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Morphology of Second-stage Juveniles and Males of *Globodera tabacum tabacum, G. t. virginiae,* and *G. t. solanacearum* (Nemata: Heteroderinae)[1]

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Abstract: Morphological comparisons with light microscopy and scanning electron microscopy were made among second-stage juveniles (J2) and males of several isolates of the three subspecies of the tobacco cyst nematode complex, *Globodera tabacum* sspp. *tabacum, virginiae,* and *solanacearum.* Observations focused on the anterior region, (including head shape, lip pattern, and stylet morphology) and the tail region (including tail shape in J2 and spicules in males). The three subspecies could not be separated on the basis of any of these characters.

Key words: Globodera tabacum solanacearum, G. t. tabacum, G. t. virginiae, juvenile, light microscopy, lip region, male, morphology, nematode, scanning electron microscopy, stylet, tail, variability.

Globodera tabacum (Lownsbery & Lownsbery, 1954) Behrens, 1975, *G. virginiae* (Miller & Gray, 1968) Behrens, 1975, and *G. solanacearum* (Miller & Gray, 1972) Behrens, 1975 comprise a complex of species. These species are identified by a combination of sometimes confusing morphological and biological characters. The tobacco cyst nematode, *Heterodera* (=*Globodera*) *tabacum* (8), was distinguished from the closely related potato cyst nematode (PCN), *H.* (=*G.*) *rostochiensis* Wollenweber, on the basis of host range (7). The TCN did not reproduce on potato (*Solanum tuberosum* L.), whereas the PCN did not reproduce on several tobacco varieties (*Nicotiana tabacum* L.). Some morphological differences between the two species were reported. The tail of the J2 was shorter, the distance of the dorsal esophageal gland opening (DEGO) to the base of the stylet of the male was shorter, and the head shape was more set off in males of *G. tabacum* than in *G. rostochiensis* (7). The lip region of *G. tabacum* females had three prominent annules, while those of *G. rostochiensis* had only two. The display of cuticular punctations between anus and vulva of adult females was parallel or with no alignment in *G. tabacum,* but perpendicular to the vulva-anus axis in *G. rostochiensis* (7).

Miller and Gray (10) distinguished *G. virginiae* from *G. rostochiensis* and *G. tabacum* on the basis of general cyst shape, posterior cyst wall pattern, shape of the female dorsal stylet knob, Granek's ratio, and the shape of the fenestra. *Globodera virginiae* reproduced on *Nicotiana acuminata* (R. Grah.) Hook., whereas *G. tabacum* did not.

Heterodera (=*G.*) *solanacearum* was distinguished from the other two TCN species, *G. tabacum* and *G. virginiae,* based on slight differences in general cyst shape, color of young cysts, posterior cyst wall pattern, shape of female stylet knobs, and shape of fenestra (11). *Globodera solanacearum* did not reproduce on *Nicotiana sanderae* W. Wats., whereas the other TCN species did. The Virginia TCN species were detected within 48 km of each other. They possibly overlap in their geographic distribution and may interbreed naturally (9,20).

The tobacco cyst nematode complex was reevaluated by Stone to consist of three subspecies, *G. tabacum tabacum* (GTT), *G. t. virginiae* (GTV), and *G. t. solanacearum* (GTS) (20).

Morphology is still the most commonly used method for identification of cyst nematodes (1). The morphology of second-stage juveniles (J2) or males of *Globodera* spp. has received little attention. Stud-

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ies on the morphology of these stages may contribute new and more reliable characters for identifying *Globodera* spp. The purpose of this research is to evaluate new characters for identification of GTT, GTV, and GTS and to describe the variability within J2 and males of the tobacco cyst nematode complex. Preliminary observations have been reported (12,13).

Materials and Methods

Isolates of TCN used in this study are listed in Table 1. All populations were reared on 'Rutgers' tomato, *Lycopersicum esculentum* L., in a greenhouse maintained at 25–30 C in either 10 or 15 cm clay pots with a 2:1 steam sterilized top soil to sand mix. After 60–70 days, nematodes were extracted by a combination of Cobb's decanting and sieving and Baermann funnel techniques.

Specimens prepared for LM were heat-killed and mounted in water on a microscope slide with a thin ring of nail polish. The posterior portion of the male body was cut to overcome difficulties in orientation and for better observation. Observations were made immediately after preparation, and specimens were photographed through a planapochromatic, bright field, compound microscope with Polaroid type 55 or 35 mm Agfa Copex film.

Specimens were prepared for SEM by placing them in a BPI dish with 10 drops of buffer (0.1 M sodium cacodylate pH 7.2) at 4 C for approximately 15 minutes (3). A sequential fixation previously described (4) was modified with various fixatives (4% glutaraldehyde, 2% formalin, 2% acrolein) and mixtures of two or more fixatives. After fixation was completed, specimens were left in a refrigerator (4–5 C) for 24–48 hours, followed by three rinses with buffer within a 15-minute period. They were postfixed in 2% osmium tetroxide, placed in a refrigerator for 8–48 hours, and rinsed three times with buffer within a 15-minute period. Specimens were dehydrated in a seven-step ethanol series, including three changes of 100% ethanol. They were either critical-point dried or freeze-dried. Stylets were extracted (3) by cutting off the anterior portion of the nematode in 0.01–0.05% sodium hypochlorite, which allowed the stylet to remain intact while the surrounding tissues were dissolved. The stylet was cleaned and placed in the central area of the cover slip using a dental root canal file with the aid of a stereoscope at ×60. The stylet was attached and fixed to the cover slip with one drop of 2.5% formalin every 2 minutes until the sodium hypochlorite was removed. Alternatively, tap water was used in place of the formalin, which removed the sodium hypochlorite crystals better than did the formalin. The excess formalin or water was drained from the coverslip with either filter paper or a micropipette, and the stylet was air-dried. The specimens were stored in a desiccator overnight, mounted on SEM stubs with double sticky tape, sputter-coated with 20 nm of gold-palladium, and observed with a Philips 505 SEM operating at 20 kV with a 20–50 nm spot size. Images were recorded with Polaroid type 55 film.

Table 1. Isolates of the tobacco cyst nematode complex, *Globodera tabacum* sspp. *tabacum* (GTT), *virginiae* (GTV), and *solanacearum* (GTS).

Isolate	Location	County, State	Origin
GTT-1 (type locality)	Hazardville	Hartford, CT	P. M. Miller
GTT-2	Windsor	Hartford, CT	P. M. Miller
GTV-1	Horton	Suffolk, VA	L. I. Miller
GTV-1-X	Horton	Suffolk, VA	M. Mota
GTV-11 (type locality)	Standard 24	Suffolk, VA	L. I. Miller
GTS-1	Fisher-Nottoway	Nottoway, VA	L. I. Miller
GTS-10 (type locality)	Watkins	Amelia, VA	L. I. Miller

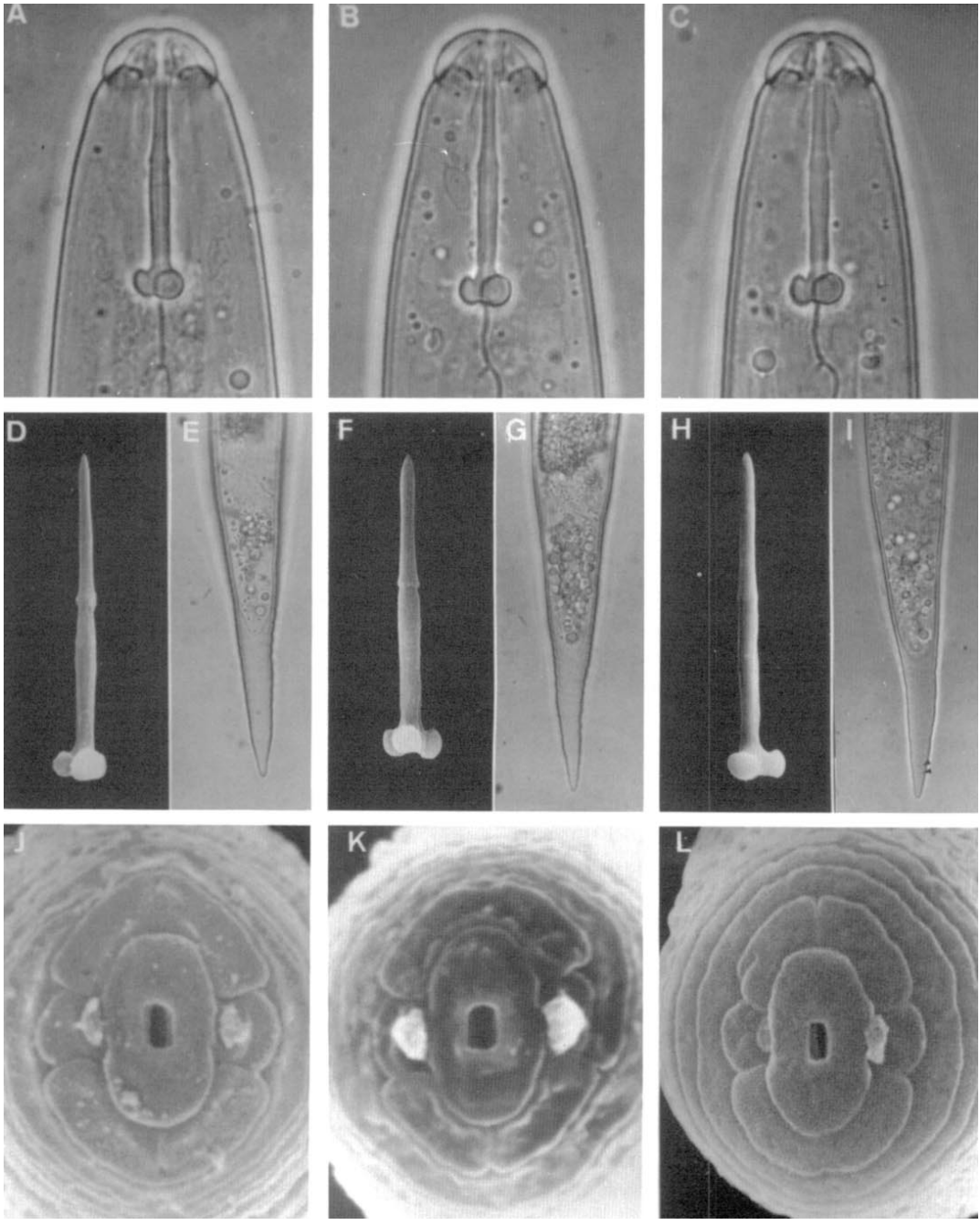

FIG. 1. Anterior region (A–C,D,F,H,J–L) and tails (E,G,I) of second-stage juveniles of certain isolates (see Table 1) of the tobacco cyst nematode complex, *Globodera tabacum tabacum* (GTT), *G. t. virginiae* (GTV), and *G. t. solanacearum* (GTS). A) LM of GTT-1. B) LM of GTV-1. C) LM of GTS-10. D) SEM of stylet of GTT-1. E) LM of tail of GTT-1. F) SEM of stylet of GTV-1. G) LM of tail of GTV-1. H) SEM of stylet of GTS-1. I) LM of tail of GTS-1; arrows indicate lipidic inclusions J) SEM of "en face" view of GTT-1. K) SEM of "en face" view of GTV-1. L) SEM of "en face" view of GTS-1. A–C, E, G, and I are lateral observations.

Results

Second-stage juveniles

Head and stylet (Fig. 1A–D,F,H): The body of J2 tapers anteriorly. The head is slightly set off and is formed by four head annules, the lip region, and an oral disk. In lateral view, it has a slight central depression that corresponds to the oral opening. The cephalic framework is heavily sclerotized. The stylet is robust with three stout, rounded knobs (Fig. 1D–F). The dorsal knob is curved anteriorly and appears anchor-shaped. The stylet knobs of some specimens of GTT were stouter than others. The lip region, in SEM (Fig. 1J–L), has an elongated elliptical oral disc containing a rectangular prestoma. In some specimens, the oral disc was more rectangular than elliptical (Fig. 1J). The oral disc is surrounded by submedial lips formed by fusion of the dorsal and ventral lip pairs. In some specimens, these lips may not be fused. The smaller lateral lips are separated from the oral disc by the amphidial openings, which are frequently obstructed with secretions. The shape of the submedial lips is rectangular to rounded (Fig. 1J–L), and this lip pattern is Stone's type 1 (19). There were no observable differences among the three subspecies, and the variability described above is present in GTT, GTV, and GTS.

Tail (Fig. 1E,G,I): The tail of J2 of all members of the TCN complex is pointed and ends in a fine rounded tip. There is only slight variability in the width of the tip. The hyaline portion of the tail is approximately one-half the tail length. Frequently, lipid inclusions are observed near the tail tip (Fig. 1I). No observable morphological differences occurred among the isolates other than natural variability.

Male

Head and stylet. (Fig. 2A–I): The head region is slightly set off from the body annules and consists of six annules, six lips, and an oral disc. The oral opening is slightly depressed in lateral view. The cephalic framework is heavily sclerotized. The stylet is robust, the cone is slightly shorter than the shaft, and narrows slightly in the middle. The knobs are rounded and sloped posteriorly, in GTV, the dorsal knob slopes more than in the other two subspecies (Fig. 2B,E). When viewed with the SEM (Fig. 2G–I), the lip region consists of a large, elliptical oral disc containing a centrally located rectangular prestoma. The oral disc is surrounded by four large submedial lips, which are rectangular or rounded, and by two small lateral lips. The amphids open between the lateral lips and oral disc. Morphological differences among males of the isolates of the three subspecies were not detected.

Tail (Fig. 3A–F): The tail of males of the TCN complex is twisted 90° in relation to the rest of the body. Two conspicuous spicules that slightly curve ventrally are present. The spicules usually protrude in live specimens, but they are usually retracted in fixed specimens (Fig. 3A–C). The head of the spicule is more enlarged in GTT (Fig. 3A,D). However, in some specimens, the head appears to be the same diameter as the shaft. The blade ends in a single tip, characteristic of males of the genus *Globodera.* The tip of the spicule may be pointed (Fig. 3D,F) or more rounded (Fig. 3E) and varies with individuals. No clear-cut differences in spicule morphology were detected among the three subspecies.

Discussion

Members of the TCN complex were analyzed by Stone (18,20), Greet (6), and Green (5). Stone compared the morphometrics of J2 and males of the three TCN species, three pathotypes (A, B, and E) of PCN, and another cyst species, *H. mexicana* nomen nudum (2). Comparisons were made also using the lip patterns of J2. Stone (20) recognized three major groups of round cyst nematodes on the basis of lip patterns of J2. One group included PCN pathotype A, another included pathotypes B and E, and a final group included the TCN complex and *H. mexicana.* Greet con-

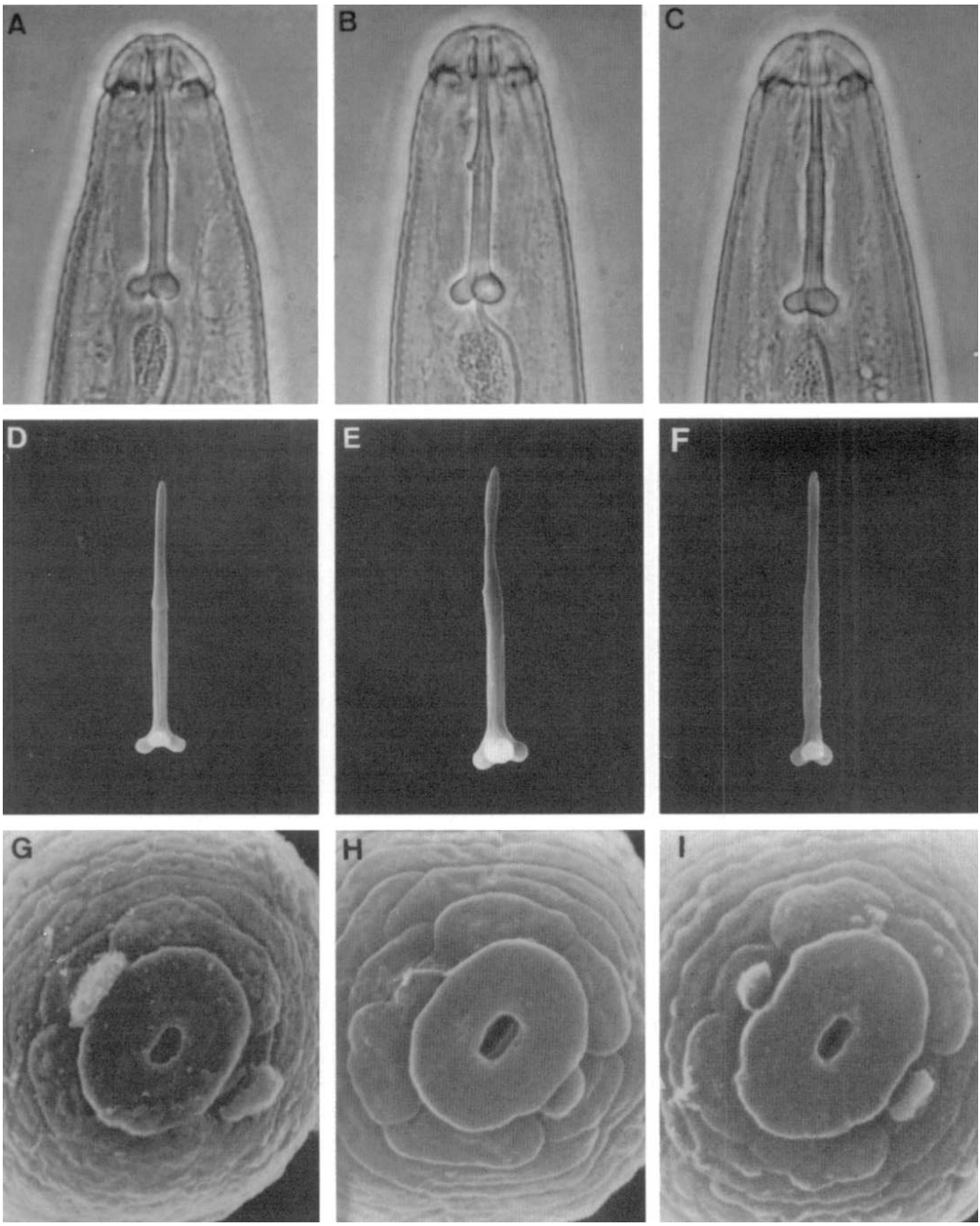

FIG. 2. Anterior region of males of certain isolates (see Table 1) of the tobacco cyst nematode complex, *Globodera tabacum tabacum* (GTT), *G. t. virginiae* (GTV), and *G. t. solanacearum* (GTS). A) LM of GTT-1. B) LM of GTV-1. C) LM of GTS-1. D) SEM of stylet of GTT-1. E) SEM of stylet of GTV-1. F) SEM of stylet of GTS-1. G) SEM of "en face" view of GTT-2. H) SEM of "en face" view of GTV-1. I) SEM of "en face" view of GTS-1. A–C are lateral observations.

sidered the TCN complex and *H. mexicana* to be indistinguishable based on polyacrylamide gel electrophoretic patterns of total proteins (6). Only *G. tabacum* had a faint grayish band that was distinct from the others. PCN pathotype A was distinct from pathotype E, and both differed from the TCN species. Miller and Gray (10,11) did

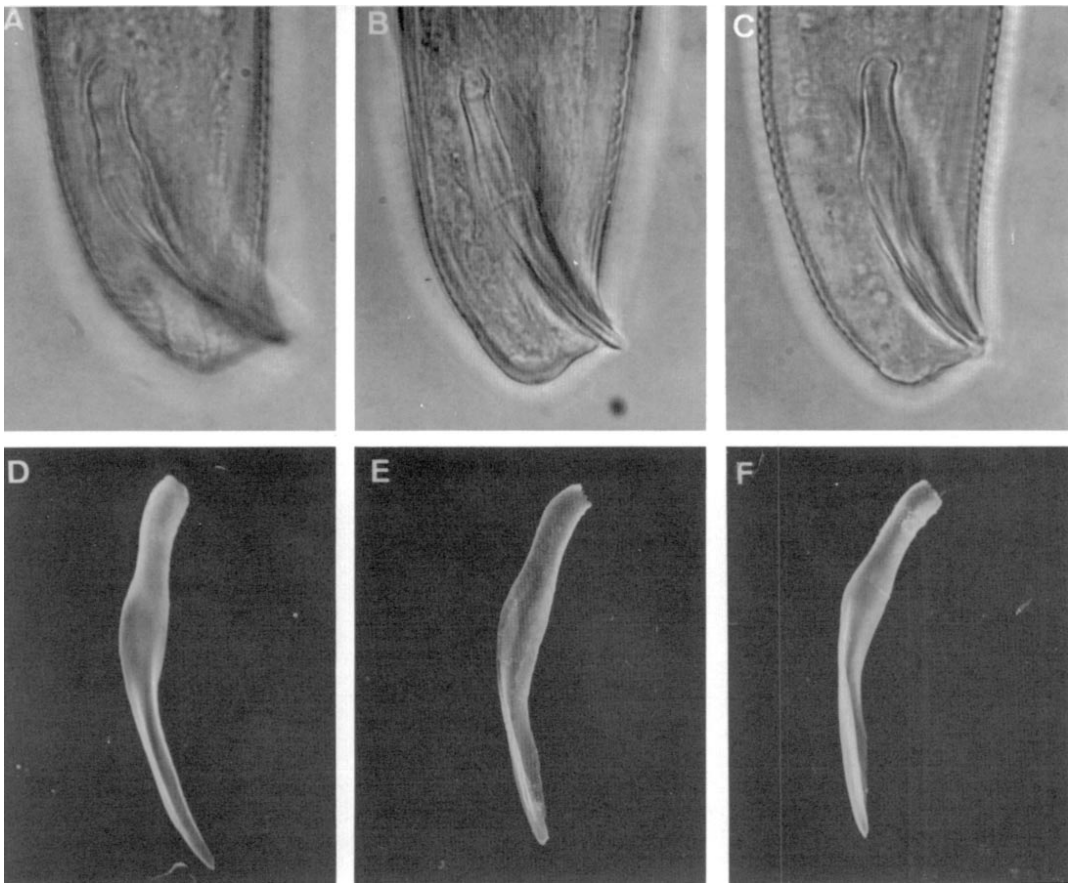

FIG. 3. Tail region of males of certain isolates (see Table 1) of the tobacco cyst nematode complex, *Globodera tabacum tabacum* (GTT), *G. t. virginiae* (GTV), and *G. t. solanacearum* (GTS), observed in lateral position. A) GTT-2. B) GTV-11. C) GTS-1. D). SEM of spicule of GTT-1. E) SEM of spicule of GTV-1. F) SEM of spicule of GTS-10.

not report morphological differences among J2 and males of *G. virginiae, G. solanacearum,* and *G. tabacum.*

Roberts and Stone (16) concluded that host range data within *Solanum* spp. failed to differentiate *G. solanacearum, G. virginiae,* and *G. tabacum.* Furthermore, Miller (9) reported that all crosses between combinations of the three species produced fertile hybrids. Because the three TCN species have very small morphological differences, similar polyacrylamide gel patterns, host range, and ability to produce viable hybrids, Stone (20) proposed ranking them as subspecies: GTT, GTV, and GTS. Although not all authors accept this scheme (17), our observations support the present status, as established by Stone.

Olsson (14) used the morphology of male spicules to distinguish between PCN species and two TCN species (*G. virginiae* and *G. tabacum*). According to this author, *G. tabacum* has a shorter and thicker spicule as compared to *G. virginiae.* Our studies, however, failed to show observable morphological differences in spicule morphology. Extracted stylets of J2 and males of the three subspecies were similar.

Lip patterns of J2 and males were not different. Othman et al. (15) reported that the submedial lip pairs of J2 were occasionally fused in *Globodera* (contrary to Stone's type 1 pattern, in which the submedial lip pairs are separate), except for *G. rostochiensis.* Our observations indicate that the submedian lip pairs of J2 are often

fused, and that this character is not useful to distinguish subspecies. The percentage of specimens with fused submedial lip pairs seems to vary according to the observer, although no study has presented actual counts of those with and without fused lips. Wouts (21) differentiated several species of *Globodera* by the shape of the J2 stylet knobs, but he did not include TCN. Our studies revealed no clear-cut differences in stylet knob morphology among the three subspecies.

In conclusion, J2 and males of several isolates of the three subspecies of the tobacco cyst nematode complex, GTT, GTV, and GTS, reared on the same plant host to avoid ecophenotypic variation were morphologically similar, suggesting that these subspecies cannot be identified on the basis of morphology of J2 and males. These results are consistent with Stone's placement of these organisms as a group of subspecies.

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