

## Differential Morphological, Structural and Biological Characteristics of Cysts in *Heterodera* Species in Korea

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Morphological (cyst shape, color, and sizes [length (L), maximum width (W), volume and “a” (L/W)], structural (vulvar cone slope angle [VCSA], surface wrinkle [VCSW], cyst wall thickness, composition, and texture) and biological characteristics (fecundity, hatching, and emergence [number of second-stage juveniles (J2) from a cyst]) in preceding *Heterodera glycines* (Hg), currently-recorded *H. sojae* (Hs) and *H. trifolii* (Ht) were examined by microscopy. Cysts were lemon-shaped, indicating the genus is *Heterodera* except for Hs that formed frequently globular cysts with significantly flatter VCSA (102.2°) with smooth VCSW than Hg (50.6°) and Ht (82.0°), but not genus *Globodera* because of the presence of vulvar cone in Hs. Ht was significantly larger in all morphological characteristics than Hg and Hs, suggesting Ht may be diagnosed differentially by cyst sizes and also host plant preferences. Hs showed smaller “a” value with more globular shape and stronger structures with more thickened and strengthened collagen-like texture of cyst wall than Hg and Ht. This suggests Hs may be diagnosed differently by structural characteristics from the others, especially Hg with similar cyst sizes. There were no significant differences in emergence (inoculum potential) among cyst nematodes due to the offset of fecundity and hatching rate; however, the inoculum potential of Hs may be not so persistent as

Hg and Ht in fields because of its lower fecundity and higher hatching rate (causing rapid inoculum loss) than the others. These characteristics of cysts provide information useful for simple and differential diagnoses and reliable management of cyst nematodes.

**Keywords** : biological characteristics, cysts, *Heterodera* spp., morphological and structural characteristics

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Cyst nematodes represent a large group of sedentary endoparasitic nematodes comprised of a variety of species. These species are typically included in three important genera which are classified by cyst shape; lemon-shaped type genus *Heterodera*, round-shaped (spherical) *Globodera* and pear-shaped *Punctodera* in the family Heteroderidae, although cyst shape alone is not a reliable characteristic for species identification (Golden, 1986; Hesling, 1982; Mulvey and Stone, 1976).

One of the typical characteristics in cyst nematodes is the formation of cysts, termed dead females, of which the female cuticle turns to leathery cyst wall, so as to protect eggs inside of the cysts from external harmful materials and harsh environments like drought (Clarke, 1968; Sharma, 1998). Thus, morphological, structural and biological characteristics of cysts have a paramount importance in differential diagnosis of cyst nematodes and development of control strategies efficient for cyst nematodes.

In Korea, a novel cyst nematode was found in soybean fields in 2016. It forms white, creamy young females, changing to brownish-black when old with a shiny appearance and lack of the typical yellow cyst phase readily seen in soybean cyst nematodes (*Heterodera glycines*). It is morphologically different from the preceding soybean cyst

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nematode *H. glycines* (Hg), and named *H. sojae* (Hs) that is distributed widely in soybean fields as Hg in Korea (Kang et al., 2016). However, other structural characteristics have not been compared in detail, which may be important in order to allow simple and reliable diagnosis of these two cyst nematodes at a species level that have similar host plant preferences and a common distribution in soybean fields (Kang et al., 2016).

The sugar beet cyst nematode, *H. schachtii*, was discovered in highland Chinese cabbage fields in Korea. It was distributed in 11.6 ha during 2011 (Kwon et al., 2016). Also, the clover cyst nematode *H. trifolii* (Ht), constituting the *H. schachtii sensu stricto* group together with *H. schachtii* and other related *Heterodera* species (Subbotin et al., 2000), was newly isolated from the highland Chinese cabbage fields in 2016, when the distribution was expanded to 156.6 ha in the highland Chinese cabbage fields, possibly occurred by either Ht alone and/or both Ht and *H. schachtii* (Mwamula et al., 2018). Determining detailed morphological characteristics of cysts would be beneficial for correct differential diagnosis of preceding Hg from currently-recorded Hs and Ht even though they have different host plant preferences (Williams, 1982). This is especially true for differentiating Hg from Hs, because of their narrow host range and common distribution in soybean fields (Kang et al., 2016; Lilley et al., 2005; Williams, 1982).

It has been generally known that the control of cyst nematodes is difficult owing to the hardened cyst wall that protects living nematodes (eggs and sometimes hatched juveniles) existing inside of cysts (Hu et al., 2018). Thus, determining the biological characteristics of Hg, Hs, and Ht cysts will aid in the development of efficient control strategies for each nematode.

In this study, microscopic techniques were used to examine: morphological (cyst shape, color, and sizes); structural (vulvar cone slope angles [VCSA], vulvar cone surface wrinkles [VCSW], and cyst wall thickness, composition and texture); and biological (fecundity [number of eggs in a cyst], hatching rate [%] and emergence [number of second-stage juveniles (j2) hatched from eggs formed in a cyst (i.e., fecundity  $\times$  hatching rate)]). These studies on morphological, structural and biological characteristics of cysts in cyst nematodes will provide useful information for reliable diagnosis of cyst nematode species and contribute to the development of control strategies efficient for the cyst nematodes.

## Materials and Methods

### Plants, nematodes and inoculum preparation and inoculation.

**Plants.** Plants for the culture of Hg and Hs, both of which were cultured in Nematode Research Center, Life and Industry Convergence Research Institute, Pusan National University (Kang et al., 2016), used in this study were cv. Lee 74 provided from National Biodiversity Center, Korea. Plants for the culture of Ht (provided from National Academy of Agricultural Sciences) were two Chinese cabbage cultivars cv. Crsingsing (Cr) and Koryeogeumdonggi (Koryeo) and three radish cultivars cv. Geumjeongdaehyeong (Geumjeong), Sokseongdaehyeong (Sokseong), and Songbaek of which the seeds were purchased in commercial markets. Seeds of the plants were sown in 9 cm (diameter)  $\times$  8 cm (depth) plastic pots filled with sandy soil sterilized at 15 psi, 121°C for 20 min, and grown at 25°C in a growth chamber. For nematode inoculum preparation, the cyst nematodes were cultured in the soybean cv. Lee 74 (for Hg and Hs) and Chinese cabbage cv. Koryeo (for Ht) for 40 days, and the cysts produced in the plants were collected on a 100-mesh screen and crushed with a tweezers to release eggs, from which second-stage juveniles (J2) were hatched by the modified Baermann funnel method (Kim et al., 2017). The J2 were suspended in sterilized distilled water (SDW) to make nematode solution containing 100 J2/ml. For inoculation, 10 ml of the nematode solution (containing about 1,000 J2) was poured around the rhizosphere of each plant. After nematode inoculation, plants were grown at 25°C for 28 days after inoculation (DAI) in a growth chamber, watering to field capacity at 2-day intervals throughout the experimental period.

**Application of microscopic techniques for examining morphological, structural and biological characteristics of cysts.** Four microscopic techniques were used in this study, including stereomicroscopy (SM), light microscopy (LM), and electron microscopy (EM) (scanning electron microscopy [SEM] and transmission electron microscopy [TEM]).

**Stereomicroscopy.** For examining cyst shape, color and sizes (length [L], maximum width [W]), 20 cysts collected randomly on 100-mesh screen from each plant of soybean cv. Lee 74 (for Hg and Hs) and from Chinese cabbage cv. Koryeo (for Ht) at 28 DAI with an initial inoculum amount of about 1,000 J2 per plant were directly observed under a stereomicroscope (Olympus SZ-ST, Tokyo, Japan). Cyst volume and “a” value were calculated by the ellipsoidal volume equation  $[4/3\pi \times (L/2) \times (W/2)^2]$  and  $L/W$ , respectively. For biological characteristics examined by SM, the 20 cysts each in three replications (plates) produced in plants was collected from 100-mesh screen and pressed

by a tweezers to release all eggs outside from the cyst in SDW, and the number of eggs from a cyst was calculated for fecundity under the stereomicroscope. All eggs obtained from each cyst were hatched by the modified Baermann funnel method up to 7 days after treatment at 25°C in the dark in an incubation chamber, in which the number of J2 per fecundity in a cyst was regarded as hatching and converted to the J2 number hatched from an egg  $\times$  100 (%) with 20 replications (cysts). Emergence of J2 from a cyst was also calculated by the number of J2 from a cyst (fecundity  $\times$  hatching rate), and this was also re-examined to confirm the number of J2 under the stereomicroscope with three replications (from three plants).

**Light microscopy.** LM was used for the examination of cyst wall thickness and composition using toluidine blue staining. For this, cyst specimens were cross-sectioned with a razor blade and fixed in Karnovsky's fixative in 0.05 M cacodylate buffer (pH 7.2) for 4 h, followed by washing with buffer solution three times for 20 min each. Samples were then post-fixed with 1.0% osmium tetroxide in 0.05 M cacodylate buffer (pH 7.2) for 2 h and washed briefly with SDW (Karnovsky, 1965). The fixed specimens were dehydrated in a series of ethanol (30%, 50%, 70%, 80%, 95% and 3 $\times$  of 100%) for 10 min each, and further dehydrated in propylene oxide twice for 15 min each. These dehydrated cyst specimens were embedded in Spurr's resin and then polymerized at 70°C for 8 h in a dry oven to make resin blocks containing the cyst specimens (Kim et al., 2017). Resin blocks were trimmed to expose cyst specimens and sectioned 1.0  $\mu$ m thick with a diamond knife (MF1758, Diatome, Nidau, Switzerland) on an MT-X ultramicrotome (RMC, Tucson, AZ, USA). The sections were mounted on glass slides, stained with 1.0% toluidine blue in ethanol for one min, and observed under a compound light microscope (AxioPhot, Carl Zeiss, Oberkochen, Germany).

**Scanning electron microscopy.** For examining cyst wall thickness and structures by SEM, cyst specimens (cut or intact) were dehydrated using the same ethanol series as LM mentioned above, and dried in a critical point dryer (EM CPD 300, Leica, Wetzlar, Germany) after transition of the specimens through 100% isoamyl acetate two times for 10 min each at room temperature (Kim et al., 2017). These dried cyst specimens were mounted on metal stubs and coated with gold using a sputter coater (EM ACE 200, Leica, Vienna, Austria). Specimens were examined for cyst wall thickness and detailed wall structure under a Supra 55VP scanning electron microscope (Carl Zeiss) at 2.0 kV

(Kim et al., 2017).

**Transmission electron microscopy.** For TEM, resin blocks as prepared for LM were sectioned 80 nm thick with the diamond knife on the MT-X ultramicrotome (RMC). Sectioned samples were mounted on 300- or 400-mesh copper grids and stained with 2.0% uranyl acetate and lead citrate for 6 min each for examining cyst wall thickness and textures under a transmission electron microscope (JEM-1010, Jeol, Tokyo, Japan) at 80 kV (Kim et al., 2017).

**Labeling cyst wall layers.** By SEM and TEM, the names of layers in cross sectioned cyst wall followed previous studies, corresponding to exo-cuticles, endo-cuticles and other structures on cyst surfaces (Hesling, 1982) and relating four cyst wall layers outermost external A (exo-cuticle) and inner B-D layers (endo-cuticle) (Baldwin, 1983). The composition of cyst wall layers was determined based on their fibrous texture to collagenous proteins and plain or granular texture to non-collagenous (homogeneous) proteins (Filshie and Rogers, 1962).

#### **Measurements of cyst size, wall thickness and VCSA.**

All the parameters of cyst sizes including length (L), maximum width (W), and cyst wall thickness were measured with the Java image processing program, ImageJ (Fortin and Battié, 2012), using the photographs from SM and LM with 20 replications, respectively. Length (L) was divided by maximum width (W) to calculate "a" values. Cyst volumes were calculated using the ellipsoidal volume equation as mentioned above. Angles between tangential lines of the opposite vulvar cone slopes (VCSA) were measured with ImageJ using SEM microphotographs with 10 replications (Fortin and Battié, 2012).

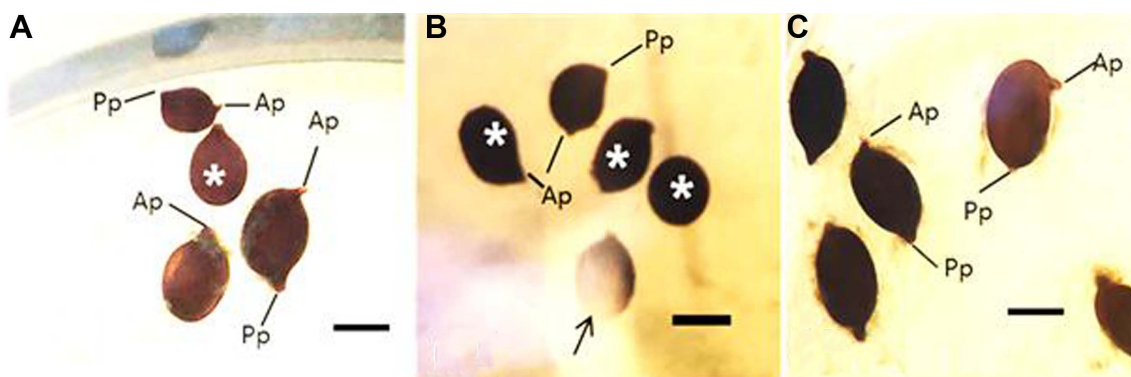
**Statistical analysis.** Data were analyzed statistically using one-way analysis of variance (ANOVA). Significant difference tests were performed using Fisher's least significant difference test with  $P \leq 0.05$  referring to Student's T distribution table.

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## **Results**

### **External gross morphology and cyst sizes observed by SM.**

External gross morphologies examined by SM were all dark-brown and lemon-shaped except for occasional globular cysts with no definitive posterior protuberances, especially in Hs (Fig. 1). Ht cysts were larger, including length (L), maximum width (W), "a" value (L/W), and cyst volume than the other *Heterodera* spp. examined in



**Fig. 1.** Stereomicroscopy of cysts in *Heterodera* spp. formed on soybean cv. Lee 74 for *H. glycines* (Hg) (A) and *H. sojae* (Hs) (B) and Chinese cabbage cv. Koryeogeumdonggi for *H. trifolii* (Ht) (C) at 28 days after inoculation with c.a. 1,000 second stage juveniles (J2) for each cyst nematode, all of which showed typical lemon shape of the genus *Heterodera*, indicated by anterior (Ap) (head region) and posterior protrusion (Pp) (vulvar cone) sometimes with no visible or blunt (flat) Pp (\*). Cyst sizes of Ht (C) were larger than those of Hg (A) and Hs (B). Arrow indicates white cyst sometimes found in Hs. Scale bars = 500 μm.

**Table 1.** Cyst sizes in *Heterodera* species produced in soybean cv. Lee 74 for *H. glycines* and *H. sojae* and Chinese cabbage cv. Koryeogeumdonggi for *H. trifolii* 28 DAI

<i>Heterodera</i> species	Sizes of cysts <sup>a</sup>			
	Cyst length (L) (μm)	Max. cyst width (W) (μm)	Cyst volume (×10 <sup>6</sup> μm <sup>3</sup> )	“a” value (L/W)
<i>H. glycines</i>	694.05 b <sup>b</sup>	474.57 b	84.22 b	1.48 b
<i>H. sojae</i>	629.01 b	469.55 b	73.12 b	1.35 b
<i>H. trifolii</i>	999.04 a	613.07 a	198.74 a	1.64 a

Values are presented as means ± standard deviations of 20 replications (cysts).

DAI, days after inoculation.

<sup>a</sup>L and W were measured by the Java image processing program, Image J, using the pictures from stereomicroscopy with 20 replications; CV was calculated using ellipsoidal volume equation of  $4/3\pi \times (L/2) \times (W/2)^2$ .

<sup>b</sup>Means followed by the same letters denote no significant differences at  $P \leq 0.05$  by the least significant difference test.

this study (Table 1, Fig. 1). Hs had the lowest “a” value because of the frequent formation of globular cysts (shorter

L compared to W) sometimes with flat or blunt terminal vulvar cone (Table 1, Fig. 1).

**Table 2.** Cyst structural characteristics in *Heterodera* species related to cyst wall thickness and VCSA of cysts produced in soybean cv. Lee 74 for *H. glycines* and *H. sojae* and Chinese cabbage cv. Koryeogeumdonggi for *H. trifolii* 28 days after inoculation

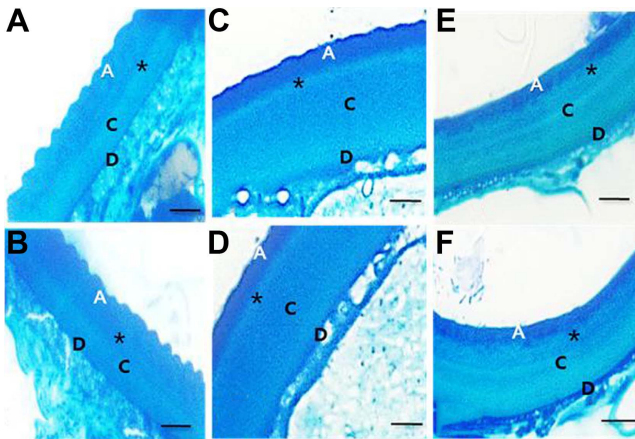
<i>Heterodera</i> species	Structural characteristics of cysts				
	Cyst wall thickness <sup>a</sup>				Vulvar cone slope angle (°) <sup>b</sup> (VCSA)
	Layer A	Layer (B + C)	Layer D	Total	
<i>H. glycines</i>	6.06 ± 1.65 a <sup>c</sup>	7.35 ± 2.07 c	0.85 ± 0.34 b	14.26 ± 1.04 b	50.6 ± 10.07 c
<i>H. sojae</i>	5.16 ± 1.43 a	18.00 ± 5.61 a	1.45 ± 0.70 a	24.61 ± 6.31 a	102.2 ± 8.43 a
<i>H. trifolii</i>	3.12 ± 0.47 b	11.00 ± 3.08 b	1.63 ± 0.66 a	15.75 ± 3.26 b	82.2 ± 18.3 b

Values are presented as means ± standard deviations of 20 replications for cyst wall thickness and 10 replications for vulvar cone slope angles.

<sup>a</sup>Cyst wall thickness was measured by the Java image processing program, Image J, using the pictures from light microscopy with 20 replications.

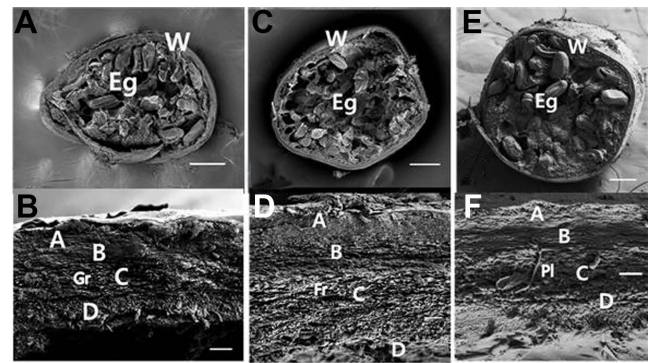
<sup>b</sup>VCSA was calculated by Image J using the pictures from scanning electron microscopy with 10 replications.

<sup>c</sup>Means followed by the same letters denote no significant difference at  $P \leq 0.05$  by the least significant difference test.



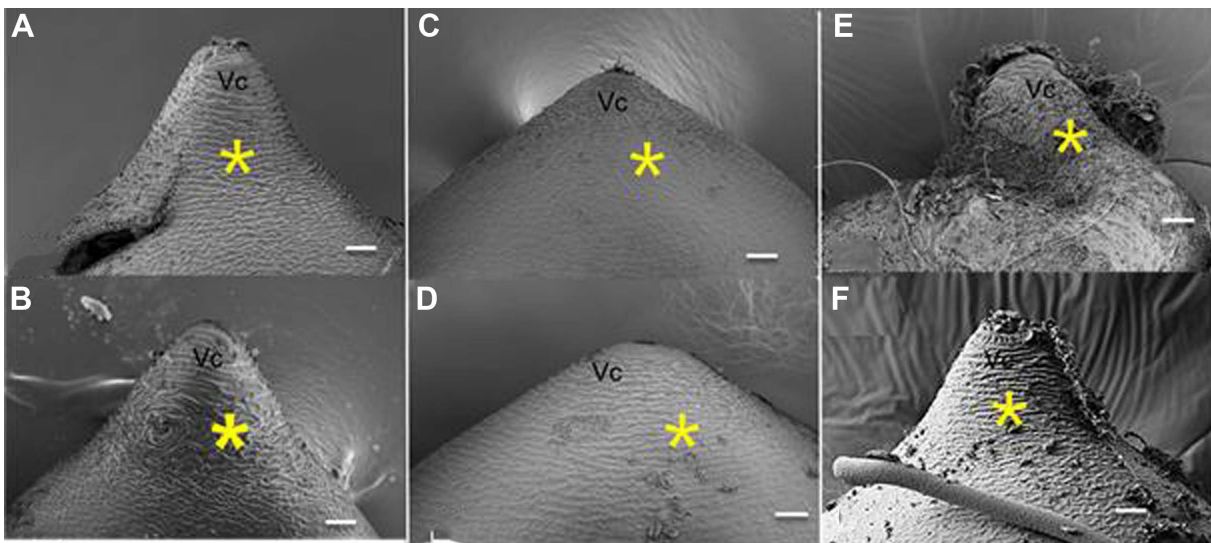
**Fig. 2.** Light micrographs of cyst wall stained with toluidine blue, showing more densely stained exo-cuticle (A) than endo-cuticle layers interior to the layer A; the layers B (\*), C, and the innermost layer D. Note more thickened cyst wall layer of C in *Heterodera sojae* (C, D) than *H. glycines* (A, B) and *H. trifolii* (E, F). Scale bars = 10  $\mu$ m.

**Cyst wall thickness viewed by light microscopy.** All cyst walls stained with toluidine blue showed three or four wall layers, including the outermost layer A (corresponding to exo-cuticle) stained more densely than endo-cuticle including the layers B, C, and D (Fig. 2). Total wall thickness was significantly greater in Hs than Hg and Ht because of the significantly more thickened layer C than those of Hg and Ht (Table 2, Fig. 2).

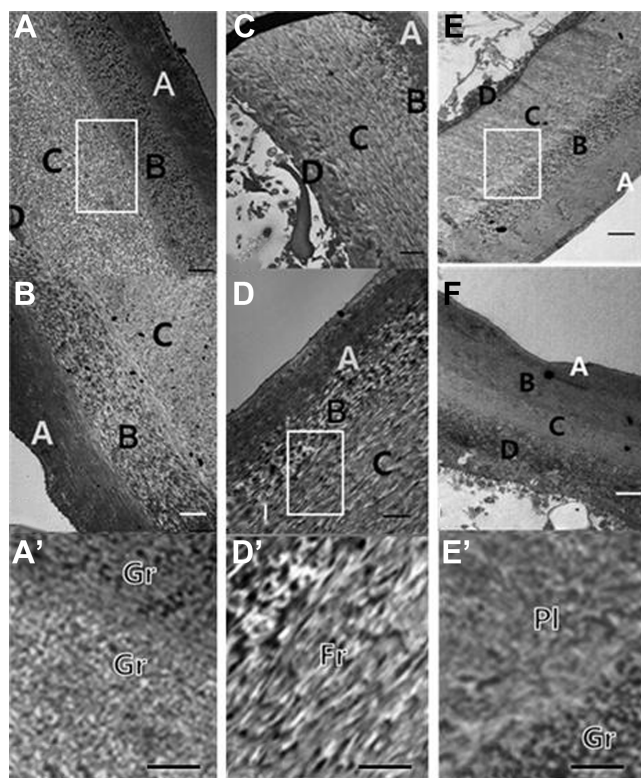


**Fig. 4.** Scanning electron micrographs of cysts, showing cross wall sections; eggs enclosed by cyst walls (A, C, E) and wall sections, showing granular (Gr) in *Heterodera glycines* (A, B), fibrous (Fr) in *H. sojae* (C, D) and plain (Pl) textures in *H. trifolii* (E, F), respectively. Scale bars = 20  $\mu$ m.

**VCSA, surface wrinkles (VCSW) and detailed structure of cyst wall cross-sections observed by SEM and TEM.** Vulvar cones observed by SEM were present at the terminal region of cysts in all nematodes examined (Fig. 3). However, VCSA was significantly more acute in the higher order of Hg (50.6°), Ht (82.0°), and Hs (102.2°) in average in our study, and the vulvar cone surface was roughly wrinkled in Hg and Ht and smoothly wrinkled in Hs, respectively (Table 2, Fig. 3). Also, SEM showed nematode eggs enclosed by the cyst wall composed of all 4 layers separated from one another, from the outermost layer A to the innermost layer D (Fig. 4). Also, the layer



**Fig. 3.** Scanning electron micrographs of vulvar cone (Vc) of cysts and cyst surface wrinkles (asterisks). Note different vulvar cone slope angles (VCSA) and cyst surface wrinkles, showing significantly more acute VCSA with roughly wrinkled surface in *Heterodera glycines* (A, B) and *H. trifolii* (E, F) than *H. sojae* (C, D) with smooth surface wrinkles. Scale bars = 20  $\mu$ m.



**Fig. 5.** Transmission electron microscopy of physical compositions of cyst wall, showing the detailed textures of cyst wall cross sections; plain (Pl) or granular (Gr) textures in *Heterodera glycines* (A, B, A') and *H. trifolii* (E, F, E') and fibrous (Fr) textures in *H. sojae* (C, D, D'). (A', D', E') Magnification of rectangular region of (A), (D), and (E). Scale bars = 2.0  $\mu\text{m}$  (A-F), 1.0  $\mu\text{m}$  (A', D', E').

C of Hs was remarkably thickened compared to those of other cyst nematodes as in LM (Figs. 2 and 4). In SEM, the texture of layer C was granular in Hg, fibrous in Hs,

and plain in Ht, respectively (Fig. 4). In TEM showing all the layers observed by SEM, the outermost layer A (exocuticle) was definitely separate from the layer B (outermost layer of endo-cuticle) in Hg and Ht but not in Hs, and the textures of layer C were granular and plain in Hg and Ht, respectively. However, those of Hs were fibrous-textured, especially in the layer C as shown by SEM (Figs. 4 and 5).

### Biological characteristics of cysts in *Heterodera* spp.

When Hs cysts were compressed with tweezers to release eggs, slightly greater pressure was required to burst cysts with plusive sound than Hg and Ht. In comparison of biological characteristics among cyst nematodes, fecundity was significantly higher and hatching rate significantly lower in Ht than Hg and Hs, respectively. Hs had significantly lower fecundity than Hg and Ht and a higher hatching rate than Ht, indicating there were no significant differences in the emergence due to the offset of biological characteristics (fecundity and hatching rate) contributing to the emergence, which was calculated and confirmed equivalent to the calculation of fecundity  $\times$  hatching of a cyst (Table 3).

### Discussion

In cyst nematodes, females turn to cysts when they are mature and protrude their bodies out of roots owing to large cysts relative to the root diameters of feeding sites, at the same time that the females die and become cysts with two different color sequences; (1) white  $\rightarrow$  yellow  $\rightarrow$  brown phase, and (2) white  $\rightarrow$  brown phase without distinct yellow phase (Hesling, 1982; Sharma, 1998). In some cyst nematodes, the yellow phase is related to the color changes inside of cyst wall, but the brown phase occurs in the cyst

**Table 3.** Cyst biological characteristics, including fecundity, hatching rate (%) and reproduction rate in *Heterodera* species produced in soybean cv. Lee 74 for *H. glycines* and *H. sojae* and Chinese cabbage cv. Koryeogeumdonggi for *H. trifolii* at 28 days after initial inoculation

<i>Heterodera</i> sp.	Biological characteristics of cysts <sup>a</sup>				
	Fecundity	Hatching rates (%)			Emergence
		3 DAT	5 DAT	7 DAT	
<i>H. glycines</i>	204.69 b <sup>b</sup>	22.37 a	29.51 a	32.14 a	65.00 a
<i>H. sojae</i>	147.74 c	18.38 a	28.48 a	34.03 a	49.90 a
<i>H. trifolii</i>	261.20 a	3.49 b	18.19 b	23.44 b	60.22 a

Values are presented as means  $\pm$  standard deviations of 20 replications.

<sup>a</sup>Fecundity: number of eggs/cyst; hatching rates (%): number of J2 hatched from 100 eggs for 3-7 days after treatment (DAT) by the modified Baermann funnel method; Emergence: (number of J2 from one of cysts produced in a plant; inoculum potential of cysts).

<sup>b</sup>Means followed by the same letters denote no significant difference at  $P \leq 0.05$  by the least significant difference test.

wall (Hesling, 1982). This suggests the second processes of wall color change (2) may have the longer multiplication time for cyst wall conversion in Hs with thickened endo-cuticle than Hg and Ht, especially the layer C.

In our study, SM was used to examine gross cyst morphology, including cyst shape, color and sizes of the three cyst nematodes (Hg and Hs from the soybean and Ht from the Chinese cabbage), showing yellow cyst phases for Hg and Ht but not for Hs with brownish cysts and sometimes white cysts. All cyst nematodes were lemon-shaped, as indicated by both anterior and posterior protuberances, although Hs had low vulvar cone superficially similar to the genus *Globodera*. Hs had a globular-like shape with frequent formation of a blunt or flat vulvar cone, but Hs should belong to the genus *Heterodera*, but not *Globodera* because it had terminal vulvar cone even with flatter than other cysts, while vulvar cones are absent in *Globodera* and *Punctodera* (Golden, 1986; Hesling, 1982; Mulvey and Stone, 1976). These suggest Hs should belong to the genus *Heterodera*, but it was more similar in shape to the genus *Globodera*. Also it was found in the molecular genetic relationships of cyst nematodes examined in the phylogenetic analyses of 28S and internal transcribed spacer rDNA sequences, in which Hs is more closely related to *Globodera* spp. than Hg and Ht, but not included in genus *Globodera* (Kang et al., 2016).

Considering the previous studies mentioned above and the morphological and structural characteristics examined in our study and the molecular characteristics of cysts in a previous study (Kang et al., 2016), it is believable that all three nematodes belong to *Heterodera* morphologically and molecular-genetically, and the morphological criteria that have long been used previously in the classification of plant-parasitic or soil-borne nematodes may be as valid as current molecular genetic taxonomic criteria at least in higher taxa ( $\geq$  genus) (Kang et al., 2016; Mwamula et al., 2018; Subbotin et al., 2000). However, molecular genetic analyses of cysts may have more validated in species classification rather than morphological and structural characteristics because species characteristics are closely exerted in genetic levels (Dudu et al., 2001).

Cyst morphology and color stage sequences are important criteria for classifying cyst nematode genera (Hesling, 1982). White cysts were frequently observed in Hs in our study with no or very rarely yellow cysts, indicating Hs progressing from white directly to brown cyst processing as shown in Hs in our and previous studies and in the genus *Globodera pallida* (Hesling, 1982; Kang et al., 2016; Narabu et al., 2016), suggesting the occasional observation of white cysts from soils that are formed during maturation

of females may be another way to identify Hs, as was reported previously in Hs (Kang et al., 2016).

Cuticle layers and structures differ depending on nematode species and life cycle stages of cyst nematodes (Bird, 1956; Cliff and Baldwin, 1985; Shepherd et al., 1972). In LM, the layer A (exo-cuticle) was stained most densely with toluidine blue in our study, indicating that cyst wall components, mostly proteins, may accumulate most in the exo-cuticle (layer A) (Clarke, 1968). The layer C of cyst wall (the middle layer of the endo-cuticle) in Hs was composed of fibrous textures observed by SEM and TEM, indicating structural proteins are strong as feather-like keratin proteins (Filshie and Rogers, 1962). This was confirmed by the fact that greater pressure was required to break cysts of Hs, somewhat with a plosive sound, than those of Hg and Ht in our study. This suggests a differential diagnosis of Hs may be possible from Hg and Ht based on these structural characteristics, especially from Hg with similar cyst sizes and distribution in soybean fields owing to similar host plant preferences as mentioned above.

Emergence is the number of J2 (infective and capable of penetration) emerged from a cyst; amount of inoculum potentials of a cyst (Agrios, 2004). The emergence was not significantly different among the cyst nematodes examined. Accordingly, the number of cysts isolated from a plant may be regarded the same as inoculum potential due to the offset of factors such as fecundity and hatching rate, which contribute to the emergence. For example, fecundity was significantly lower in Hs than Hg and Ht, while hatching rate was significantly lower in Ht than Hg and Hs, respectively (Table 3). Among the factors contributing to the emergence, fecundity is related to nematode reproduction, suggesting it is more related to genetic status of host plants, for which the reproduction was inhibited in resistant plants that are governed by resistance genes and poor nutritional status related to the expression of resistance genes (Kim et al., 1987, 1999; Li et al., 2010). However, hatching rates are influenced more by abiotic and biotic soil factors, especially for Ht belonging to *H. shachtii sensu stricto* group (Subbotin et al., 2000), which are enhanced in the presence of root exudates from host plants, compared to fecundity as shown in our study (Table 3) and as shown in a previous study (Williams, 1982). These aspects suggest that the fecundity is governed more by the genetic factors of plants than environmental ones that are more related to the hatching rates, both of which contribute emergence (inoculum potential), respectively (Williams, 1982).

No inhibition of nematode egg hatching occurs in distilled water (Adamu et al., 2003), indicating a full inoculum potential is maintained and eventually ex-

pressed in SDW. Thus, all three nematodes may have the identical final emergence (final inoculum potentials). Thus, Hs may be equivalent in emergence to Hg and Ht shortly after exposure of cysts to soil environments; however, the emergence of Hs is minimized in the long term due to its rapid loss because of lower fecundity and higher hatching rate (causing rapid exhaustion of inoculum potential). On the other hand, emergence of Ht may be maintained in a long-term because it has the highest fecundity among the cyst nematodes, and slow loss of inoculum potential by lowered hatching rate, indicating the emergence continues for a long period of time compared to other nematodes examined. This is due to Ht cysts that were exposed to field conditions adjacent to host plant roots maintain hatching because fecundity of Ht is high and the inoculum amount (the number of J2) is gradually lost due to a lower hatching rate compared to Hg and Hs. This is supported more by the aspects that egg hatching is stimulated by root exudates released continuously from host plants (Williams, 1982). These suggest, in field conditions with host plants grown, Ht may be losing slowly because of high inoculum potential (fecundity) and its slow exhaustion due to lower hatching rate, although it is enhanced by the hatching factors in soil especially such as root exudates (Williams, 1982).

Considering all of the aspects discussed above, the information on morphological, structural and biological characteristics of cysts in *Heterodera* spp. may be used for reliable diagnosis of cyst nematodes and for development of control strategies relevant to each nematode species. Especially, the combined morphological such as cyst wall strength and biological characteristics of cysts such as fecundity and hatching rate may provide more reliable and useful information for cyst nematode control than either characteristic alone.

### Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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