# Scanning Electron Microscopy of Vulval Cones of Heterodera glycines and Three Related Cyst Nematode Species<sup>1</sup>

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Abstract: Vulval cones of four closely related Heterodera species—H. glycines (races 1-5), H. lespedezae, H. schachtii, and H. trifolii—were examined using scanning electron microscopy. Numbers of dorsal and ventral radial ridges, total radial ridges, perineal ridges, and preanal ridges were useful in differentiating the five races of H. glycines and the other three species. Most of the populations differed significantly (P < 0.01) using the Waller–Duncan k-ratio t-test for mean separation of the five characters. H. glycines races 2, 4, and 5 were most similar. H. schachtii and H. trifolii were most dissimilar to each other and to H. lespedezae and H. glycines. Two additional qualitative characters were also useful in differentiating the populations. The shallower, shorter radial ridges of H. glycines provided a basis for separation from the other three species. Width and smoothness of the perineal ridges were useful in differentiating both races and species.

Most keys for the cyst nematodes rely on cyst characters to differentiate species. Cooper (1) first used vulval cone morphology in a 1955 key, which established the terminology used in more recent keys. Cyst characters were used to subdivide *Heterodera* into five groups (13), and all but the most closely related cyst nematodes can be identified using cysts (16).

H. glycines Ichinohe is believed to have evolved from H. schachtii Schmidt (19,20). The close taxonomic relationship between H. glycines and H. schachtii was demonstrated when a high frequency of successful matings between H. schachtii males and H. glycines females resulted in fertile offspring (17). The mitotically parthenogenetic species, H. lespedezae Golden & G. Cobb and H. trifolii Goffart, are also believed to have evolved from the amphimictic *H.* schachtii during the genetic isolation caused by their changed reproductive state and probably are related subspecifically (11,18). All four species belong in the schachtii group of *Heterodera*.

Separation of H. lespedezae or H. trifolii from H. glycines cysts is difficult using light microscopy (4,10,13-16,21), but scanning electron microscopy (SEM) has proven useful in studies of some Heterodera sensu lato vulval cone structures (7,15). Mulvey (15) included the four species in this study, but found the previously reported differences in vulval cones useful only for grouping morphologically similar species of Heterodera. Hirschmann and Triantaphyllou (11) were able to distinguish populations of H. trifolii using SEM. SEM of white female and cyst vulval cones was used successfully to differentiate Globodera pallida (Stone, 1973) Behrens, 1975 which had been considered a pathotype of *Heterodera* (sic *Globodera*) rostochiensis (6,14).

Techniques that differentiate closely related species of *Heterodera* may be useful in identifying *H. glycines* races. The purpose of this study was to determine whether SEM of vulval cones could facilitate separating *H. glycines* races and differentiating the closely related species—*H. glycines*, *H. lespedezae*, *H. schachtii*, and *H. trifolii*.

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## MATERIALS AND METHODS

Populations studied: Five populations of H. glycines were selected from cultures at the University of Illinois. The populations represent five races of H. glycines as determined by host range studies (5,12). Race 1 (HgR1) and race 2 (HgR2) were originally obtained from North Carolina. Race 3 (HgR3), race 4 (HgR4), and race 5 (HgR5) were originally collected in Illinois, Tennessee, and Hokkaido, Japan, respectively. All populations were cultured on soybean (Glycine max (L.) Merr. cv. Williams 82). Single populations of H. schachtii and H. trifolii from California and H. lespedezae from North Carolina were cultured on sugar beet (Beta vulgaris L.), white clover (Trifolium repens L. cv. Dutch White), and striate lespedeza (L. striata (Thunb.) Hook. & Arn. cv. Kobe), respectively. All cultures were maintained in a greenhouse at 22-28 C.

Preparation and SEM of cysts: Tan or brown cysts were selected individually, immersed in distilled water, and sonicated for 40 seconds. Water in the sonicator was changed several times until it appeared clear. Cysts in water were chilled to 4 C, and the water was replaced with chilled 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 (PB). Fixed cysts were refrigerated 2 weeks at 4 C. Cysts then were rinsed in cold buffer for at least 12 hours, postfixed in buffered 2% osmium tetroxide at room temperature, rinsed in PB for another 12 hours at 4 C, and dehydrated in an 8-step graded ethanol dehydration series over a 3-day period at room temperature. Specimens were transferred to modified beam capsules (2) and critical-point dried in a Samdri-790 drier, using carbon dioxide.

At least 80 dried cysts from each population were cut in half, and the vulval cones were transferred to the sticky side of silver tape mounted on double sticky tape on two SEM stubs. The stubs then were sputter coated with gold or gold–paladium. Cysts were viewed on an ISI DS-130 scanning electron microscope operated at 10–15 kV. Forty specimens were photographed in top view and 20 specimens in side view. Before recording the data, photomicrographs of all specimens from all populations were shuffled to produce a completely randomized experimental design. The number of perineal and preanal ridges and the number of radial ridges per dorsal and ventral vulval half were recorded for lateral view and top view, respectively. Analysis of variance, and a Waller-Duncan k-ratio t-test with  $k = 500 \ (P < 0.01)$  were used to determine significant differences among populations in the number of perineal, preanal, and dorsal and ventral radial ridges. Thirty-eight specimens of all populations except H. lespedezae were analyzed for radial ridges. Because of extensive fenestration which often eliminated the radial ridges in H. lespedezae cysts, 35 specimens were analyzed for dorsal, 33 for ventral, and 30 for total radial ridges for this species. Twenty specimens were analyzed for perineal and preanal ridges of all populations except HgR4 for which 19 specimens were available.

#### RESULTS

Terminology for vulval cone morphology, except for the term preanal ridge, was defined previously (1,8,11) and is illustrated in Figure 1. Preanal ridges extended anteriorly from the anal basin to the area where the normal zig-zag or reticulate pattern resumed. Perineal ridges were found between the vulva and anus. Radial ridges occurred along the edge of the smooth semifenestrae in the unfenestrated cyst. The mean number of perineal ridges per population varied from a low of 4.1 for H. lespedezae to a high of 8.0 for HgR3 (Table 1). Ranges for these two populations overlapped slightly, H. lespedezae having 2-6 ridges and HgR3 5-13. The preanal ridge means varied from 1.3 for H. schachtii to 7.1 for HgR3. The ranges of 3-11 preanal ridges for HgR3 and 0-5 for H. schachtii also overlapped. H. schachtii was separable from all other populations using preanal ridges. Using perineal and preanal ridges, 19 of the 28 possible comparisons of the eight populations were significantly differ-



FIG. 1. Diagramatic view of the *schachtii* group cyst vulval cone.

ent (Table 1). Eleven comparisons were different if perineal ridges alone were used, and 13 were different if preanal ridges alone were used. Only 14 comparisons were different if perineal and preanal ridges per individual were summed, as opposed to 19 if numbers of these ridges were used separately.

The range of means for radial ridges was larger than for perineal and preanal ridges (Table 1). HgR3 had the lowest mean of 9.5 for dorsal radial ridges. HgR2 had the lowest means of 11.4 for ventral radial ridges and 21.2 for total radial ridges. H. trifolii had the highest means of 15.3 dorsal, 19.7 ventral, and 35.0 total radial ridges. H. trifolii was separable from all other populations using ventral and total radial ridges. When using dorsal and ventral radial ridges, 19 of 28 possible comparisons were differentiated. If the number of dorsal and ventral radial ridges were added together, 17 comparisons of all combinations of the eight populations could be differentiated. In HgR1, HgR4, and H. schachtii, the number of radial ridges on the dorsal and ventral halves of the vulva were not different. In the other five populations, the ridges per half were different according to a paired *t*-test. For either ventral or dorsal radial ridges, 17 comparisons were different, although not all of the same comparisons were different.

 TABLE 1. Number of perineal, preanal and radial ridges on vulval cones of Heterodera glycines races 1-5,

 H. lespedezae, H. schachtii, and H. trifolii.

	Perineal† ridges mean ± S <sub>r</sub> (range)	Preanal† ridges mean $\pm S_{\bar{x}}$ (range)	Radial ridges‡		
Species and race			Dorsal mean $\pm S_{\tilde{s}}$ (range)	Ventral mean $\pm S_{\bar{x}}$ (range)	Total mean $\pm S_{\tilde{s}}$ (range)
H. glycines (HgR1)	$5.3 \pm 0.4$ (2-8)	$4.4 \pm 0.6$ (0-10)	$11.6 \pm 0.4$ (3-17)	$11.4 \pm 0.4$ (5-17)	$23.0 \pm 0.7 \\ (12 - 31)$
H. glycines (HgR2)	$5.6 \pm 0.4$ (0-9)	$5.7 \pm 0.6$ (2-11)	$9.8 \pm 0.6 \ (1-16)$	$11.4 \pm 0.4$ (5–16)	$21.2 \pm 0.9 \ (1031)$
H. glycines (HgR3)	$8.0 \pm 0.4$ (5-13)	$7.1 \pm 0.5$ (3-11)	$9.5 \pm 0.6 \ (1{-}15)$	$12.3 \pm 0.4$ (6-18)	$21.8 \pm 0.8 \ (1232)$
H. glycines (HgR4)	$5.0 \pm 0.7$ (0-12)	$6.3 \pm 0.9$ (0-15)	$11.1 \pm 0.4$ (2–16)	$11.9 \pm 0.4$ (5–16)	$\begin{array}{c} 23.0  \pm  0.7 \\ (7\text{-}31) \end{array}$
H. glycines (HgR5)	$5.8 \pm 0.6 \ (1{-}11)$	$6.8 \pm 0.8$ (2-16)	$10.1 \pm 0.5 \ (5{-}15)$	$11.8 \pm 0.4$ (7–16)	$21.9 \pm 0.7 \ (13-29)$
H. lespedezae	$4.1 \pm 0.3$ (2-6)	$6.1 \pm 0.6$ (2-12)	$14.1 \pm 0.6 \ (7{-}21)$	$16.6 \pm 0.6 \ (1228)$	$\begin{array}{c} 30.3\pm0.8\ (20{-}42) \end{array}$
H. schachtii	$\begin{array}{c} 6.0\ \pm\ 0.3\ (3-8) \end{array}$	$1.3 \pm 0.3$ (0-5)	$15.1 \pm 0.4$ (9–20)	$15.7 \pm 0.6 \ (10{-}21)$	$30.8 \pm 0.9 \ (21{-}40)$
H. trifolii	$6.9 \pm 0.4 \ (5{-}10)$	$3.6 \pm 0.5$ (0-8)	$15.3 \pm 0.4$ (8–21)	$19.7 \pm 0.4 \ (14-25)$	$35.0 \pm 0.7$ (23-42)
Waller-Duncan LSD $(k-ratio = 500)$	1.54	2.14	1.61	1.51	2.57

 $\dagger n = 20$  for perineal and preanal ridges of all populations except *H. glycines* race 4 where n = 19 for preanal ridges.  $\ddagger n = 38$  for radial ridges of all populations except *H. lespedezae* where n = 35 for dorsal, n = 33 for ventral, and n = 30 for total radial ridges.



FIGS. 2-5. Scanning electron micrographs of vulval cones of *Heterodera glycines* races. 2) Race 1,  $\times$  560. 3) Race 2,  $\times$  730. 4) Race 3,  $\times$  550. 5) Race 4,  $\times$  660.

When using the four different quantitative characters, numbers of dorsal radial ridges, ventral radial ridges, perineal ridges, and preanal ridges, 24 of a possible 28 population comparisons could be differentiated at the 0.01 significance level using the Waller-Duncan k-ratio t-test. HgR3 differed from the other races in number of perineal ridges. HgR1 and HgR3 differed in number of preanal ridges but were not different from the other three races. HgR1 differed from HgR2 and HgR3 in number of dorsal radial ridges, but HgR2, HgR3, HgR4, and HgR5 were not different. There were no differences among H. glycines races in numbers of ventral radial ridges or total radial ridges. H.

schachtii was different from all other species in number of preanal ridges, and *H. trifolii* was different in the number of ventral and total radial ridges. *H. glycines* was in a different grouping for radial ridges from the other three species—*H. schachtii*, *H. lespedezae*, and *H. trifolii* (Table 1). *H. lespedezae* and *H. trifolii* were separable using ventral radial and total radial, perineal, and preanal ridges.

Qualitative differences in vulval cones were apparent among the four species. *H.* glycines (Figs. 2-6) often lacked radial ridges, and those that were present were shorter and less pronounced than radial ridges on *H. schachtii*, *H. lespedezae*, and *H.* trifolii (Figs. 7-9). *H. lespedezae* had many



FIGS. 6-9. Scanning electron micrographs of vulval cones. 6) Heterodera glycines race 5, ×690. 7) H. schachtü, ×950. 8) H. lespedezae, ×670. 9) H. trifolü, ×620.

more fenestrated individuals than the other species. Populations also differed in the prevalence of straight or zig-zag perineal ridges (Figs. 10-17). HgR3 (Fig. 12) had a majority (17/20) of individuals with very straight perineal ridges, and all individuals had very thin ridges. HgR4, HgR5, H. schachtii, H. lespedezae, and H. trifolii (Figs. 13-17) had mostly zig-zag perineal ridges, but all were easily differentiated from HgR1 and HgR3 based on the appearance of the perineal ridges. HgR1 (Fig. 10) had a majority (18/20) of individuals with broad perineal ridges, most of which were also straight. HgR2 (Fig. 11) was variable, having zig-zag and straight perineal ridges of different widths.

#### DISCUSSION

H. glycines races 1–5, H. lespedezae, H. schachtii, and H. trifolii were separable into six groups based on vulval cone morphology. The four species were readily identifiable using SEM, with H. schachtii and H. trifolii the most easily distinguishable. HgR2, HgR4, and HgR5 were morphologically similar, whereas HgR1 and HgR3 were dissimilar. HgR3 could be distinguished from HgR2, HgR4, and HgR5, whereas HgR1 could be distinguished from HgR2 and HgR5 using dorsal radial ridges. The ranges of the values whose means can be used to differentiate these populations generally overlap ranges of other popula-



FIGS. 10-13. Scanning electron micrographs of vulval cone dorsal views of *Heterodera glycines* races. 10) Race 1,  $\times$ 580. 11) Race 2,  $\times$ 760. 12) Race 3,  $\times$ 560. 13) Race 4,  $\times$ 680.



FIGS. 14–17. Scanning electron micrographs of vulval cone dorsal views. 14) Heterodera glycines race 5, ×610. 15) H. schachtii, ×730. 16) H. lespedezae, ×680. 17) H. trifolii, ×620.

tions. This is also true of some juvenile morphometrics which are usually used to identify the species in this group.

When using light microscopy to study cysts, it is extremely difficult to differentiate among H. lespedezae, H. trifolii, and H. glycines (4,10,13-16,21). However, H. schachtii is easily separated from the other three species because it has molar-like anal bullae which are absent in the other species. SEM of ventral and total radial and perineal and preanal ridges provided a method for differentiating between H. trifolii and H. lespedezae. The identification of two closely related Heterodera species using SEM is in contrast to previous work with Meloidogyne perineal areas (3). Many of the subsurface characters most often used to differentiate Meloidogyne groups were not visible with the SEM technique, and no additional information was obtained from the cuticular surface using SEM.

The number of perineal ridges was first used for differentiating species in the subgenus Globodera of Heterodera sensu lato (6,9). Green (7) also used number of perineal ridges as one of the characters to subdivide 15 species of Heterodera into groups, reporting that both H. schachtii and H. trifolii had means of 7.0 perineal ridges. Means of 6.0 ridges for H. schachtii and 6.9 for H. trifolii in the present study agree closely with previous findings. Hirschmann and Triantaphyllou (11) reported ranges of 2-6, 3-7, 7-11, and 9-12 ridges for four populations of *H. trifolii* having four different chromosome numbers. The range of 5-10 perineal ridges reported here for H. trifolii overlap those of all four populations studied by Hirschmann and Triantaphyllou. The H. lespedezae range of 2-6 perineal ridges reported previously (11) agrees with our observations.

*H. lespedezae* was described as having as many as six straight parallel ridges anterior to the anus (11). We have called these ridges preanal ridges. In the present study, up to 12 were found in this species, and they were apparent to some degree in all of the species and races studied.

The perineal and preanal ridge mor-

phology of *H. lespedezae* was more similar to H. glycines than was H. trifolii. When using the quantitative characters, number of dorsal and ventral radial ridges, and number of perineal and preanal ridges, the five races of H. glycines were most similar to one another. However, the qualitative features, smoothness and width of preanal ridges, provided additional characters for identification of the races. H. schachtii and H. lespedezae were the next most similar, with H. lespedezae being more like H. glycines than were H. trifolii and H. schachtii. The extensive fenestration of H, lespedezae cysts was a qualitative difference not found in the other species, but this may have been due to differences in population growth patterns. Lespedeza may have reached its carrying capacity earlier than hosts of other species and may have harbored older cysts in a culture of the same age as the other species. H. schachtii and H. trifolii were the most morphologically dissimilar populations of those studied here.

SEM of vulval cones was an effective method of differentiating H. glycines, H. lespedezae, and H. trifolii. Certain populations of H. trifolii and H. lespedezae vary cytogenetically and morphologically (11). Comparisons of other populations of these two species would provide more information concerning intraspecific and interspecific variation. Hirschmann and Triantaphyllou (11) found similar differences in numbers of ridges and appearance of vulval cones among H. lespedezae, H. galeopsidis Goffart, and four populations of H. trifolii, indicating that H. lespedezae and H. trifolii could be considered subspecies in a parthenogenetic species complex. Data from electrophoresis studies of these two species support the proposal of a parthenogenetic species complex (18), but our SEM data indicate that H. lespedezae and H. trifolii are more dissimilar than are H. glycines and H. lespedezae. SEM of H. glycines races revealed differences which allowed placement of the five races into three groups. SEM studies of more populations of each race might clarify those characters associated with a particular race.

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