

Revised hypotheses for phylogenetic homology of the stomatostylet in tylenchid nematodes

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Received: 26 July 2004; revised: 6 September 2004

Accepted for publication: 6 September 2004

Summary – Molecular phylogenetic systematics suggest that Tylenchida share an immediate ancestor with Cephalobina. This relationship has implications, contradicting classical views, for evolution of the stomatostylet and homology with the cephalobid stoma. Emerging evidence from comparative TEM and 3D modelling is the basis for hypothesising that the tylenchid stylet, specifically the cone and shaft, is homologous with cuticle associated with arcade syncytia of the cephalobid gymnostom; furthermore, the stylet knobs and associated m1 protractors, are prostegostom. The guiding apparatus through which the stylet moves is lined by epidermal syncytia and is homologous with the cephalobid cheilostom. Junctional complexes associated with the epidermal syncytia of the cheilostom and adjacent gymnostom arcade cells occur in both cephalobids and tylenchids, but in the latter the membrane complexes are folded and modified as the guide ring. Testing of the hypothesis requires clearer phylogenetic resolution as well as additional ultrastructural and developmental observations of representatives of tylenchids and outgroups.

Keywords – Cephalobina, cheilostom, development, evolution, fine structure, gymnostom, stegostom, stoma, Tylenchida.

Tylenchida Thorne, 1949, including most species of plant-parasitic nematodes, are characterised by adaptation of the stoma as a protrusible stomatostylet of extracellular collagenous cuticle enclosing a narrow lumen. Typically the stylet consists anteriorly of a tapering cone, followed by a cylindrical shaft and one dorsal and two sub-ventral stylet knobs from which protractor muscles extend anteriorly. The stylet moves through a cuticle-lined channel (guiding apparatus) consisting anteriorly of a vestibule (the hub of a hexaradiate framework) and posteriorly of a vestibule extension; the stylet is attached to the channel near the base of the vestibule extension by a junctional complex specialisation of folded cell membranes (guide ring). The stylet is structurally and functionally linked to the pharynx, which is a source for digestive enzymes and muscular pumping; during feeding the stylet lumen is the conduit through which enzymes can be injected into the host and host cytoplasm ingested (Dropkin, 1969).

Evolution of the highly specialised tylenchid feeding system is pertinent to broader questions of the evolution of plant parasitism (Baldwin *et al.*, 2004). The stylet has been previously proposed to have evolved through a morphological transformation series including the open stomas of Cephalobina, Rhabditina and Diplogastrina

(Thorne, 1961; Andr assy, 1962, 1976; Goodey, 1963; De Grisse, 1972). These classical proposals of morphological evolution have included attempts to suggest homologies of specific rhabdia (thickenings of stoma linings) among these stomas to components of the stomatostylet and guiding apparatus. Stomatostylet components ascribed to rhabdia include the framework, vestibule, vestibule extension, cone, shaft and knobs (for terminology see Fig. 1 in Baldwin and Hirschmann, 1976). Thorne (1961), for example, suggested that among the cephalobid rhabdia, the cheilorhabdion is homologous to the tylenchid framework whereas the prorhabdion corresponds to the stylet cone, the meso- and metarhabdia to the shaft, and the telorhabdion to the stylet knobs (Fig. 1).

A broadly held classical view of phylogenetic relationships, consistent with the morphological transformation from the open stoma to the tylenchid stomatostylet, was that of a unique shared ancestry of Diplogastrina and Tylenchida (Steiner, 1933; Thorne, 1961; Goodey, 1963; Andr assy, 1976; Maggenti, 1981), but this view is inconsistent with SSU rRNA phylogenies which support a Diplogastrina-Rhabditina clade that excludes Tylenchida. Instead Tylenchida share a unique clade with Cephalobina Andr assy, 1974 (Blaxter *et al.*, 1998; Dolin-

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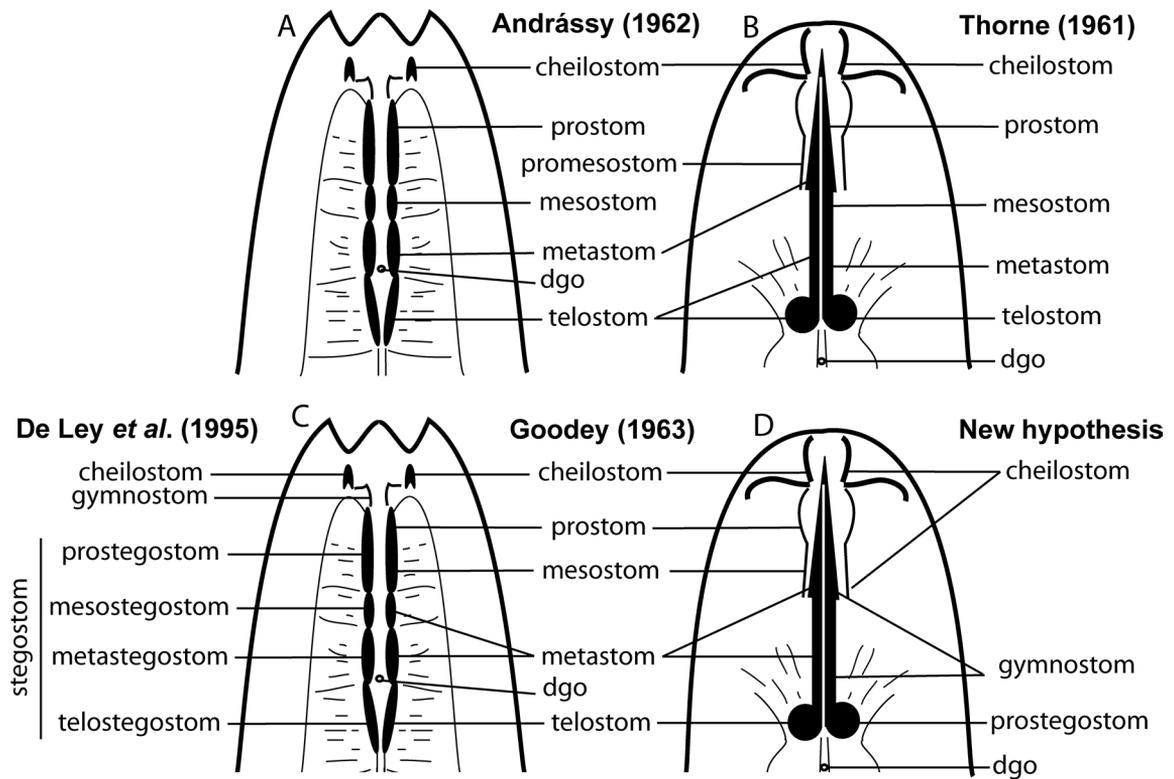


Fig. 1. Diagrammatic representation of hypotheses of homology of components of the stoma of *Cephalobina* to the stomatostylet of *Tylenchida*. A: ‘Cephalobus-Type’ of Andr ssy (1962), with regions designated identical to those of Thorne (1961) for ‘Eucephalobus’; B: ‘Tylenchus-Type’ of Andr ssy, represented by left column labels, and ‘Rotylenchus’ of Thorne (1961), represented by right column labels; C: Cephalobid with terminology designations of De Ley et al. (1995) represented by left column labels and those of Goodey (1963) represented by right column labels; D: Tylenchid stomatostylet with terminology designations of Goodey (1963) represented by left column. Terminology represented by right column labels show homologies proposed herein. (Abbreviation: dgo = dorsal gland orifice.)

ski et al., 2001), partly consistent with the earlier hypothesis of Siddiqi (1980). Subsequent phylogenies including many additional taxa and other genes further support that the Tylenchida and Cephalobina are sister taxa (Subbotin, Thomas, and Nadler, pers. comm.) and thus suggest that the stomatostylet arose from a narrow, open stoma, like that which characterises extant *Cephalobina*, thereby effectively rejecting previous hypotheses of homology.

It is generally accepted that there are three criteria by which to propose and evaluate hypotheses of homology: conjunction, similarity, and congruence (Patterson, 1988). Conjunction and similarity are primarily used to propose initial hypotheses of homology, while congruence with other hypotheses, such as through a well-supported phylogenetic tree, is a means by which to test homologies. Similarity, as a criterion for proposing hypotheses of homology, can be further categorised as compositional, topo-

logical, developmental, functional, or genetic (Panchen, 1994). Classical views on stoma morphology are based upon relatively unsophisticated observations of compositional and topological similarity of secreted structures (the rhabdia and stylet components). Previously, limited resolution of light microscopy and lack of developmental information prevented more detailed and meaningful interpretations of homology. Furthermore, the classical hypotheses of stylet homology fail a test of congruence with well-supported phylogenetic trees, making an alternative hypothesis necessary. Here, we collate and re-evaluate previous TEM, together with new information, into a more viable and testable hypothesis of homology as it pertains to the evolution of the stomatostylet.

The proposal to extend interpretations of stoma homologies in *Cephalobina* to *Tylenchida* is a crucial step in understanding evolution of the stomatostylet. Fine struc-

ture and developmental observations are providing new insight for interpreting cellular organisation of the stoma of Cephalobina in relation to additional Secernentea* (De Ley *et al.*, 1993, 1995; Baldwin & Eddleman, 1995; Baldwin *et al.*, 1997; Dolinski *et al.*, 1998; Dolinski & Baldwin, 2003). These are more recently being extended through 3D modelling of electron micrographs, electron tomography, 4D developmental observations, and confocal microscopy (Bumbarger & Baldwin, unpubl.; Burr & Baldwin, unpubl.). Key to understanding homologies with the stomatostylet is that the stoma of Cephalobina includes, posterior to the cheilostom, a cuticle-lined region, nearly invisible by light microscopy, that is not surrounded by pharynx (stegostom) but instead by a thin stack of two arcade syncytia (gymnostom) (Baldwin & Eddleman, 1995; De Ley *et al.*, 1995); homologues of these arcade syncytia, and thus the gymnostom, are well supported in Rhabditina and Diplogastrina (Albertson & Thomson, 1975; Wright & Thomson, 1981; Baldwin *et al.*, 1997; De Ley & Blaxter, 2002).

Topological and developmental information on stylet components (framework, vestibule, vestibule extension, cone, shaft, and knobs) that could be informative for establishing homologies has not yet been adequately researched. There is a body of classical literature on fine structure of several tylenchid species that predates and thus excludes interpretation that considers fine structural understanding of stoma characters and their homologies in other Secernentea (De Grisse, 1972; Baldwin & Hirschmann, 1976; Shepherd & Clark, 1976; Chen & Wen, 1980; Shepherd *et al.*, 1980; Endo, 1985; Endo & Nickle, 1993; Endo *et al.*, 1997). These studies provide a starting point for proposing new hypotheses of homologies of the stomatostylet components with parts of the stoma, as also understood from fine structure, in Cephalobina.

Comparative observations, homologies

We hypothesise that the tylenchid stylet, specifically the cone and shaft, is homologous with the cuticle lining of the gymnostom of Cephalobina. Unlike extant hypotheses that compare the stylet to the entire stoma,

* In this paper we use Secernentea for continuity with previous literature. We acknowledge, however, a strong justification for a clade Chromadorea within which the clade Secernentea (syn. Order Rhabditida *sensu* De Ley & Blaxter, 2002) is embedded (De Ley & Blaxter, 2002).

including all of the stegostom, herein only the stylet knobs are proposed to be homologous with the lining of the anterior end of the cephalobid stegostom. This anterior region was designated by De Ley *et al.* (1995) as the prostegostom and equivalent to the prostom of Thorne (1961) and Andr assy (1962) and the prostom + mesostom of Goodey (1963) (Fig. 2). The tylenchid vestibule, associated cephalic framework and vestibule extension are surrounded by epidermal* syncytia as is the cheilostom of Cephalobina. A guide ring surrounding the stylet shaft consists of junctional complexes between syncytia. These complexes are reminiscent of, and may be homologous with, the ring of junctional complexes between the epidermal syncytium of the cheilostom and the arcade syncytium of the gymnostom of Cephalobina (Van de Velde *et al.*, 1994; Baldwin & Eddleman, 1995; De Ley *et al.*, 1995).

STYLET KNOBS, STEGOSTOM

In Cephalobina, the stegostom is characterised by a triradiate lumen lined by thickened cuticle (Fig. 3A). Each apex of the lumen is associated with a marginal cell elongate in the anterior-posterior axis and, between the apices, are three sectors occupied by a stack of radial muscle cells in the dorsal and two subventral positions (Figs 2A; 3A). The stack of muscle cells defines, from anterior to posterior, the pro-, meso-, meta- and telostegostom (De Ley *et al.*, 1995) (Figs 1; 2A). In the prostegostom each sector includes a single interradiial cell, m1; posteriorly in the mesostegostom, similar interradiial cells are designated m2. In contrast, the sectors posterior to these each include a pair of adradial muscle cells in a pattern that apparently extends into the procorpus (De Ley *et al.*, 1995; Dolinski *et al.*, 1998).

As in Cephalobina, the anteriormost cells of the stegostom in Tylenchida include three elongate marginal cells. These are separated by three large interradiial cells, the stylet protractor muscles (Fig. 3B, C). The protractor muscles, designated m1 (defining the prostegostom), attach to the thickened cuticle lining of the stoma, *viz* the three stylet knobs (Figs 2B; 3B, C). The protractor muscles of Tylenchida differ from the radial muscles of Cephalobina in that they show a complex pattern of anterior branching and attach near the junction of the body wall and cephalic framework (Baldwin & Hirschmann, 1976) (Figs 2B; 4D). They also differ by possessing a

* We use epidermal as a synonym of hypodermal. For a review of terminology see Bird and Bird (1991, p. 4).

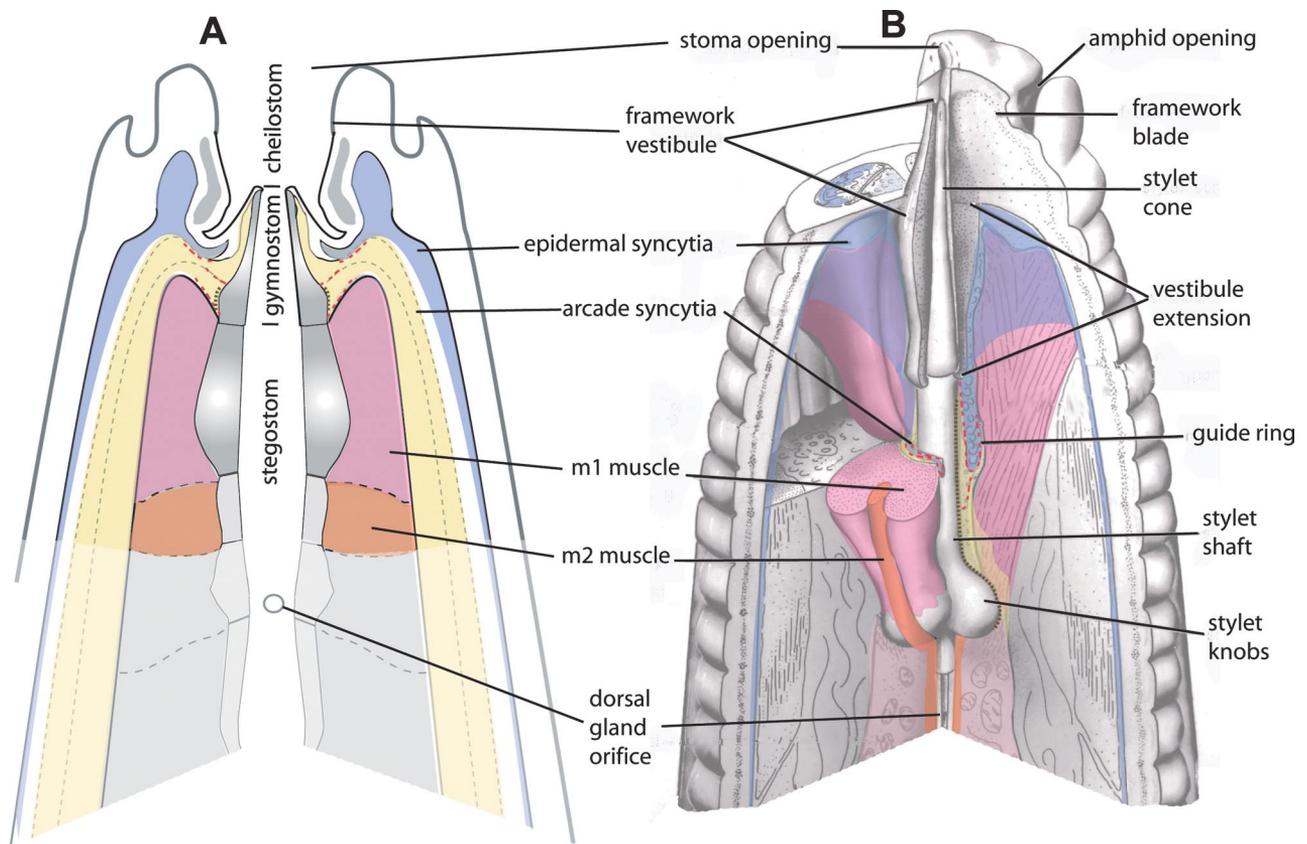
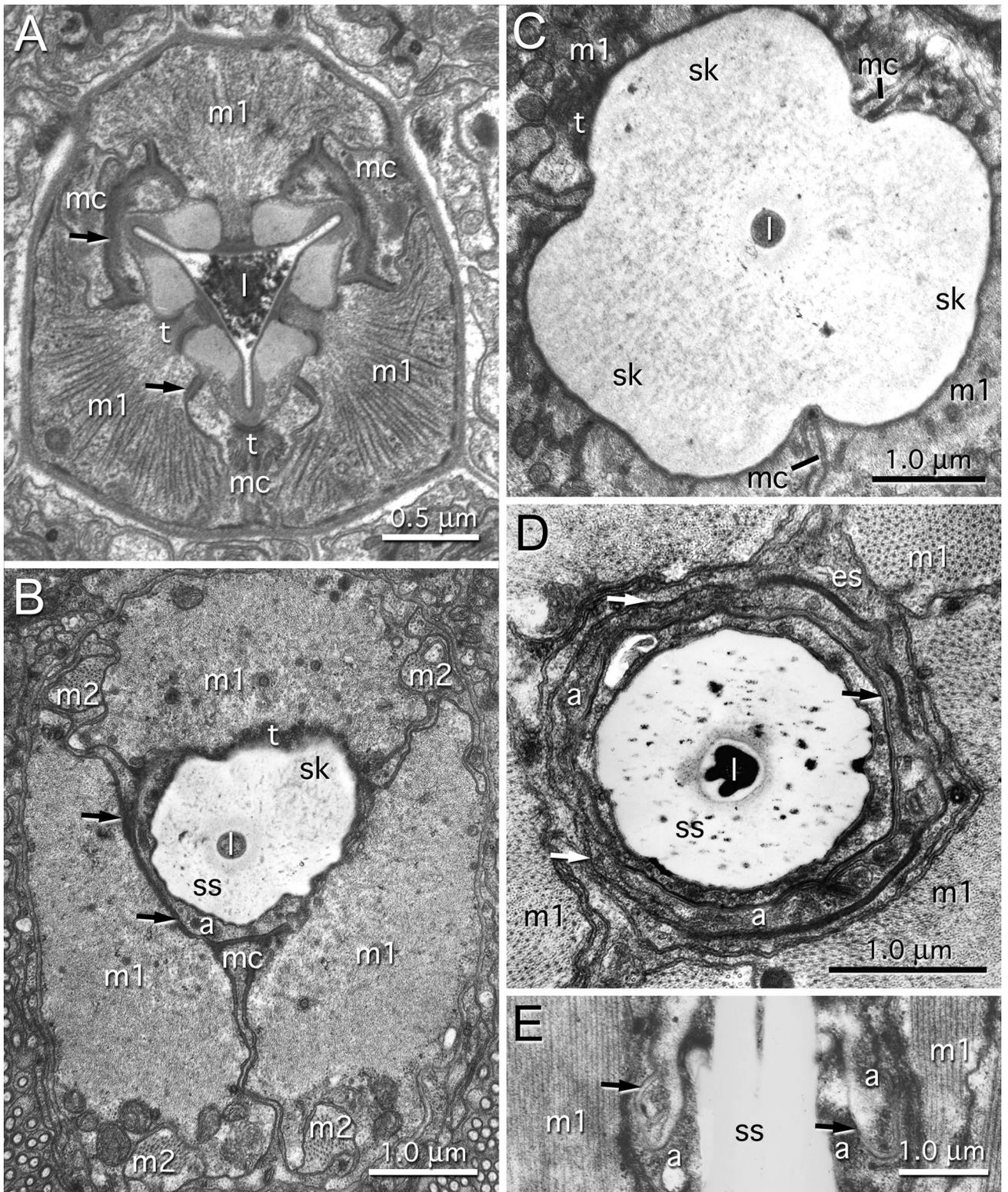


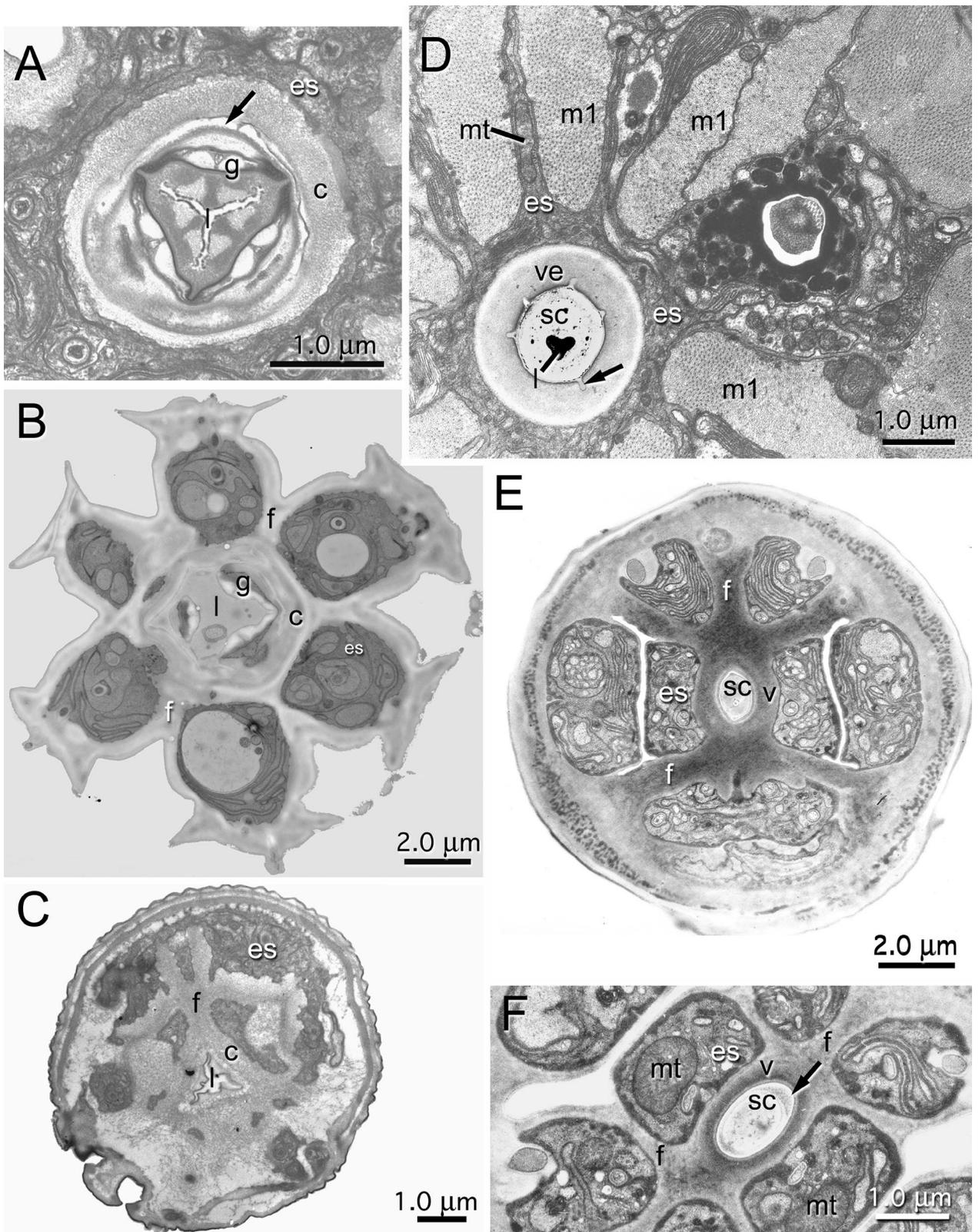
Fig. 2. Diagrammatic representation of the components of the cephalobid stoma (A) in relation to the tylenchid stomatostylet (B). Colours corresponding between A and B represent a hypothesis of homology. Broken red lines indicate the presence of junctional complexes; broken brown lines represent tonofilaments attaching arcade syncytia to stoma/stomatostylet cuticle. 'A' is adapted from Baldwin and Eddleman (1995) and 'B' is modified, and partially redrawn, from Baldwin and Hirschmann (1976).

broad, noncontractile region that includes the nucleus, and which extends posteriorly into the procorpus (Baldwin & Hirschmann, 1976), vs a much narrower posterior process as in those of *Cephalobina* (Dolinski *et al.*, 1998). Posterior to the tylenchid stylet knobs a second set

of radial muscles, m2 (defining the mesostegostom), attaches to the narrow pharyngeal lumen, but extends anterior as narrow muscular processes closely associated with protractors. Curiously, m2 comprises a pair of adradial cells on the dorsal side, whereas on each subventral

Fig. 3. Transmission electron micrographs through the anterior stegostom of representatives of *Cephalobina* and *Tylenchida*. Sections are transverse unless otherwise indicated. A: *Zeldia punctata* female with the stomatal lumen (l) surrounded by three interradial muscles (m1) separated by three marginal cells (mc) (t = tonofilaments). Arrows denote junctional complexes; dorsal is toward the top of the figure; B: *Meloidogyne incognita* male near junction of stylet shaft (ss) with stylet knobs (sk), the former flanked by arcade syncytia (a). The three protractor muscles (m1) are separated by narrow marginal cells (mc). (Abbreviations: l = stylet lumen, m2 = anterior contractile region of m2. Arrows denote junctional complexes; dorsal is toward the top of the figure); C: Stylet knobs (sk) of *M. incognita* male. (Abbreviations: l = lumen, m1 = protractor muscles, mc = marginal cells, t = tonofilaments. Dorsal is toward the top of the figure); D: Stylet shaft (ss) of *M. incognita* male surrounded by junctional complexes (arrows) between syncytia including arcade (a) and epidermis (es). (Abbreviations: m1 = protractor muscles); E: Longitudinal section through region corresponding to 'D' including stylet shaft (ss) surrounded by junctional complexes (arrows) associated with adjacent arcade syncytia (a). Figures D and E are printed from TEM negatives, parts of which are used in Baldwin and Hirschmann (1973) and for B, C, and E, parts of which are used in Baldwin and Hirschmann (1976).





side there is only a single interradiated m2 cell (Figs 2B; 3B). These muscle cells may be discernible with light microscopy (Coomans & van Bezooijen, 1968), thereby allowing us to evaluate efficiently these features throughout Tylenchida.

Although further investigations of the tylenchid corpus may reveal homologies of the metastegostom and telostegostom, we suggest that, if present at all, these are not components of the stomatostylet. Reviewing TEM of a moulting tylenchid (Endo, 1985) suggests that during development the stylet knobs and protractors develop in pharyngeal tissue independent from other more anterior components of the stylet (our interpretation of published micrographs). Our new interpretation of homology recognises the consistent position of the dorsal gland orifice posterior to the stylet knobs and m1 muscles (Tylenchida) and homologue to the stylet knobs (m1 muscle region) in Cephalobina (Fig. 2). This new hypothesis contrasts with previous, less parsimonious hypotheses, where the dorsal gland orifice in Cephalobina occurs well anterior to the putative homologue of the stylet knobs (Thorne, 1961; Andr assy, 1962; Goodey, 1963) (Fig. 1).

STYLET CONE AND SHAFT, GYMNSTOM

In Cephalobina, the gymnoston is enclosed by thickened cuticle surrounded by a stack of two arcade syncytial rings that occur anterior to the stegostom and the pharynx (Fig. 2A) (De Ley *et al.*, 1995). As in Rhabditina, the arcade cytoplasm is characterised (between moults) by multiple small vesicles and the near absence of mitochondria, endoplasmic reticulum, and other organelles (Wright & Thomson, 1981). Junctional complexes occur between the two arcade syncytia. Posteriorly, at the point of attachment and junctional complex between the second arcade syn-

cytium and the stegostom, there is a distinctive electron dense band of tonofilaments (Fig. 2A). Delimiting the anterior end of the gymnoston, a junctional complex occurs between the anterior arcade syncytium and adjacent epidermal syncytium of the cheilostom. The transition is further distinguished by an abrupt discontinuity and change in associated cuticle lining of the stoma, the cuticle anterior to this boundary being a labial invagination continuous with the body wall and separate from the cuticle lining the gymnoston, stegostom and pharynx (De Ley *et al.*, 1995). The cuticle lining associated with the gymnoston protrudes anteriorly to the gymnoston and into the cheilostom lumen (Van de Velde *et al.*, 1994; Baldwin & Eddleman, 1995; De Ley *et al.*, 1995) (Figs 2A; 4A). This extension of the cuticle associated with the gymnoston protruding into the cheilostom lumen occurs in other Secernentea, and is particularly striking in Diplogastrina (Baldwin *et al.*, 1997).

Herein we hypothesise that the cone and shaft of the tylenchid stylet together represent the homologue of the cuticle lining/lumen of the gymnoston (Fig. 1). Although the stylet may extend far anteriorly (where it moves freely through the guiding apparatus), it is significant that its anterior point of attachment is with arcade syncytial tissue that surrounds the shaft (Figs 2B; 3D, E). As in Cephalobina this arcade syncytial tissue includes vesicles, but between moults has very few mitochondria, endoplasmic reticula, and other organelles. Posteriorly, this arcade tissue forms, in conjunction with abundant tonofilaments, a junctional complex with the stegostom (consisting of protractor muscles and marginal cells) analogous to that observed between the gymnoston and stegostom in Cephalobina (Figs 2; 3A-C). What is visible with light microscopy as the guide ring that

Fig. 4. Transverse transmission electron micrographs through the cheilostom and gymnoston of representatives of Cephalobina and Tylenchida. A: *Zeldia punctata* during fourth moult, showing a portion of the cheilostom (c) and surrounding epidermal syncytium (es). Within the stoma is a portion of the adult and moulting cuticle of the fourth-stage, protruding from the more posterior gymnoston (g). Arrow indicates stoma space between the cheilostom and gymnoston cuticle (l = lumen of the gymnoston); B: *Acrobeles complexus* female showing vestibule of the cheilostom (c) and its relationship to the hexaradiate framework (f) that is surrounded by epidermal syncytium (es). Cheilostom lumen (l) encloses an anterior protrusion of the more posterior gymnoston (g); C: Anterior terminus of *Zeldia punctata* female including a portion of the cheilostom (c) surrounding the stoma lumen (l). The very shallow cephalic framework (f) is surrounded by the epidermal syncytium (es); D: Anterior portion of vestibule extension (ve) in *Meloidogyne incognita* male. Vestibule extension is enclosed by epidermal syncytium that includes mitochondria (mt) and extends between branches of the protractor cells (m1) that extend anteriorly from the stegostom. Arrow indicates stoma space between the cheilostom and stylet cone (sc) cuticle. (Abbreviation: l = lumen of the stylet cone); E: Cephalic framework (f) of *M. incognita* male. Vestibule (v) encloses stylet cone (sc). (Abbreviation: es = epidermal syncytium); F: A portion of the cephalic framework (f) slightly anterior to that shown in D. Epidermal syncytium (hs) includes large mitochondria (mt). Arrow indicates stoma space between the vestibule (v) and stylet cone (sc). Figures D and E printed from TEM negatives, parts of which are also used in Baldwin and Hirschmann (1976).

surrounds the stylet shaft is known from TEM to be a ring of folded junctional complexes between syncytia. These are considered analogous to junctional complexes characterising the gymnostom and form between the epidermal and anterior arcade syncytia as well as between the two arcade syncytia in *Cephalobina* (Figs 2; 3D, E).

The stylet cone and shaft, originating within the gymnostom and extending anteriorly into the cheilostom as the stylet (gymnostom cuticle) elongates, is reminiscent of a similar morphology in *Cephalobina* and other Secernentea (see above). This interpretation appears to be consistent with light microscope observations of stylet formation during moulting (Roman & Hirschmann, 1969) and with TEM observations of cone and shaft formation within two arcade syncytia. These two syncytia lie posterior to the developing guiding apparatus* (cheilostom) through which the stylet extends, but to which it remains unattached (Endo, 1985).

VESTIBULE, VESTIBULE EXTENSION, CHEILOSTOM

The cheilostom of *Cephalobina* occurs anterior to the gymnostom and may also enclose the anterior extension of the gymnostom (Figs 2A; 4A). Since the cuticle lining of the cheilostom is continuous with that of the body wall, the underlying tissue is epidermis, as is also the case for the body wall. Reconstruction through 3D modelling of cells and syncytia of the anterior end of *Cephalobina* suggest that the epidermis of the head region primarily consists of two, highly convoluted and interdigitating, syncytial rings (Bumbarger & Baldwin, unpubl.). Anteriorly, the cheilostom region of *Cephalobina* is surrounded by a hexaradial cuticular framework, although in some taxa this framework may be very shallow and may follow the dome shape of the anterior end of the head (Fig. 4B, C).

In Tylenchida, we hypothesise that the cheilostom is represented by the guiding apparatus and associated structures, including the vestibule, framework, and vestibule extension, all of which are continuous with the body wall cuticle. The vestibule and framework are similar in form to that of *Cephalobina*, although in Tylenchida they are typically much more elongate along the anterior-posterior axis (Figs 2; 4B-E). The vestibule extension is continuous with, and extends posteriorly to, the vestibule (Figs 2B; 4D). The syncytium enclosing the guiding apparatus includes some large mitochondria and is similar in this respect to the labial epidermis of other Secernentea (Wright & Thomson, 1981) (Fig. 4D, F). It pervades

* Equivalent to stomatal wall in Endo (1985).

the region above the framework, forming a sheath around the vestibule extension that extends between the anterior branches of the stylet protractors (Figs 2B; 4D-F).

Conclusions

Relative to the simple stoma of most *Cephalobina*, the stomatostylet of Tylenchida is extremely complex. Whereas homologies are difficult to establish based on light microscopy alone, a re-evaluation of TEM of Tylenchida, in conjunction with a newer understanding of the stoma structure in *Cephalobina*, has provided a basis for plausible hypotheses of homology of cheilostom, gymnostom and prostegostom between the two groups. Further testing of the hypotheses requires clearer phylogenetic resolution. This resolution provides the basis for interpreting representatives used as morphological models to address questions such as that of the independent evolution of the stylet in Tylenchida and some Aphelenchida (Baldwin *et al.*, 2004). Due to the highly derived morphology of the tylenchid stylet (and the possibility that it has further diverged within the tylenchids), it is proposed that a choice of taxa basal to the tylenchid clade, such as *Aphelenchus* (see Blaxter *et al.*, 1998 and Baldwin *et al.*, 2004 for phylogenies including this genus), a choice that would be made in the light of more clearly resolved phylogenies, might be most informative for comparison with *Cephalobina*.

Other approaches for testing the hypotheses include 3D ultrastructural and developmental reconstruction of representatives of Tylenchida and outgroups. Particularly complex tissues include the epidermal and arcade syncytia, their junctional complexes and tonofilament-rich connections with cuticle, and the relationship of all these structures during stoma/stomatostylet development. It is not known if some cells that participate during stoma/stomatostylet formation undergo transformation or death in the adult, perhaps obscuring their relationship to the completed structures as seen between moults.

Acknowledgements

The authors acknowledge electron micrographs archived and used for comparative reconstruction in the Baldwin laboratory, including those from previous work by Drs Dolinski, Zhang and Eddleman.

This work was supported by the US National Science Foundation grants DEB-0228692, DEB-9712355 and DEB-0206569.

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