

## A Comparison of Low-Temperature and Ambient-Temperature SEM for Viewing Nematode Faces

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**Abstract:** Faces of lesion nematodes *Pratylenchus teres* (populations RTB and JK) and *P. zae* or the bacterivore *Distolabrellus veechi* were observed on frozen specimens with low-temperature scanning electron microscopy and as chemically fixed, critical-point dried specimens with conventional scanning electron microscopy. Amphidial secretions were preserved in chemically fixed but not cryofixed lesion nematodes. Overhanging liplets of chemically fixed *D. veechi* may be artifactual because they appeared as variably filled, mostly empty membranes when cryofixed. The diagnostically useful lips of the frozen lesion nematodes exhibited six sectors of variable prominence that were absent in chemically fixed specimens. This variability may be due to different degrees of muscle contraction captured during cryofixation, which occurs in milliseconds. This is the first evidence that rarely observed lip sectors in *Pratylenchus* may be something other than an artifact of shrinkage.

**Key words:** amphid, cryofixation, *Distolabrellus veechi*, glutaraldehyde, face patterns, low temperature scanning electron microscopy, lip sectors, *Pratylenchus teres*, *Pratylenchus zae*, Rhabditida, scanning electron microscopy, taxonomy, Tylenchida.

Scanning Electron Microscopy (SEM) of nematodes has become an essential component of new descriptions in many higher taxa (Corbett and Clark, 1983; Sauer, 1985). Conventional SEM, which requires chemical fixation, dehydration, and critical-point drying, has been extensively applied to obtain face views that were used either to support the creation of new lesion nematode species among morphometrically similar populations (Duncan et al., 1999) or to justify the synonymization of two species (Hernández et al., 2001). However, little information is available on face pattern variability (Anderson and Townshend, 1985), especially as influenced by conditions of fixation (Eisenback, 1991).

Timed freezing has been used in the past to preserve stages in cellular processes such as synaptic vesicle exocytosis (Heuser et al., 1976; Velasco and Pécot-Dechavassine, 1993) observed by the freeze-etching technique. The relatively new method of cryofixation has the advantage of processing organisms to a more life-like state because the process occurs in milliseconds and the specimens remain fully hydrated (Wergin et al., 1993). As a result, taxonomically significant morphological differences have been seen in cryofixed mites not visible in those chemically fixed (Wergin et al.,

2000a). Therefore, a study was undertaken to compare and contrast cryofixed nematode anterior ends to those prepared by conventional procedures, to detect possible artifacts, new characters, or functionally important aspects of morphology. The genera selected for systematic comparison were *Pratylenchus* and *Distolabrellus* based on unexpected preliminary observations.

### MATERIALS AND METHODS

**Culture:** Lesion nematode *Pratylenchus teres* population JK, from Jan Kempdorp, South Africa, and population RTB from Rustenberg, South Africa, with different host preference; and *P. zae* from Columbus, Ohio, were cultured at 28 °C on excised Iowa Chief corn roots on Gamborg's B5 Medium (Huettel and Rebois, 1985) and extracted on a Baermann funnel (Southey, 1986). *Distolabrellus veechi* LKC10 was isolated from Salisbury, Maryland microplot soil in December 1997 and cultured on *Escherichia coli* OP50 bacteria-spotted NGM agar dishes at 20 °C. Nematodes from cultured dishes were synchronized by an egg preparation protocol using bleach and observing plates over time (Stiernagle, 1999) to enrich for adults. Voucher specimens were deposited in the collections of the USDA Nematode Collection, Beltsville, Maryland.

**Scanning electron microscopy:** Two to four 100-mm-diam. petri dishes containing a minimum of 1,000 live nematodes were washed with distilled water from the agar surface. Distilled water was necessary to avoid salt crystals. For conventional preparation, nematodes were chemically fixed in 3% glutaraldehyde in 0.05M phosphate buffer, dehydrated in an ethanol series, critical point dried (CPD), mounted *en masse* on aluminum SEM stubs (Wergin and Stone, 1981), and observed in a Hitachi S-570 SEM at 10 kV. For low-temperature observations, samples of nematodes *en masse* were placed on approximately 0.5-cm squares of Whatman no. 1 filter paper from which any excess water was wicked away. The paper was supported on the specimen holder composed of a 1.5-cm-wide, 3.0-cm-long, and 1.0-mm-thick copper plate. The mounted specimen was placed

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on a  $-196\text{ }^{\circ}\text{C}$  liquid nitrogen-precooled 14-mm square brass tube within the cryo-work chamber made from an 8-inch  $\times$  10-inch  $\times$  1 1/2-inch styrofoam box (Fig. 1). This contact freeze immobilization method cryofixes the nematodes milliseconds after contact with the metal surface and preserves their natural biological positioning and actions (Figs. 2–3 in Wergin et al., 2000b). Frozen samples were etched at  $-90\text{ }^{\circ}\text{C}$  to remove surface water in an Oxford CT 1500HF Cryotrans system, and observed in a Hitachi S-4100 field emission SEM (Wergin et al., 1993). Six to 10 individuals were photographed for each population and treatment.

## RESULTS

In all cases nematodes treated with conventional glutaraldehyde fixation, dehydration, and CPD were puckered and shrunken compared to cryofixed specimens. Both head and body showed signs of collapse.

With conventional preparation *Distolabrellus* exhibited 6 lips, from which liplets overhung the stoma (Fig. 2A). However, the majority of cryofixed specimens had no liplet overhang, but high, nearly hemispherical lips with the labial sensillum surrounded by spoke-like seams of a membrane on the surface of the cuticle (Fig. 2D); one cryofixed liplet was partially inflated, with two pairs of liplets merged (Fig. 2C). In one specimen, liplets were fully inflated over the stoma (Fig. 2B). Thus, variable liplet inflation occurred both between individuals and among lips in a single individual.

Low-temperature preparation of lesion nematodes showed six sectors of variable prominence within the diagnostically useful lips (Fig. 2F–H, J–L, N–P). These prominent sectors were not seen in conventionally prepared specimens (Fig. 2E, I, M), which either displayed submedial lip sectors fused around the central oral aperture into a panduriform shape as in *P. teres* (Fig. 2E, I)

or unfused sectors as in *P. zaeae* (Fig. 2M). Secretions from amphids on either side of the oral aperture were preserved in all conventionally prepared lesion nematode specimens (Fig. 2E, I, M) but not in any of the cryofixed nematodes (Fig. 2F–H, J–L, N–P).

## DISCUSSION

The rhabditid genus *Distolabrellus* was described with 6 lips uniquely characterized by large protrusions, described as liplets, that overhung the stoma (Anderson, 1983). It should be noted that the term liplets was originally used for flat, apparently rigid cuticular plates at the opening of some adenophorean nematode stoma (Grootaert and Wyss, 1979). The liplet morphology *sensu* Anderson was reproducible with conventionally prepared specimens in this study, where liplets were slightly more distorted than their underlying lip sectors. In cryofixed specimens, lip sectors were highly expanded while liplets were usually dramatically contracted compared to these structures when conventionally fixed. The change in liplets is striking compared to the conventionally fixed structure. While osmotic differences are likely to be involved, the variation between liplet inflation and collapse may represent a real condition in the living organism.

A different type of variation occurred in the cryofixed lesion nematode lip region. Instead of differences in elevation of lips, these atypical cryofixed lips were laterally retracted so six sectors were apparent. With conventional fixation, some other plant-parasitic Tylenchida also show this sectoring (Sauer, 1985). The high frequency of lateral lip indentations revealing lip sectors with cryofixation may be due to contraction of muscles attached to the cephalic framework within the head. Differences in killing time (microseconds with cryofixation compared to 10 to 60 minutes for chemical fixation) might result in changes in appearance due to differences in degree of muscle contraction independent of osmotic distortion of lip structures. Fast-freezing has been shown to preserve rapid changes in muscle contraction (Nassar and Sommer, 1992). This sectoring was exceptionally reported in the lips of conventionally fixed *Pratylenchus* and *Hirschmanniella*, but interpreted as an artifact of overall shrinkage (Anderson and Townshend, 1985; Fortuner and Maggenti, 1991). In our cryofixed, sectorized lesion specimens, no distortion of cuticle other than the lateral contraction of the lips occurred. The rare, flat faces of cryofixed *Pratylenchus* (Fig. 2F, J, N) could represent resting, live nematodes at the time of fixation, or individuals that died before fixation.

In summary, well-defined lip sectors seen in cryofixed *Pratylenchus* spp., and occasionally reported in chemically fixed nematodes, may represent muscle contraction of live nematodes. However, osmotic factors probably account for the differences in inflation of the liplets of conventionally fixed and cryofixed *Distolabrellus*.

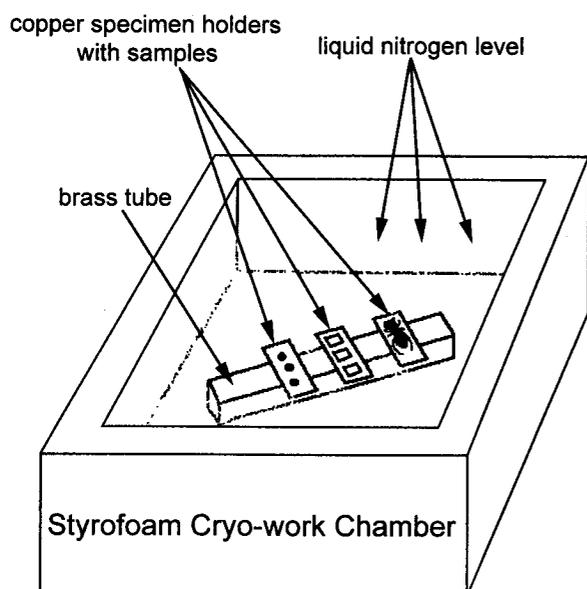


Fig. 1. Styrofoam cryo-work chamber for LT-SEM cryofixation.

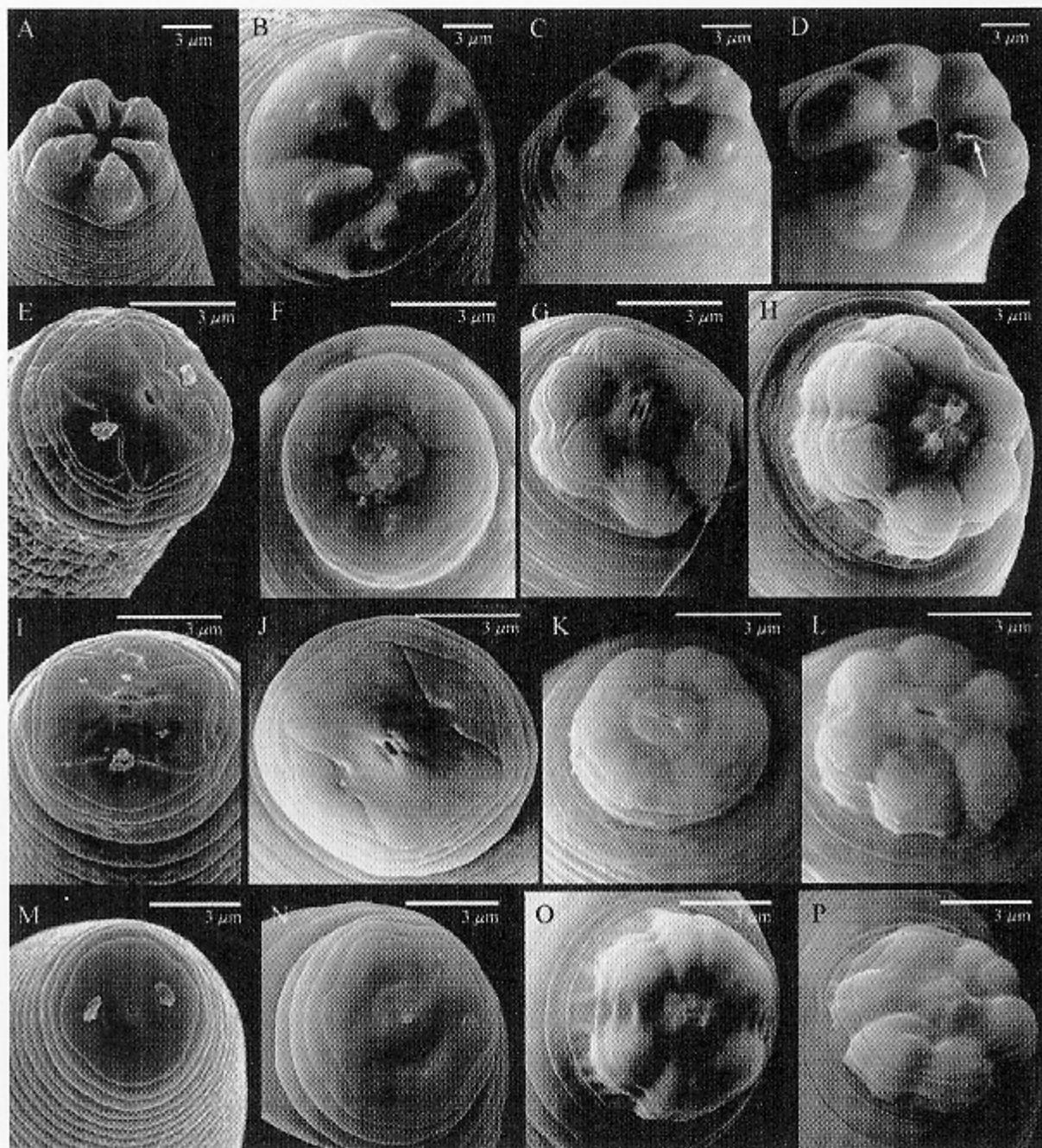


FIG. 2. *Distolabrellus veechi* SEM face views. A) Conventional preparation (chemical fixation, dehydration, and critical point drying). B) Cryofixation with fully inflated liplets. C) Cryofixation with partially inflated liplet. D) Low-temperature preparation (cryofixation) illustrating deflated liplet (arrow). *Pratylenchus teres* JK population; SEM face views. E) Conventional preparation. F) Cryofixation with uncontracted face. G) Cryofixation with partially contracted face. H) Cryofixation with fully contracted face. *Pratylenchus teres* RTB population; SEM face views. I) Conventional preparation. J) Cryofixation with uncontracted face. K) Cryofixation, partially contracted face. L) Cryofixation with fully contracted face. *Pratylenchus zoei*; SEM face views. M) Conventional preparation. N) Cryofixation with slightly contracted face. O) Cryofixation with partially contracted face. P) Cryofixation with fully contracted face.

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