Bertin Technologies, Montigny-le-Bretonneux, France). After centrifugation at 12,000 × g for 15 min at 4°C, the aqueous phase and interphase containing total RNA and genomic DNA, respectively, were obtained. After nucleic acid extraction, DNase I was not treated for genomic DNA conservation. First strand cDNA was synthesized from 250 ng total RNA using Superscript™ III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), by priming with oligo (dT). Thirty five cycles of PCR amplifications were performed using Ex Taq DNA polymerase (Takara Korea Biomedical Inc., Seoul, Korea), at 98°C for 10 s, 55°C for 30 s, and at 72°C for 30 s. PCR products were visualized in the 1.5% agarose gel.

For *CO*I and ITS sequencing, DNA was extracted from cysts following a method described by Iwahori et al (2000). Briefly, 10 cysts from each monoxenic population were separately transferred into a drop of water on a glass slide and crushed gently with a piece of filter paper (smaller than 2 mm × 2 mm in size) and small tweezers, by exertion of pressure onto the filter paper. The crushed cyst contents were then transferred into the 0.2 ml PCR tubes containing Proteinase K solution (2 M KCl_2, 10 mM Tris HCl, 1 M $MgCl_2$, 10% [w/v] Triton-X 100, 20.6 mg/ml Proteinase K) and DNA was extracted using PCR cycler (PTC-200, MJ Research, CA, USA), at 60°C for 30 minutes and at 94°C for 10 minutes of the lysis process. Polymerase chain reaction (PCR) was then performed to amplify ITS rRNA (Joyce et al., 1994) and *CO*I gene (Derycke et al., 2005) of the 2 cyst-forming nematodes, *H. trifolii* and *H. schachtii*, using the universal primer sets as indicated in Supplementary Table 2. The PCR reaction mixture composed of 15 µl PCR premix (Ready-2×-Go with Taq plus; Nanohelix™, Daejeon, Korea), 4 µl template DNA, 0.5 µl forward primer, 0.5 µl reverse primer, and 30 µl sterilized deionized water. PCR reaction was conducted with a thermal cycler (PTC-200, MJ Research) and the program was as follows: 35 cycles of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C for amplification of ITS rRNA, and 35 cycles of 30s at 94°C, 30s at 53°C, and 45s at 72°C for *CO*I gene.

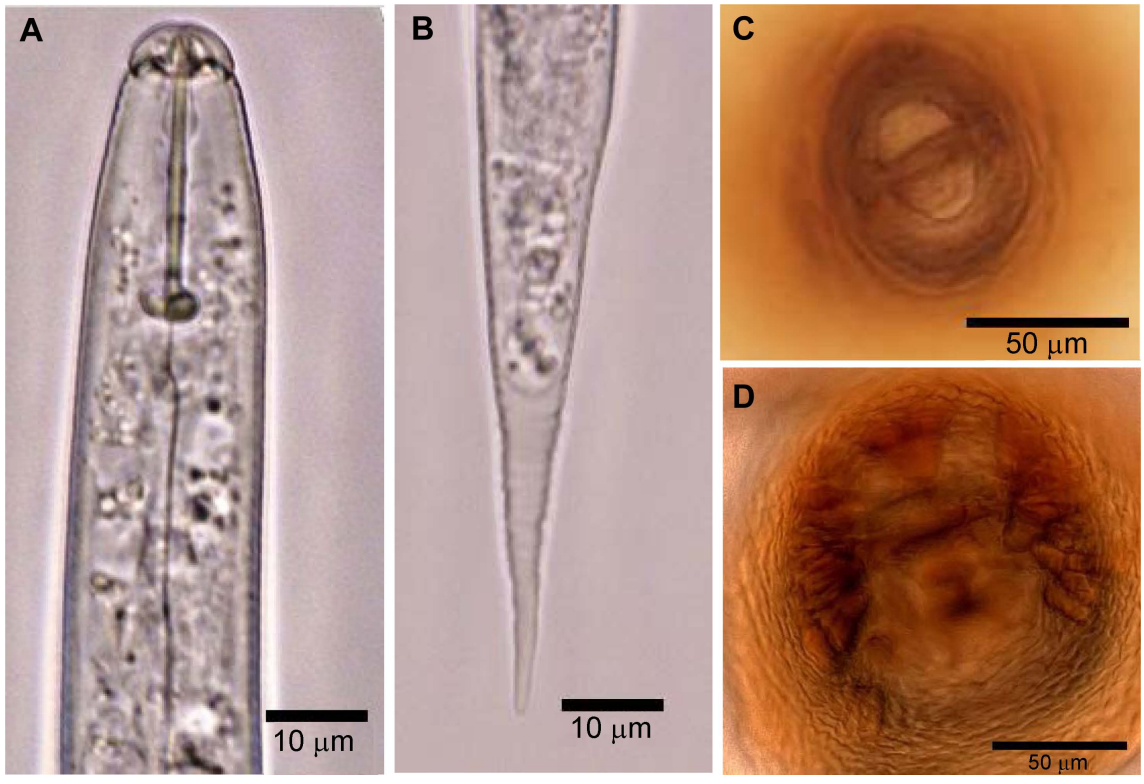

Fig. 1. Photomicrographs of *Heterodera trifolii* juveniles and vulval cone. (A) Head region. (B) Tail region. (C) Top view showing the fenestra region. (D) Prominent but scattered bullae.

The PCR products were confirmed with electrophoresis equipments (Mupid-eXu, Advance, Tokyo, Japan) and gel documentation system (Major Science, New Taipei City, Taiwan).

Phylogenetic analysis. The PCR products were purified using PCR purification kit (PCR and Gel clean up, Macherey-Nagel, Duren, Germany) with a provided manual. DNA sequence analysis (n=10) of two population was done at GenoTech Corporation (Dajeon, Korea). Outgroup taxa for each dataset was taken based on references from previous studies (Sekimoto et al., 2017; Subbotin et al., 2001; Vovlas et al., 2015). The newly obtained sequences of ITS rRNA and *COI* gene were aligned using ClustalX 1.83 program with datasets of 'Schachtii' group species, registered in National center for biotechnology information. Bayesian analysis of the sequence datasets was performed using MrBayes 3.1.2 (Kang et al., 2016; Sekimoto et al., 2017; Vovlas et al., 2015). The general time reversible substitution model (GTR+I+G) was chosen for this analysis and was run with four chains for 1×10^6 generations. The posterior probability of phylogenetic tree was estimated with MCMC (Markov Chain Monte Carlo) method and consensus tree was generated with a 50% majority rule. The trees were edited and visualized using Dendroscope 3.5.7. Intraspecific and interspecific sequence variation was analyzed using Mega 6.0 program (Tamura et al., 2013).

Statistical analysis. Morphometric data of both J2s and vulval cones of the two nematode species were subjected to analysis of variance according to the general linear model procedure using the SAS statistical package (SAS Institute Inc. Cary, North Carolina, USA). Arithmetic means were compared using Tukey's HSD (Honest Significant Difference) test at $P \leq 0.05$ to test the differences or similarities between measurements of the different morphological characters used in identification. Additionally, arithmetic means, standard deviation and measurement range were calculated for all morphometric characters and comparisons with morphometrics extracted from other similar studies were made (Supplementary Table 2, 3, 4 and 5).

Results

Description: *H. trifolii* (YS503).

Infective second stage Juveniles (J2s) (n = 20). Average body length was 601.9 ± 20.6 µm (561.9-630.8) and body posture was generally straight or slightly curved when heat

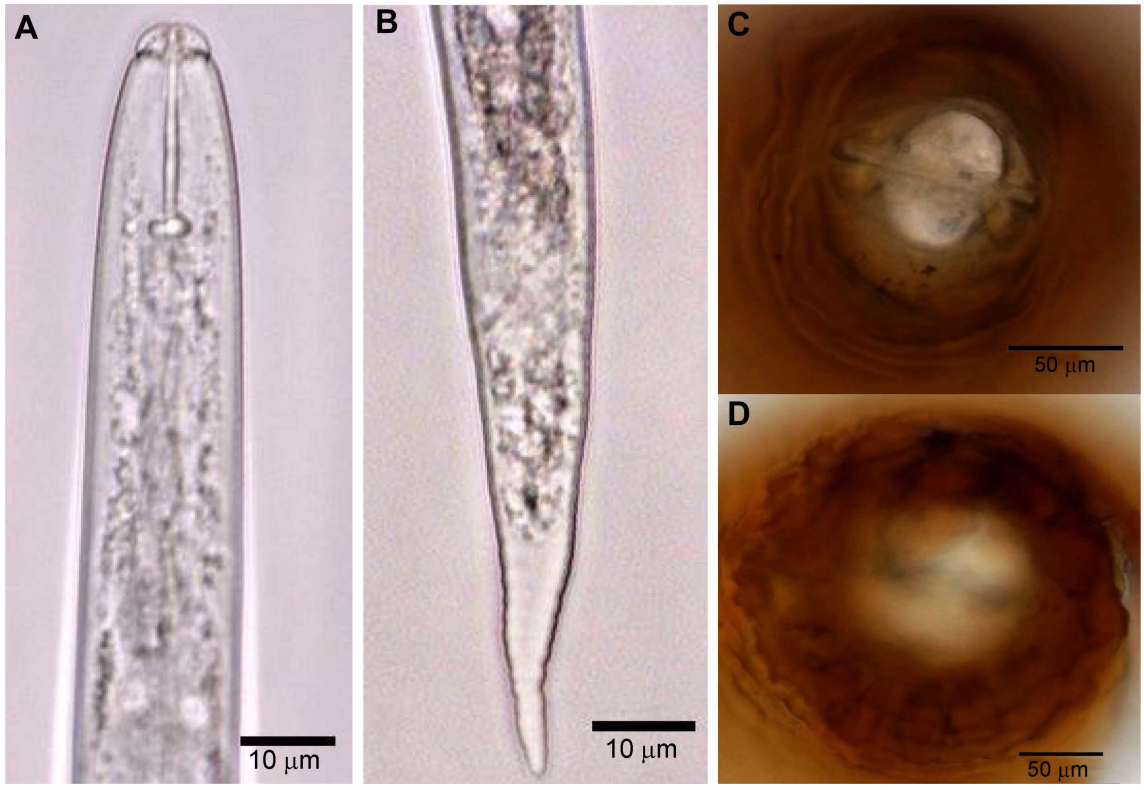

Fig. 2. Photomicrographs of *Heterodera schachtii* juveniles and vulval cone. (A) Head region. (B) Tail region. (C) Top view showing the fenestra region. (D) Numerous prominent molar shaped bullae.

killed. Body tapers posteriorly into a conoid finely rounded tail terminus. Head offset from the body, about 4.7 ± 0.5 µm (4.0-5.4) long and 10.7 ± 0.5 µm (9.9-11.8) wide, heavily sclerotized and has 4 annules. The stylet appears strong and robust with deeply concave anterior directed knobs (Fig. 1), measuring 3.7 ± 0.4 µm (3.2-4.7) long and 7.0 ± 0.5 µm (6.3-7.5) wide. Dorsal gland orifice located 4.7 ± 0.5 µm (4.0-5.4) posterior to the stylet knobs. Excretory pore located at 132.6 ± 2.9 µm (127.9-136.5) from the head tip. Well-developed esophageal glands clearly visible, subventrally overlapping the intestine and extending well posterior to esophago-intestine valve. Lateral field marked with 4 incisures covering approximately 1/4 of the body width, not areolated. The tail hyaline part forms approximately 55% of the tail length (38.2 ± 2.1 µm (35.5-41.6).

Cysts (n = 10). Cysts appear light to dark brown in color and generally lemon shaped with protruding neck and vulval regions, aligned with zigzag cuticular pattern; measuring 910.0 ± 160 µm (650.0-1100.0) in length and 500.0 ± 99.2 µm (350.0-601.0) in width, with a length to width ratio of 1.8 ± 0.2 (1.7-2.1). Top cone structures ambifenestrate with fenestra length and width of 50.9 ± 4.9 µm (47.1-58.0) and 36.0 ± 2.2 µm (33.4-38.3), respectively. A well-developed vulval bridge separates the semifenestrae (Fig. 1). Beneath the vulval bridge lies a well-developed underbridge, 112.7 ± 10.3 µm (105.2-127.8) long and 35.5 ± 5.6 µm (29.8-44.7) wide. Underbridge strongly pigmented, heavier, with bifurcate ends. Long vulval slit (47.3 ± 2.8 µm (44.0-50.8). Prominent bullae present, often appearing irregular in shape and scattered. The observations are in agreement with the descriptions in Abawi et al. (1973), Mulvey (1957), Turner and Subbotin (2013) and Wouts and Baldwin (1998). No males were intercepted in the soil samples.

***Heterodera schachtii* (GC147).**

Infective second stage Juveniles (J2s) (n = 20). The J2s generally cylindrical in shape with average body length of

Table 2. Morphometric comparison of second-stage juveniles (J2s) in *Heteroderma trifolii* and *Heteroderma schachtii*

J2 character	*H. trifolii* (YS503)	*H. schachtii* (GC147)
n	20	20
L	601.9 ± 20.6^{a} (561.9-630.8)	484.2 ± 11.1^{b} (473-504.2)
Max body width	22.6 ± 0.9^{a} (21.3-23.7)	20.3 ± 0.9^{b} (19.1-21.8)
Anal body width	15.9 ± 0.7^{a} (14.8-16.9)	14.4 ± 0.7^{b} (14.2-15.8)
Stylet length	26.3 ± 1.0^{a} (25.3-27.6)	22.1 ± 0.9^{b} (21.3-24.3)
DGO to stylet base	5.3 ± 1.0^{a} (3.9-6.7)	4.0 ± 0.3$_{b}$ (3.5-4.4)
Lip height	4.7 ± 0.5^{a} (4.0-5.4)	4.4 ± 0.3^{a} (3.9-4.9)
Lip width	10.7 ± 0.5^{a} (9.9-11.8)	9.5 ± 0.2^{b} (9.1-9.9)
Anterior end to median bulb valve	93.7 ± 5.0^{a} (86.1-100.6)	76.4 ± 0.4^{b} (75.8-77)
Anterior end to pharyngeal intestine junction	139.6 ± 3.8^{a} (137.3-142.4)	126.2 ± 1.0^{b} (124.8-131.1)
Anterior end to pharyngeal gland overlap	189.9 ± 10.4^{a} (172.4-203.6)	169.3 ± 15.2^{b} (138.4-184.4)
Anterior to median bulb base	100.3 ± 5.7^{a} (96.5-110.2)	82.9 ± 1.0^{b} (81.3-84.3)
Anterior to excretory pore	132.6 ± 2.9^{a} (127.9-136.5)	105.6 ± 1.9^{b} (104.2-110.2)
Tail length	69.2 ± 5.7^{a} (63.6-75.9)	49.9 ± 1.8^{b} (48.2-53.2)
Hyaline length	38.2 ± 2.1^{a} (35.5-41.6)	29.2 ± 2.1^{b} (26.7-34.3)
a	26.7 ± 1.6^{a} (24.2-29.6)	23.8 ± 0.9^{b} (22.9-25.1)
b	4.5 ± 0.2^{a} (4.1-5.2)	4.2 ± 0.1^{b} (3.7-4.6)
b’	3.3 ± 0.2^{a} (3.1-3.5)	2.9 ± 0.3^{b} (2.6-3.4)
c	8.8 ± 0.7^{b} (7.9-9.9)	9.6 ± 0.4^{a} (9.4-10.0)
c’	4.4 ± 0.4^{a} (3.8-4.9)	3.5 ± 0.2^{b} (3.2-3.8)
L/Anterior end to median bulb valve	6.5 ± 0.4^{a} (6.0-7.3)	6.3 ± 0.1^{a} (6.2-6.5)
H/ST	1.5 ± 0.1^{a} (1.4-1.6)	1.3 ± 0.1^{b} (1.2-1.6)
Tail/H	1.8 ± 0.1^{a} (1.6-2.1)	1.7 ± 0.1^{a} (1.6-1.9)

All measurements are in µm and in the form mean ± standard deviation (range). (n = number of specimens, L = total body length, a = body length/greatest body width, b = body length/distance from anterior end to junction of pharynx and intestine, b' = body length/distance from anterior end to posterior end of pharyngeal glands, c = body length/tail length, c' = tail length/body width at anus, H/ST = Hyaline length/ Stylet length, and Tail/H = Tail length/Hyaline length.).

The same letters (a, b) denote no significant differences at $P \leq 0.05$ in a row, by Tukey’s HSD test.

484.2 ± 11.1 µm (473-504.2). Body appears nearly straight when heat killed. Head region offset and moderately sclerotized, with 4 head annules. Lip height 4.4 ± 0.3 µm (3.9-4.9) with a width of 9.5 ± 0.2 µm (9.1-9.9). Robust stylet with distinct round basal knobs (Fig. 2). Stylet length 22.1 ± 0.9 µm (21.3-24.3). A prominent median bulb visible, with elongated esophageal glands overlapping intestine ventrally. Lateral region marked with 4 incisures covering approximately 1/4 of the body width. Dorsal gland orifice opens at 4.0 ± 0.3 µm (3.5-4.4) below the stylet base. Excretory pore located at 105.6 ± 1.9 µm (104.2-110.2) from the head tip. The body tapers into a conical tail region. Tail length 49.9 ± 1.8 µm (48.2-53.2) and anal body width 14.4 ± 0.7 µm (14.2-15.8), with tail length to anal body width ratio of approximately 4. Hyaline length 29.2 ± 2.1 µm (26.7-34.3), making up approximately 58% of the tail length. Phasmid visible on the tail and the tail tapers abruptly into a rounded tail terminus (Fig. 2).

Cysts (n = 10). Cysts appear dark brown in color and lemon shaped with neck and vulval regions appearing as protuberances at the respective cyst body ends. The cyst measures 795.8 ± 97.1 µm (639.5-882.6) in length and 510.8 ± 75.1 µm (378.5-600.4) in width, forming a length to width ratio of 1.6 ± 0.1 (1.5-1.6). Cone top structures equally ambifenestrate with a robust underbridge measuring 102.3 ± 10.8 µm (93.7-120.8) long and 16.6 ± 2.4 µm (12.4-18.4) wide. A prominent vulval bridge separates the semifenestrae, with a fenestra length and width of 42.2 ± 3.6 µm (36.1-45.3) and 28.2 ± 4.0 µm (24.3-33.8), respectively (Fig. 2). Vulval slit length measures 37.4 ± 5.2 µm (30.3-42.2). Molar shaped bullae prominently present, positioned underneath the vulval bridge. The observations are in agreement with the descriptions in Abawi et al. (1973), Amiri et al. (2003), Mulvey and Golden (1983), Turner and Subbotin (2013) and Wouts and Baldwin (1998). No males were equally intercepted in the soil samples.

Morphometrical comparisons between *H. trifolii* and *H. schachtii*. Statistical comparisons of the morphometric data of J2s and cyst cones between the two species (*H. trifolii* and *H. schachtii*) are listed in Table 2 and 3, respectively. The mean body length of *H. trifolii* J2s is significantly larger than *H. schachtii*. Correspondingly, lip width, maximum body width, anal body width, stylet length, tail length, hyaline length and distances from anterior end to excretory pore, anterior end to the median bulb valve, anterior end to the pharyngeal-intestine junction and anterior end to the end of pharyngeal gland overlap are significantly larger in *H. trifolii* compared to *H. schachtii*. However, lip height and the ratios: body length to the distance from anterior end to median bulb valve and tail length to hyaline length do not significantly differ between the 2 species. Contrary to J2 body length, cyst length and width do not differ significantly between *H. trifolii* and *H. schachtii*. However, fenestra length and width, vulval slit length, vulval bridge width and underbridge width show significant differences between *H. trifolii* and *H. schachtii*. A comparison with morphometric measurement ranges extracted from other similar studies is shown in Supplementary Table 2, 3, 4 and 5 for *H. trifolii* and *H. schachtii*, respectively.

Molecular characterization. The two procedures involving the use of two different primer sets yielded positive results. When nucleic acids were extracted from a group containing mixed field population (TB372), both *CO*I genes and ITS regions were clearly amplified with primers of the two

Table 3. Morphometric comparison of vulval cone in *Heteroderma trifolii* and *Heteroderma schachtii*

Cyst character	*H. trifolii* (YS503)	*H. schachtii* (GC147)
n	10	10
Cyst length	910.0 ± 160[a] (650.0-1100.0)	795.8 ± 97.1[a] (639.5-882.6)
Cyst width	500.0 ± 99.2[a] (350.0-601.0)	510.8 ± 75.1[a] (378.5-600.4)
Cyst length/cyst width	1.8 ± 0.2[a] (1.7-2.1)	1.6 ± 0.1[a] (1.5-1.6)
Fenestra length	50.9 ± 4.9[a] (47.1-58.0)	42.2 ± 3.6[b] (36.1-45.3)
Fenestra width	36.0 ± 2.2[a] (33.4-38.3)	28.2 ± 4.0[b] (24.3-33.8)
Vulval bridge width	7.4 ± 0.7[a] (6.9-7.9)	4.5 ± 1.2[b] (3.1-5.9)
Vulval slit length	47.3 ± 2.8[a] (44.0-50.8)	37.4 ± 5.2[b] (30.3-42.2)
Underbridge length	112.7 ± 10.3[a] (105.2-127.8)	102.3 ± 10.8[a] (93.7-120.8)
Underbridge width	35.5 ± 5.6[a] (29.8-44.7)	16.6 ± 2.4[b] (12.4-18.4)

Letters indicate similar and dissimilar average length. All measurements are in µm and in the form mean ± standard deviation (range). (n = number of specimens).

The same letters (a, b) denote no significant differences at $P \leq 0.05$ in a row, by Tukey's HSD test.

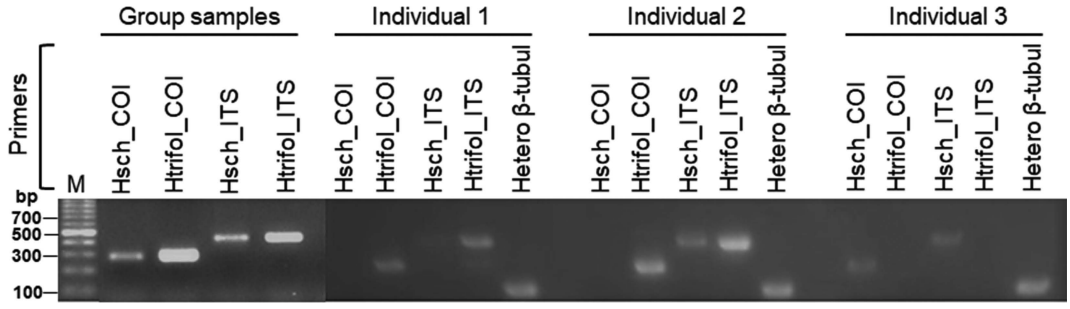

Fig. 3. PCR patterns of *CO*I gene and ITS region of *Heteroderma trifolii* and *Heteroderma schachtii* amplified with species-specific primers designed in this study (M: 100 bp DNA ladder). Primers of Hetero β-tubul were designed at the conserved region of beta-tubulin from the two *Heterodera* species. The information of each primer is available in Supplementary Fig. 1 and Supplementary Table 1.

Heterodera species (Fig. 3, see lanes of group samples), suggesting that *Heterodera* population collected from the Chinese cabbage field does not consist of a single species, but a mixture of two species, *H. trifolii* and *H. schachtii*. To identify the species, nucleic acids were prepared from 3 individuals isolated from monoxenic cultures (YS503 and GC147). In individual 1 and 2 (YS503), *CO*I and ITS of *H. trifolii* were predominantly amplified with *H. trifolii*-specific primer sets (Fig. 3, see lanes of individual 1 and 2), whereas those of *H. schachtii* were strongly detected in individual 3 (GC147) after amplification with *H. schachtii*-specific primer sets (Fig. 3, see lanes of individual 3), suggesting that individual 1 and 2 are *H. trifolii*, while individual 3 is *H. schachtii*. These results further confirm the mixture of the two species of *Heterodera* in the Chinese cabbage fields of Korea.

Phylogeny: ITS rRNA and *CO*I gene. ITS region and *CO*I gene were successfully amplified as indicated in Fig. 4 from YS503 and GC147 population and their sequence information was also obtained and submitted to the GenBank data base under accession numbers; MG682350, MG682348 for *H. trifolii* and MF043911, KY775592 for *H. schachtii*. ITS sequence dataset included 22 sequences of 'Schachtii' group species of the genus *Heterodera*, containing newly obtained sequences in Korea. The phylogenetic tree as inferred from Bayesian analysis of the dataset with GTR+I+G substitution model is shown in Fig. 5. The result showed that YS503 (MG682350) population was indistinguishable from others because the resolving power of

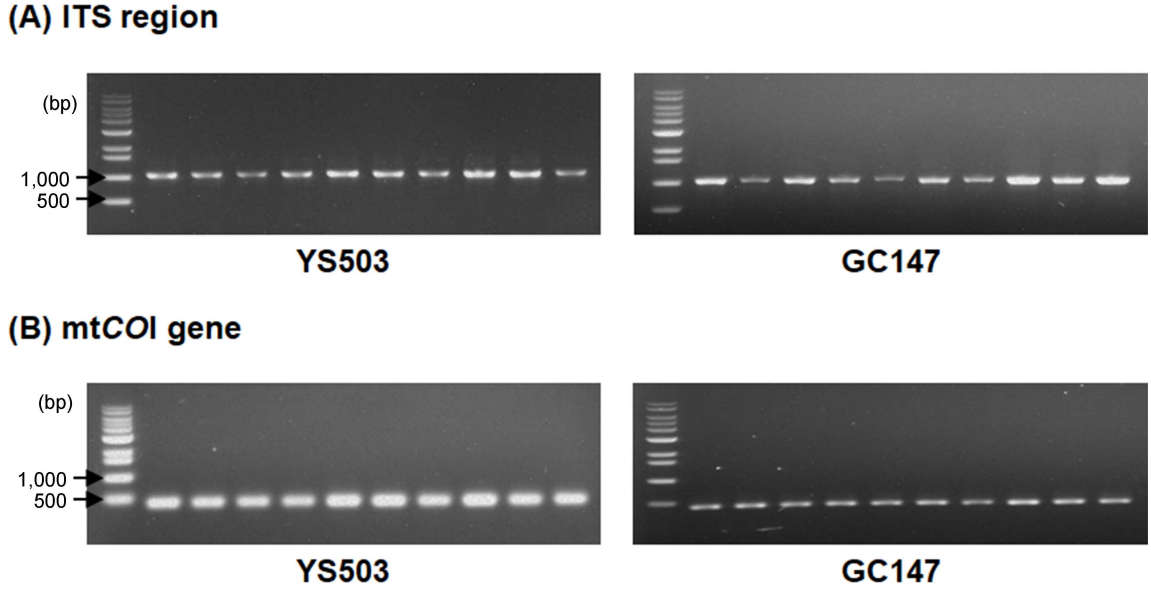

Fig. 4. PCR patterns of ITS region (A) (approximately: 1,025 bp) and COI gene (B) (approximately: 450 bp) of *Heteroderma trifolii* (YS503) and *Heteroderrma schachtii* (GC147) amplified with universal primers (DNA ladder: Bioneer 1 kb DNA ladder). The information of each primer is available in Supplementary Table 1.

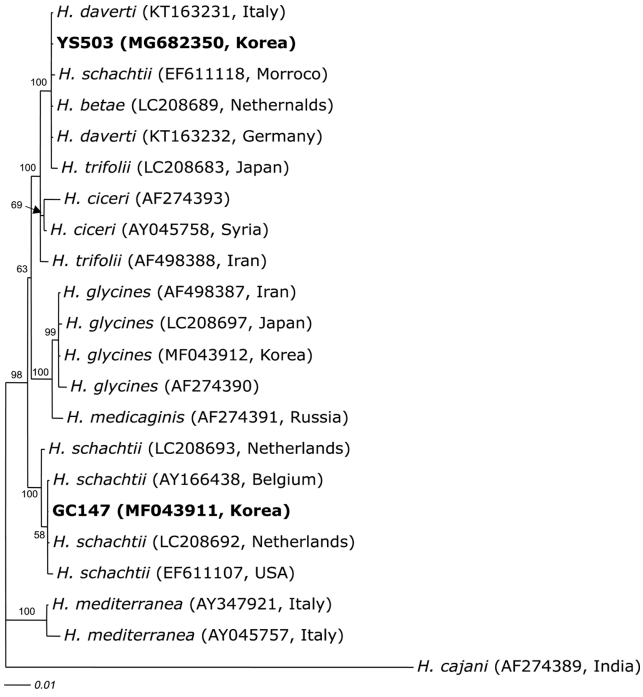

Fig. 5. Phylogenetic relationships between *Heterodera* species as inferred from Bayesian analysis of internal transcribed spacer (ITS) sequence dataset with general time reversible substitution model (GTR+I+G). The newly obtained sequences were represented in bold. Posterior probability values more than 50% are given for appropriate clade.

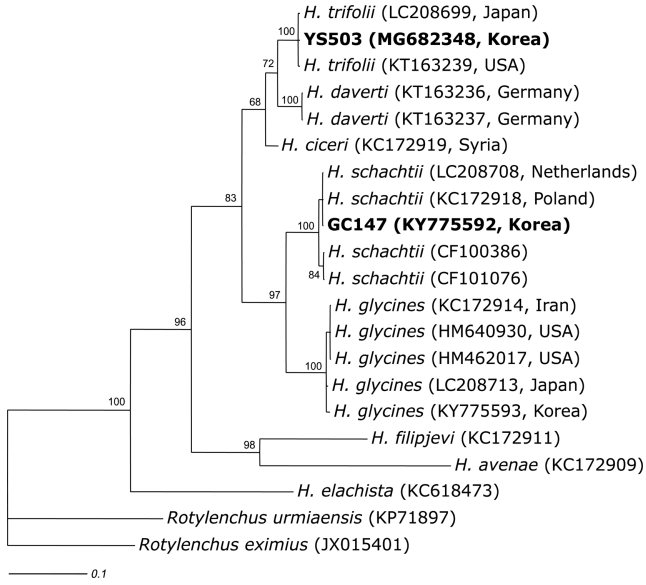

Fig. 6. Phylogenetic relationships between *Heterodera* species as inferred from Bayesian analysis of mitochondrial DNA cytochrome c oxidase subunit I (*CO*I) sequence dataset with general time reversible substitution model (GTR+I+G). The newly obtained sequences were represented in bold. Posterior probability values more than 70% are given for appropriate clade.

the clade was very low. Thus, analysis of intraspecific and interspecific sequence variation of ITS rRNA was not conducted. On the other hand, GC147 population belongs to *H. schachtii* clade according to Fig. 5. Intraspecific sequence variation of ITS rRNA between GC147 (MF043911) population and the published *H. schachtii* sequence data (EF611118) was 0.0-0.3% while interspecific variations between GC147 (MF043911) population and closely related species, *H. glycines* (LC208697) and *H. medicaginis* (AF274391) were 2.0% and 2.1%, respectively. In this analysis, GC147 population was identified as *H. schachtii*.

*CO*I sequence dataset included 21 sequences of *Heterodera* species, containing newly obtained sequences of *H. trifolii* and *H. schachtii* in Korea. The phylogenetic tree as inferred from Bayesian analysis of *CO*I gene sequence dataset with GTR+I+G substitution model is shown in Fig. VII. The results showed that YS503 (MG682348) and GC147 (KY775592) population belong to *H. trifolii* and *H. schachtii* clades, respectively. Intraspecific *CO*I sequence variation between YS503 (MG682348) population and *H. trifolii* of previous studies was 0.0% whereas interspecific *CO*I sequence variation between YS503 (MG682348) population and closely related species, *H. ciceri* (KC172919) and *H. daverti* (KT163236) were 4.0% and 4.8%, respectively. Intraspecific *CO*I sequence variations between GC147 (KY775592) population and *H. schachtii* of reference data were 0.0-0.5% while interspecific variations between GC147 (KY775592) population and closely related species, *H. glycines* (LC208713), *H. trifolii* (LC208699), and *H. ciceri* (KC172919), were 7.0%, 9.6%, and 10.8%, respectively. According to this analysis, YS503 and GC147 populations were identified as *H. trifolii* and *H. schachtii*, respectively.

Discussion

The morphometric analysis of the J2s and cyst cones confirms earlier reports that *H. trifolii* can be differentiated from *H. schachtii* based on biometric data (Amiri et al., 2003; Mulvey and Golden, 1983; Turner and Subbotin, 2013; Wouts and Baldwin, 1998). In general, the biometric data of *H. trifolii* J2s is significantly higher than that of *H. schachtii* (Ambrogioni and Irdani, 2001; Steele and Whitehand, 1984). More notably, total body length, stylet length and Tail length have always been highlighted as the main measurements essential in differentiating between the two species (Steele and Whitehead, 1984); with reports that J2s of *H. trifolii* are longer (> 500 μm) than *H. schachtii* J2 (< 500) (Turner and Subbotin, 2013; Wouts and Baldwin,

1998). Our study further points out more detailed significant differences among most of the measured J2 characters between the two species. Thus, a detailed morphometric analysis of the various J2 characters is integral for reliable differentiation of the two nematode species. Contrary, cysts of both species are similar in size**.** However, Fenestra and vulval slit length are significantly higher in vulval cones of *H. trifolii* and previous studies highlight their usefulness in differentiating between *H. trifolii* and *H. schachtii* in mixed populations (Abawi et al., 1973). A comparison between morphometric data of the described *H. trifolii* and *H. schachtii* populations in Korea and populations from other countries (Italy, Sweden, Netherlands, Germany, USA, Turkey, Jordan and Syria) showed remarkable similarities in most of the measurements, though *H. trifolii* populations from Germany are relatively longer compared to other populations especially from USA**.** Biometric data, especially the general body length of plant parasitic nematodes are known to vary according to several environmental factors, including geographical localities and differences in the host plants between the isolated populations (Tarte and Mai, 1976). This could explain the comparative morphometric measurement differences among the populations used in comparison, as all were isolated from different geographical locations, environmental conditions and host varieties.

The molecular characterization allows the differentiation of *H. trifolii* from *H. schachtii* populations. Morphological differentiation of the cyst nematode species belonging to *H. schachtii sensu stricto* group is not only difficult but also time consuming and requires specialized taxonomic skills (Amiri et al., 2001). Thus, molecular identification has increasingly become common for efficiency purposes (Amiri et al., 2003), and *H. schachtii sensu stricto* group primers for the ITS region of ribosomal gene have been developed to differentiate the species within the *schachtii* group from other nematode species (Amiri et al., 2001; Waeyenberge et al., 2009). However, there is limited interspecific variation in the ITS regions of the *H. schachtii* group and thus, species-specific identification based on sequences of ITS-rRNA genes within the group is sometimes impossible (Amiri et al., 2002). In this study, unlike *CO*I gene highly species-specific amplification, bands of ITS amplified with primer set being species-specific to *H. schachtii* were faintly detected in individual 1 and 2 putatively identified as *H. trifolii* (Fig. 3, lanes of Hsch_ITS in individual 1 and 2). As shown in Fig. 2A, 2B, sequences of ITS from the two species are more similar to each other than those of *CO*I, thereby exhibiting non-specific amplification in individual 1 and 2. However, band intensities of Htrifol_ITS were distinguishably stronger than those of Hsch_ITS, and *CO*I genes of *H. trifolii* were only amplified with Htrifol_COI in individual 1 and 2 samples, suggesting identification of individual 1 and 2 as *H. trifolii*. In individual 3, both *CO*I gene and ITS region were amplified with only primer sets which are specific to the sequence of *H. schachtii*, thus strongly showing that individual 3 is *H. schachtii*. Sequence information of ITS and *CO*I gene analyzed in this study also confirms the coexistence of two *Heterodera* species in Chinese cabbage fields in Korea (Fig. 3, 4, 5, 6). Generally, although non-specific bands were slightly visualized by amplification with Hsch_ITS primer set, species-specific primer sets designed in this study can be useful for molecular identification of two *Heterodera* species. Additionally, in the present study, both coding gene (beta-tubulin and *CO*I) and non-coding region (ITS) were efficiently amplified from nucleic acids including genomic DNA and total RNA extracted with TRI Reagent® (MRC) from individual cyst sample. In fact, genomic DNA is technically difficult to be isolated from a single cyst due to its limited size. However, when extraction of both RNA and DNA from respective aqueous phase and interphase was done after homogenization with TRI Reagent® (MRC) without treatment with DNase I, beta-tubulin, *CO*I gene and ITS region were predominantly amplified as shown in Fig. 3. These results also suggest that the modified TRI Reagent® (MRC) manual can be a proper method for molecular identification of nematode species from a single cyst.

Contrastingly, there exist different levels of intraspecific variation in the ITS-rDNA sequences within 'Schachtii' group and this has an effect on cluster analysis (Madani et al., 2007). Indeed, the phylogenetic relationships of the ITS sequences showed two sub-branches of the *H. schachtii* populations; the sequence of *H. schachtii* (EF611118) population was clustered together with closely related 'Schachtii' group species like *H. betae* (LC208689), *H. daverti* (KT163232), *H. trifolii* (LC208683), including the Korean population of *H. trifolii* (YS503; MG682350). The second sub-branch consisted of four *H. schachtii* species (LC208693, AY166438, LC208692, EF611107), populations available in the Genbank (https://www.ncbi.nlm.nih.gov), together with Korean population of the same species (GC147; MF043911). The populations were not grouped according to their geographical location and these results are in agreement with Madani et al. (2007) and Caswell-Chen et al. (1992), who indicated that the genetic similarity of *H. schachtii* populations is not correlated with their geographical proximity. Indeed in the study done by Madani et al. (2007), sequences of two ITS clone variants from the same population did not cluster together in Maximum Par-

simony trees. Nonetheless, Sekimoto et al. (2017) recently showed that *H. trifolii* can easily be distinguished from *H. schachtii* using the *CO*I mtDNA gene and certainly our results are in agreement with the findings. The *CO*I mtDNA gene is able to differentiate between the 2 species without a further application of restriction enzymes and therefore, the gene can be considered as a reliable molecular target, with a precise detection of the two species either individually or in mixed populations**.** Indeed, the phylogenetic analysis of the *CO*I gene sequences precisely grouped *H. schachtii* and *H. trifolii* in separate individual sub-branches with bootstrap of 100%. *H. schachtii* sub-branch consisted of the *H. schachtii* species; GC147 (KY775592), LC208708, KC172918, CF100386 and CF101076 while *H. trifolii* sub-branch consisted of *H. trifolii* species; YS503 (MG682348), LC208699 and KT163239. However, a combinative comparison between morphological, morphometrical and genetic variations is always the most ideal approach in identification and separation of the closely related species (Amiri et al., 2001).

H. schachtii has already been identified as a serious setback in Chinese cabbage fields in Korea, and efforts in devising proper management and control strategies are underway (Kim et al., 2016; Kwon et al., 2016). However this study highlights the coexistence of *H. schachtii* and *H. trifolii* in Chinese cabbage fields and thus, management strategies should be formulated to target both pathogens. Both species have a relatively similar host range hence co-existence is not coincidental but a true possibility as noted by Abawi et al (1973). Conclusively, the presence of *H. trifolii* in Korea is reported here for the first time.

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