14 General Morphology of Cyst Nematodes

James G. Baldwin[1] and Zafar A. Handoo[2]
[1]*University of California, Riverside, California, USA;* [2]*USDA, ARS, BARC-West, Beltsville, Maryland, USA*

14.1 Introduction

Within Tylenchomorpha, including most plant-parasitic nematodes, cyst nematodes (Heteroderinae) are morphologically distinctive among Hoplolaimidae[1] consistent with adaptations for sedentary parasitism and the capacity for dormancy or suspended development. Most important among these adaptations is the cyst, a structure that evolved within heteroderids[2] and that has been defined as 'a persistent tanned sac which retains eggs and is derived from some or all components of the mature female body wall' (Luc *et al.*, 1986). Although this type of cyst is unique to heteroderids, convergent morphological adaptations for sedentary parasitism throughout diverse groups of Tylenchomorpha, including *Meloidogyne*, *Rotylenchulus*, *Tylenchulus* and *Nacobbus*, have been the basis for many misunderstandings of relationships. However, these controversies have been largely addressed by molecular phylogenetics and by detailed morphology that underscores the uniqueness of features particular to cyst nematodes of Heteroderinae. These unique features throughout the group point to specialized functions and taxon-specific expressions that are crucial for classical identification and for understanding phenotypic evolution of the group (Baldwin and Mundo-Ocampo, 1991).

The life history of heteroderids, described in detail by Raski (1950), is consistent with other

[1] We follow De Ley and Blaxter (2002) with placement of Heteroderinae within Hoplolaimidae.
[2] Although some heteroderids (Heteroderinae) lack cysts, this feature is pleisiomorphic within the group (Baldwin and Schouest, 1990; Baldwin, 1992).

Tylenchomorpha with respect to having five (juvenile and adult) stages separated by four moults (Fig. 14.1). The embryo, first stage and first moult occur exclusively within the egg and, potentially following a period of dormancy, hatching is as an infective second-stage juvenile (J2) (see Chapter 3, this volume). While J2 destined to be males or females all include a body wall, digestive, reproductive, nervous and secretory-excretory system, these systems differ in J2 and subsequent stages based on adaptations of particular phases of the life history with respect to the stage being active or dormant, migratory or sedentary, sexually immature or mature and parasitic or free-living. Once the J2 establishes a feeding site in the host the J2 begins to swell and then undergoes successive moults. Adults are sedentary obese egg-producing females and, in most cases, also present are migratory non-feeding males. Earlier juvenile stages developing as males are swollen and sedentary but development proceeds with metamorphosis to a vermiform fourth-stage juvenile (J4) coiled within the sac-like ovoid casing of earlier stages from which it emerges as an elongate migratory adult (Figs 14.1 and 14.7). Adult males do not feed and die soon after mating. Adult females mature, die and transform into cysts.

14.2 Egg and Embryo

Eggs vary little among Heteroderinae and generally they are not distinctive from those of other Tylenchomorpha. Although suggestions of taxon-specific variability in size are often included in species descriptions, studies suggest size is probably influenced by external factors and it has not proven to be useful to identification. Notably some species of *Cactodera* (Heteroderinae) have diagnostic surface punctations, as seen with light microscopy, and they are further

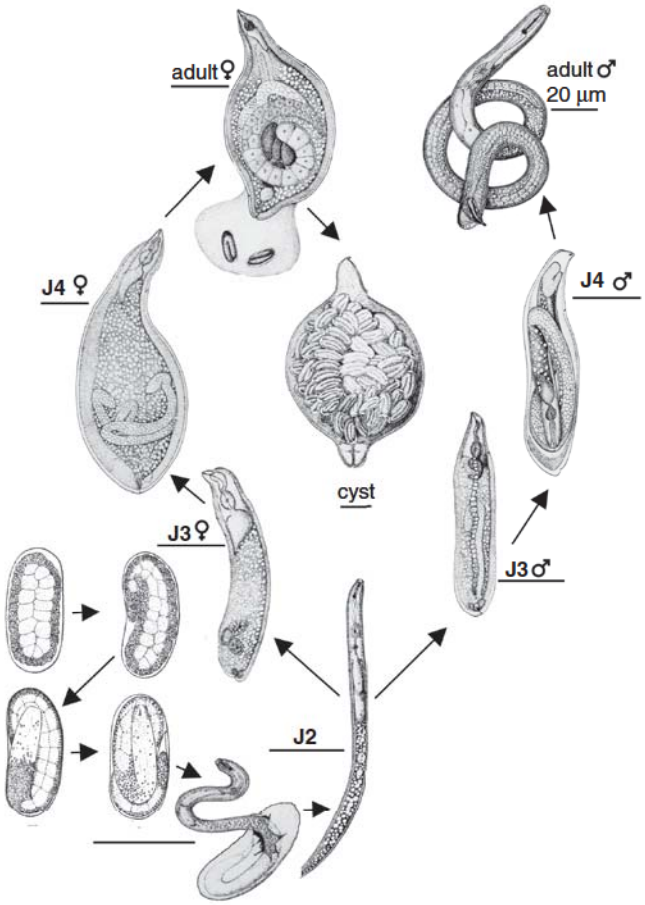

Fig. 14.1. Life cycle of cyst nematode (*Heterodera schachtii*) on sugar beet. (Adapted from C. Papp in Raski, 1950.)

shown by scanning electron microscopy (SEM) to be tubercle-like protuberances. Eggs are transparent such that within them embryonic, first-stage juveniles (J1) and pre-hatch J2 can be examined (Figs 14.1 and 14.2B). Within the egg, the J1 has three to five folds and not until after the first moult does a stylet form and cuticle striation is expressed.

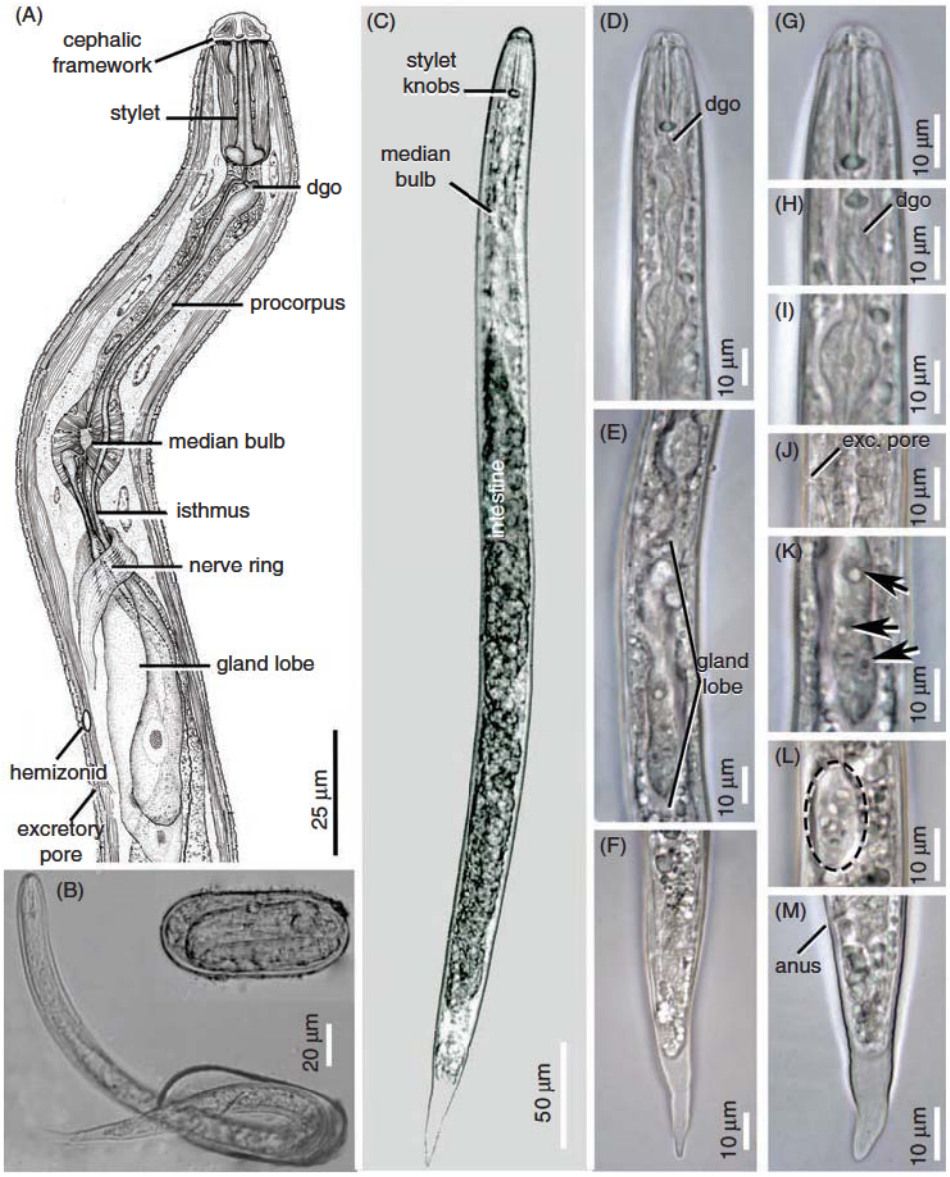

Fig. 14.2. Second-stage juvenile of cyst nematodes. (A) Anterior end including pharyngeal region of *Heterodera glycines* (modified from Endo, 1979, courtesy of USDA Nematode Laboratory). (B) Egg and hatching juvenile of *H. sojae* (courtesy of Kang *et al*., 2016 and *Journal of Nematology*). (C) Entire juvenile, lateral view. (D) Anterior end including median bulb. (E) Pharyngeal region including gland lobe. (F) Tail region showing hyaline tip. (G) Stylet enlarged from 2D. (H) Enlargement of dorsal gland orifice (dgo) from 2D. (I) Median bulb enlarged from 2D. (J) Excretory pore. (K) Gland lobe showing position of nuclei enlarged from 2E. (L) Primordium. (M) Tail region showing anus.

14.3 Second- to Fourth-stage Juveniles

Eggs with J2 ready to hatch may occur within cysts, or in some species they may also be deposited within eggsacs (Figs 14.1 and 14.6F); after hatching J2 may be found within host roots or in the soil. Recognition of distinctive heteroderid J2 in the soil is often the 'first alert' to then examine adjacent roots for the association of females and cysts and the more specific morphological identification they afford. Where dormancy occurs, it is as the pre-hatch J2 within the cyst (see Chapter 3, this volume). Upon hatching the J2 morphology, including somatic musculature and cuticular features conferring strength/flexibility, reflects the temporary role of the J2 as a free-living migratory stage, whereas its fully formed protrusible stylet and robust digestive system are indicative of an infective role in penetrating the host and establishing a feeding site (Fig. 14.3). Once a feeding site is established the J2 becomes sedentary and undergoes significant changes as it swells and commences development of the reproductive system, and the rapid sequence of moults through J3 and J4 to the adult. Generation time is highly variable among species and dependent on environmental conditions, but often it is completed in less than 30 days.

The heteroderid J2 is distinguished by its size of 300–700 µm, overall shape including a rounded head continuous with the body contour and tail that tapers to a point; the tail also includes a notable hyaline region, the presence of which is

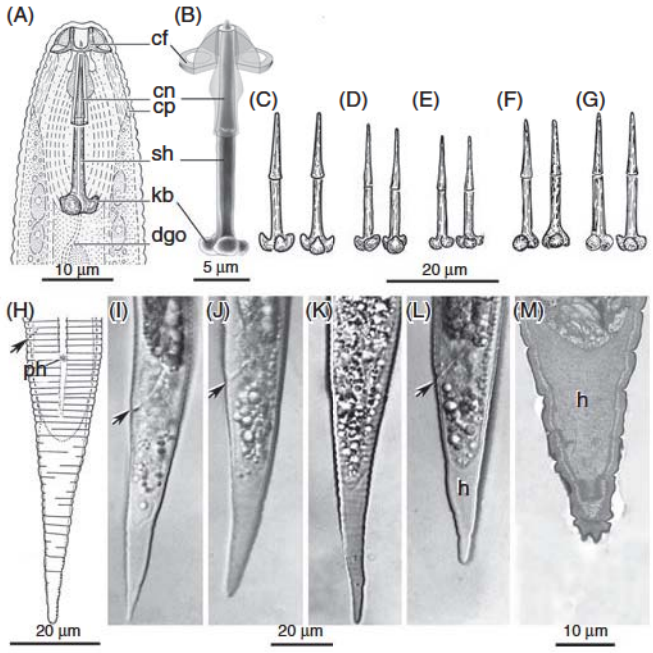

Fig. 14.3. The stylet and tail region of second-stage juveniles. (A) Diagrammatic interpretation of stylet components (after Hirschmann, 1956). (B) 3D dorsal view of stylet in relation to labial framework and vestibule extension (after Baldwin from Subbotin *et al.*, 2010). (C–G) Diagrammatic interpretation of stylets (left is lateral view, right is dorsal view). (C) *Heterodera mani*. (D) *H. trifolii.* (E) *H. zeae* stylet. (F) *Globodera rostochiensis*. (G) *G. pallida*. (H) Diagrammatic interpretation of tail (after Wouts and Weischer, 1977). (I) *H. zeae* tail. (J) *H. schachtii* tail. (K) *H. trifolii* tail. (L) *H. glycines* tail. (M) Transmission electron micrograph of *H. glycines* tail (after Endo, courtesy USDA Nematode Laboratory). Arrow indicates position of anus. Abbreviations: cf = cephalic framework, cn = cone of stylet, cp = cephalid, dgo = dorsal gland orifice, sh = shaft of stylet, h = hyaline region of tail, kb = knobs of stylet, ph = phasmid. C–G after Wouts and Baldwin (1998), I–L after Mulvey and Golden, 1983, courtesy of USDA Nematology Laboratory and *Journal of Nematology*.

distinctive in heteroderids relative to outgroups. The size and shape, tail and hyaline region length may be useful in species diagnostics (Fig. 14.3). The body wall of the J2 comprises an outer layer of cuticle underlain by epidermis (= hypodermis) and internally by a layer of platymyarian somatic muscles (Fig. 14.4). The cuticle is superficially striated and striae are interrupted on each lateral side with a field (lateral field) marked by what appears in the light microscope as the diagnostic feature of three *vs* four longitudinal lines, the 'lines' being an expression of incisures between alae (in this case small longitudinal elevations) (Figs 14.4 and 14.5I, J). Interspecific variations in the patterns include those of the anterior and posterior termini and extent of areolation of lateral lines. Post infection, cuticular striations diminish through developmental stages and lateral

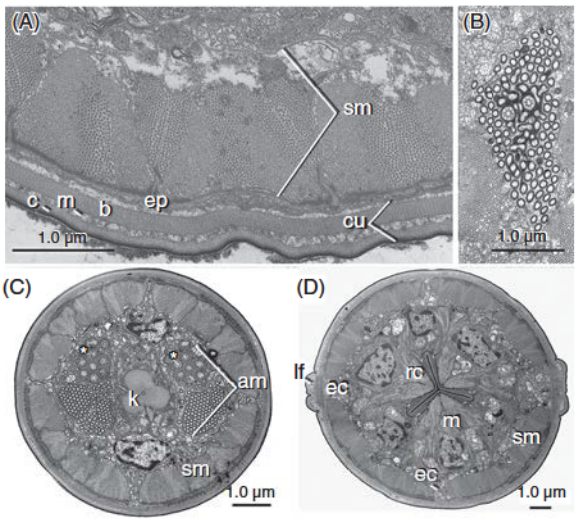

Fig. 14.4. Transmission electron micrographs of transverse sections of second-stage juveniles of *Heterodera glycines* (after Endo, courtesy of USDA Nematology Laboratory). (A) Body wall showing platymyarian somatic muscles (sm), epidermis (ep) and cuticle (cu) comprising cortex (c), medial (m) and basal (b) layers. (B) amphid including microvilli of the finger cell. (C) Level of the stylet knobs (k) including amphids (am). Asterisks (*) denote position of ciliary region of amphid sensilla. (D) Level of the median bulb including radial muscle (rc) and marginal (m) cells. Note epidermal chords (ec) and lateral field (lf).

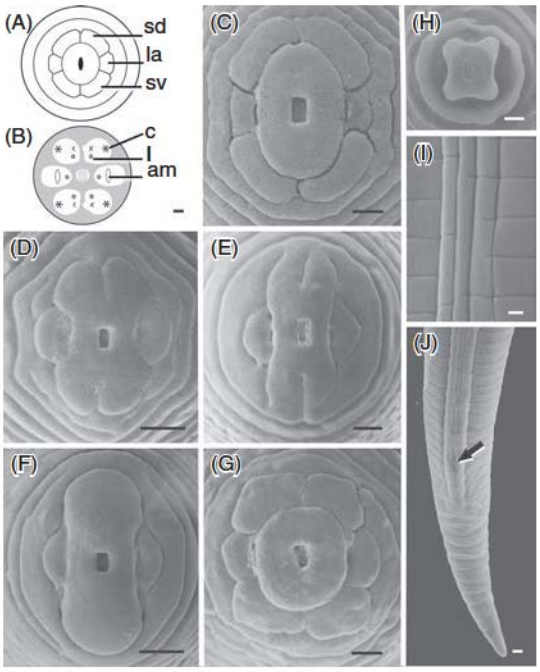

Fig. 14.5. Surface patterns of second-stage juveniles, unless otherwise indicated. (A) Diagrammatic representation of the basic pattern of the lip region including labial (la), subdorsal (sd) and subventral (sv) lips. (B) Transverse view of sensilla of the labial region including cephalic (c) and labial (l) papillae and amphid (am) opening. (C) *Cactodera cacti* lip pattern. (D) *Heterodera glycines* lip pattern. (E) *H. trifolii* lip pattern. (F) *H. avenae* lip pattern. (G) *H*: *cruciferae* lip pattern. (H) female lip pattern of *H. glycines*. (I) Lateral field of *Punctodera chalcoensis*. (J) Lateral view of tail of *H. fici.* Arrow indicates phasmid opening. (A–D, F–J after Baldwin in Subbotin *et al*., 2010). All scale bars are 1.0 µm.

lines are lost early in the sedentary transformations. By J3 there is evidence of transformation of surface patterns from striated towards the more irregular patterns characteristic of adults (Hesling, 1978).

Transmission electron microscopy (TEM) reveals that the body wall cuticle comprises three basic layers of a cortex (with a thin osmophilic external region), a 'spongy' medial and an internal striated basal layer; this basic three-layered pattern, widespread throughout the Chromodorea, is interpreted as plesiomorphic (Fig. 14.4A) (Baldwin, 1992). The three cuticle layers persist through the sedentary J2 and J3, but it is unknown to what extent they are expressed through the J4 in transition to the highly modified cuticle that characterizes adult females.

A key expression of surface cuticular patterns in J2 is that of the lip region, particularly as viewed with SEM, and specific variations of these patterns often have diagnostic value (Fig. 14.5) (Stone, 1975; Othman *et al*., 1988; Baldwin and Schouest, 1990). Components of these patterns are demarcated by lines of shallow indentations. The basic lip pattern, widespread throughout Heteroderinae, is a labial disc surrounded by six lip sectors, two lateral as well as subdorsal and subventral pairs (Fig. 14.5A). Variations occur through partial or complete fusion of the labial disc with certain lip sectors and/or with fusion of adjacent lip sectors (Fig. 14.5C–H). The head region also varies by the number of additional annules as well as the possibility of partial or complete additional longitudinal incisures posterior to the lips.

The cellular/syncytial epidermal layer underlies the cuticle and during moults it plays a key role in dissolution and secretion of the cuticle (Fig. 14.4C). It occurs as a thin 'interchordal' region and especially laterally, as well as (to a lesser extent) dorsally and ventrally, it expands to thickened chords that extend most of the length of the J2 (Fig. 14.4D). Cuticle adjacent to lateral chords and expressing the lateral field may be slightly thickened and layering may be modified. Anteriorly the epidermis underlies and secretes a cuticular cephalic framework (see description below) surrounding the stoma creating a conduit for the protrusible stylet and anterior point of attachment of stylet protractors (Figs 14.2, 14.3A, B and 14.5B). Posteriorly, the epidermis extends to the tail tip but in the J2 it terminates just anterior to the cuticular hyaline region (Fig. 14.3H–M). The epidermis, particularly of J2, is closely associated with specialized cells that form cuticularized ducts that penetrate the cuticle including the secretory-excretory duct and the rectum/anus, as well as with the socket cells that form cuticular connections of sensory structures. During the J4 and final moult the epidermis plays a key role in formation of secondary sexual cuticular structures associated with the developing vulva/vagina and male cloaca.

The innermost component of the body wall is the single layer of longitudinally spindle-shaped overlapping platymyarian somatic muscle cells organized in four fields separated by the epidermal chords (Fig. 14.4A–C). As is the case for platymyarian muscles, the contractile region underlies the epidermis, whereas a non-contractile region including the cell nucleus expands into the body cavity and one or more innervation processes extend from the non-contractile region to synapses with neurons. The contractile region is composed of bands of thin and thick myofilaments organized consistent with obliquely striated musculature (Fig. 14.4A). Whilst somatic muscles are clearly essential during the migratory stages, once a feeding site is established somatic musculature deteriorates apparently then being primarily limited to the head region of feeding stages and subsequently being re-established in migratory males.

The digestive system, including the stomatostylet, pharynx, intestine, rectum and anus, is well-formed beginning in the J2 infective stage (Fig. 14.2). Detailed information on digestive and other systems are given in an excellent book chapter 'Nematode morphology, sensory structure and function', by Baldwin and Perry (2004). The stylet, appearing in the first moult primarily as a product of arcade syncytia, is an expression of the stoma as a protrusible needle-like cuticular structure that includes a lumen through which food passes posteriorly into the pharynx. However, prior to a role in feeding, the J2 stylet has an important role in perforating the egg shell and hatching (Doncaster and Shepherd, 1967; see Chapter 3, this volume). The J2 stylet is somewhat flexible (Zunke and Eisenback, 1998), but how this flexibility functions in relation to its role in hatching or feeding needs to be further explored. Anteriorly the stylet is a cone that tapers to a point and thus is adapted to

penetrating the egg shell and then host cells (Fig. 14.2A, C, D, G and 14.3A–G). Posterior to the cone the stylet also includes a columnar shaft that further posteriorly merges into three (dorsal and two subventral) enlarged knobs (Fig. 14.4C). The stylet knobs are embedded in and produced by the anterior end of the pharynx from which three corresponding stylet protractor muscles extend anteriorly to the cuticularized cephalic framework and body wall. Contraction of the protractors moves the stylet anteriorly through the guiding cuticular conduit including a tube-like vestibule that posteriorly is continuous with a vestibule extension; the vestibule, lying just posterior to the lip region and stoma opening, is anchored by six cuticular radii extending from the vestibule/hub to the body wall and it is the combination of the vestibule and radii that comprise the cephalic framework. Contraction of stylet protractors counteracts elasticity of the adjacent alimentary tract; thus, the stylet retracts when protractors relax. Details of the J2 stylet may be useful in species diagnosis. These include stylet length, from 16–28 µm, overall robustness, and details of the size and shape of stylet knobs, including rounded or anchor-shaped (Fig. 14.3A–G). These features, however, often vary intra-specifically so that assessing differences typically requires examining a significant number of individuals and consideration in the context of additional diagnostic features.

Posterior to the stylet is the pharynx, including corpus, isthmus and glandular lobe; the corpus is further divided into a procorpus and metacorpus (Fig. 14.2). The lumen of the stylet is continuous with the cuticularized lumen of the pharynx; the stylet protractor muscles posteriorly are expressed as non-contractile regions that primarily comprise the procorpus. Within the procorpus, which is non-muscular, the lumen is round in cross-section and anteriorly it is penetrated by a cuticularized dorsal gland duct (dgo) (Figs 14.2A, D and 14.3A). This duct is the anterior terminus from the gland lobe and its position relative to the base of the stylet knobs (4–12 µm) varies among species and thus has diagnostic value.

Posterior to the procorpus the corpus enlarges to an oval muscular median bulb (metacorpus) and its anterior end is demarcated by 'constraining muscles' (Endo, 1984) (Fig. 14.2A, I). Within the median bulb the cuticularized lumen is triradiate and surrounded by non-muscular marginal cells peripheral to the apicies. Between the apicies and marginal cells are muscular radial cells oriented such that upon contraction they open the triradiate lumen (Fig. 14.4C). This opening creates pressure that results in ingesting food through the stylet and pharynx lumen. In subventral positions near the posterior end of the median bulb are a pair of cuticularized ducts associated with anterior processes of subventral glands; these open into the lumen of the median bulb. Relative to J2 the median bulb increases in size in J3, J4 and it reaches maximum size in adult females (Fig. 14.6A, B).

The median bulb, with its triradiate lumen, posteriorly merges with the narrower elongate isthmus that is primarily comprised of dorsal and subventral gland processes embedded within a framework of constraining and radial muscles (Fig. 14.2A). Associated with radial muscles, the cuticularized lumen of the isthmus is triradiate suggesting that, as in the median bulb, the lumen can be opened by contracting muscles. The pharyngeal lumen extends posteriorly into the gland lobe where it merges with the intestinal lumen. The transition with the lumen is a pair of cells that comprise the pharyngeal-intestinal valve. Within the gland lobe the dorsal gland predominates anteriorly and its large nucleus typically occurs at about the level of the pharyngeal-intestinal valve. Posteriorly, the lobe is primarily composed of a pair of subventral glands and typically the nucleus of one subventral gland is positioned anteriorly to the other (Fig. 14.2K). All the pharyngeal glands appear to be robust and functional in feeding juvenile stages although they may change in size and shape; Endo (1984) describes changes in apparent activity of subventral glands in the transition from J2 to J3.

The cuticle lining of the digestive system ends posteriorly at the pharyngeal-intestinal valve where it transitions to the intestinal lumen. The intestine is composed of large epithelial cells and in cyst nematodes the lumen is poorly defined; unlike most other nematodes it apparently lacks a border of microvilli (Endo, 1988; Borgonie *et al.*, 1995). The intestine terminates posteriorly in a cuticle-lined rectum and anus that appears to be present and functional throughout all stages (Figs 14.2A and 14.3H–J, L); an exception is the last steps of the J4 moult to the male in which the anus is not observed and the region is

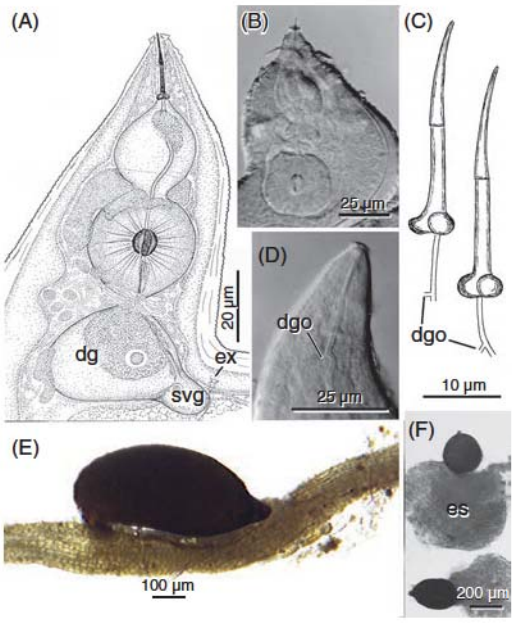

Fig. 14.6. Females of cyst nematodes. (A) Diagrammatic representation of anterior end of *Vittatidera zeaphila* showing pharynx including dorsal (dg) and subventral (svg) glands in relation to excretory pore (ex). Modified from Bernard *et al*. (2010), courtesy of *Journal of Nematology*. (B) Anterior end of female (*Heterodera zeae*) including pharyngeal bulb. (C) Diagrammatic representation of lateral view of stylet showing variation in shape (left, *H. trifolii*; right, *H. galeopsidis*; after Hirschmann and Triantaphyllou, 1979). (D) Anterior end of female (*H. zeae*) including dorsal gland orifice (dgo) (photographs for B and D modified from Mulvey and Golden (1983), courtesy of USDA Nematology Laboratory and *Journal of Nematology*). (E) Feeding on root surface *(H. goldeni)*. (F) Female of *V. zeaphila* with large eggsac (es).

transformed by the development of a cloaca and male secondary accessory structures.

The reproductive system of the infective J2 is an oval primordium positioned about in mid-body (Fig. 14.2L). It is composed of a sheath of two epithelial (cap) cells that enclose a pair of germ cells. By J3 of developing females the primordium develops into two gonad branches that become anteriorly directed as they migrate posteriorly in the J4 and attach to the developing vagina and vulva by the final moult. However, in males only a single gonad branch develops and by the final moult it has migrated posteriorly, joining with the cloaca lining and associated accessory structures (Raski, 1950) (Fig. 14.7).

The nervous system of Heteroderinae, as understood by light microscopy, is apparently conserved among species and is not known to vary among juvenile stages with respect to a nerve ring encircling the anterior portion of the isthmus as well as labial and caudal sensory organs (Figs 14.2A, 14.3H, 14.4B, C and 14.5B, J). Additional commissures are embedded within the interchordal epidermis and these may be characterized, including in J2, as hemizonid (anterior to the excretory pore), hemizonion (posterior to the excretory pore), cephalids (near the lip region) and caudalids (in the tail region) (Hirschmann, 1956) (Figs 14.2A and 14.3A). Although the position of these structures relative to numbers of annules is often reported in descriptions, they are not known to be of reliable diagnostic value. Additional commissures occur surrounding the rectum and within the pharynx, but these are not readily observed by light microscopy.

Surrounding the stoma opening are six inner labial sensilla expressed with external openings and thus presumed to be chemoreceptive. In addition four slightly elevated cephalic papillae, probably mechanoreceptors, occur peripherally and submedially in the lip region together with a pair of lateral oval amphid openings (Fig. 14.5B). Each amphid opening leads posteriorly to a cuticle-lined canal with seven sensilla receptors and enclosed by a socket cell. Posteriorly the sensilla of each amphid are embedded in a sheath cell that also encloses two finger cells, characterized by microvilli termini and presumed to be thermal receptors (Fig. 14.4B, C). In the tail region of J2 a pair of phasmids, each with a single opening occurring on each lateral side, are the predominant sensory organs (Figs 14.3H and 14.5J). These include a single sensillum that penetrates a duct, opening to the outside (Baldwin, 1985). In some cases near the opening, the duct has a small ampulla that is visible with light microscopy as a conspicuous 'lens-like' structure,

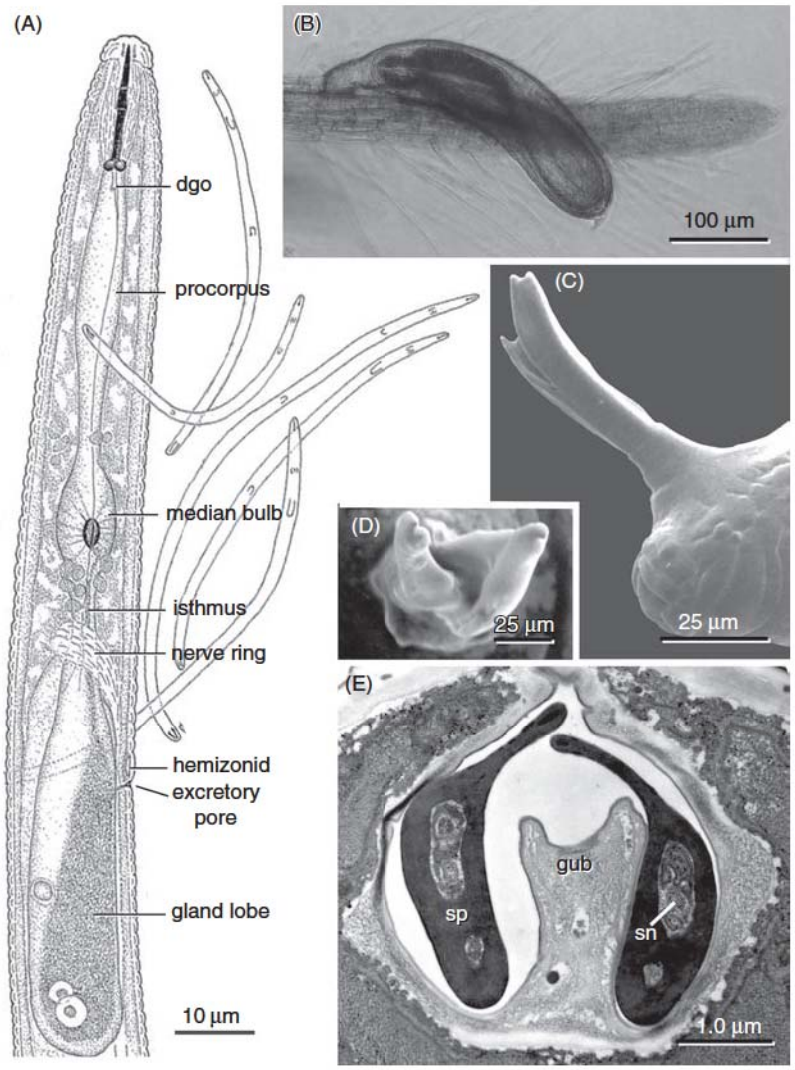

Fig. 14.7. Males of cyst nematodes. (A) Anterior end of male of *Vittatidera zeaphila* (modified after Bernard *et al*. (2010), courtesy of *Journal of Nematology*). (B) Fourth-stage male (*Heterodera schachtii*) (by permission, Ole Becker). (C) Scanning electron micrograph of lateral view of spicules showing bifid tip (*H. schachtii*). (D) Scanning electron micrograph of ventral view of spicule tips (*H. schachtii*). (E) Transmission electron micrograph of transverse view of spicules (sp) with embedded sensilla (sn) and in relation to gubernaculum (gub).

but more often the opening is small; some species are characterized by having obscure (absent?) phasmid openings. Although phasmids are expressed in J2, there is evidence that they deteriorate once a feeding site is established and they are not expressed in J3 and J4 (Carta and Baldwin, 1990). In J2 the position of the phasmids relative to the anus and tail terminus may vary among species and this position may have some diagnostic value.

The secretory-excretory system in J2 is expressed as a ventral cuticle-lined duct opening at about the level of the pharyngeal isthmus (Fig. 14.2A), although the position varies more specifically among species and in some cases it may have diagnostic value in both J2 and adults.

The duct leads to a cell body and single lateral canal, the latter extending in association throughout much of the length of the lateral chord. The status of the excretory system, including the canal and pore, is not known in J3 and J4.

14.4 Males

Most species of Heteroderinae, reproducing by amphimixis, have abundant males; more rarely species are facultative parthenogenetic such that males are rare (Triantaphyllou and Hirschmann, 1980). As is the case for other taxa of Tylenchomorpha with enlarged sedentary adult females, Heteroderinae is characterized by a striking level of sexual dimorphism (Figs 14.1, 14.7 and 14.8). As noted above, in cyst nematodes once J2 establish a feeding site, subsequent juvenile stages are sedentary until the final moult in which the J4 undergoes a metamorphosis resulting in the migratory adult male emerging from the cuticle of the sedentary juvenile stage (Figs 14.1 and 14.7). These males are vermiform with little tapering

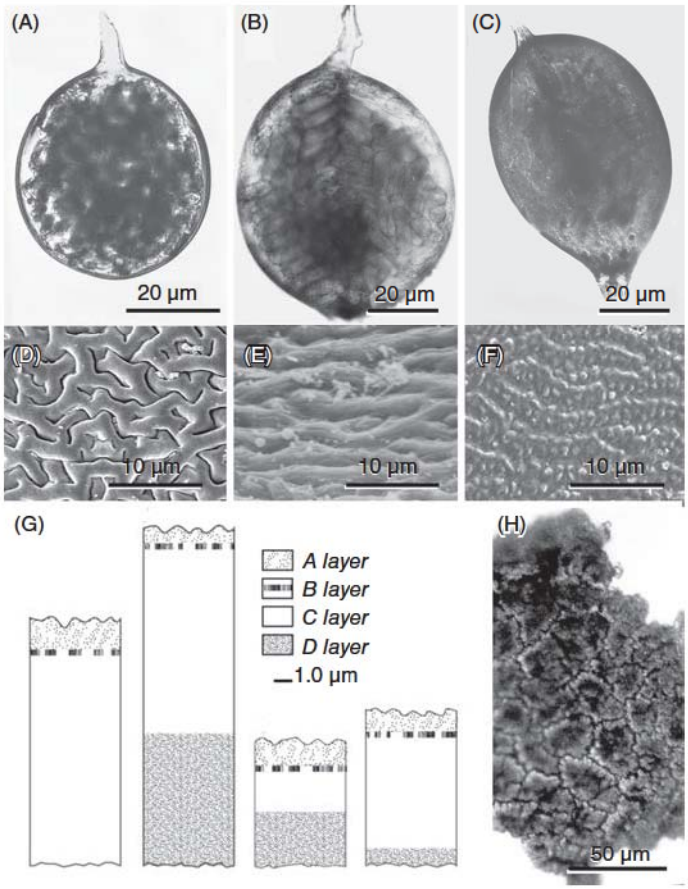

Fig. 14.8. Features of cysts. (A–C) Profiles. (A) Spherical lacking cone. (B) Nearly spherical but with small cone. (C) Lemon-shaped with pronounced cone. (D–F) Scanning electron micrographs of cyst surface patterns. (D) Rugose from mid-body (*Globodera tabacum*). (E) Striated from mid-body (*Punctodera punctata*). (F) Punctate posterior to mid-body (*G. tabacum*) of zigzag cuticular pattern. (G) Diagrammatic representation of cuticular layering without (far left) and with D layer. (H) subcrystalline layer (*Heterodera avenae*). (A–C and H by Mulvey and Golden (1983), courtesy of USDA Nematode Laboratory and *Journal of Nematology*; D–G after Subbotin *et al.* (2010).)

along the body length and rounded at both the anterior and posterior end. They are highly variable in size, even within a given species or isolate, but generally they range from 900–1600 µm. Males are present in most species of cyst nematodes and, in contrast to stationary females, they must be mobile for successful mating. They live about 10 days and are commonly found in soil or within the gelatinous matrix of an eggsac at the posterior terminus of the female.

The mobility of the males is reflected in the well-developed body wall where the cuticle, epidermis and platymyarian muscles are almost identical to those of J2. The lip patterns and lateral field with three to four lines and with or without areolation, are external expressions of the body wall. The tail region of males is characterized by a nearly terminal cuticle-lined cloacal opening through which protractable spicules may protrude (Fig. 14.7C–E), but otherwise the tail lacks caudal alae. This is in contrast to many species with vermiform females where in corresponding males caudal alae are present and may be functionally important in wrapping around the female for transferring sperm during copulation; in the case of the nearly globose sedentary females of cyst nematodes caudal alae would not likely to be functionally important. The nervous system of males is likely to play an important role in locating females and in mating. Morphologically, the system resembles that of J2 with respect to the nerve ring, hemizonids, hemizonian and other interchordal commissures as well as sensory organs of the lip region (Fig. 14.7A). In contrast to J2, phasmids apparently are not expressed in males of many species (Carta and Baldwin, 1990; Sturhan, 2016); however, males are distinctive by the presence of sensilla that extend within each spicule and that are exposed to the exterior through a minute pore (Clark *et al.*, 1973) (Fig. 14.7C). The excretory system, including the ventral position of the cuticle-lined pore, is not known to differ from that of the J2 (Fig. 14.7A).

Although males of cyst nematodes are not known to feed they have a well-developed digestive system (Fig. 14.7A). The cephalic framework and stylet are larger than those of J2 and, while the stylet may be used less often for diagnostics compared to J2, stylet length and shape of knobs may vary among species, as may the distance of the dorsal gland orifice from the base of the stylet knobs. Remarkably, considering the putative absence of feeding, the pharynx and pharyngeal-intestinal transition of males differs little from that of infective J2.

The male reproductive system includes a single testis that extends anteriorly from a vas deferens that connects posteriorly in a cloaca shared with the digestive system. Posteriorly the cloaca is enlarged to enclose a pair of spicules that protract and retract by attached muscles and are guided by a thickening of the dorsal wall, the gubernaculum (Fig. 14.7C–E). The gubernaculum includes a partition that separates the spicules from one another (Clark *et al.*, 1973). Spicules typically extend through a tubular sheath (tubus) and this sheath may vary with respect to its terminal position as well as the presence or absence of surface annulation. Each spicule has a concave surface that faces the other such that the two spicules together provide a conduit through which sperm pass. Spicules, while having a high degree of intraspecific variation, may vary among species with respect to overall length and details of the tip (Clark *et al.*, 1973) that may be rounded, flat, bifid or tridentate. Cyst sperm are amoeboid with filopodia and although diversity of form has not been broadly studied there is some evidence of morphological variation among taxa (Shepherd *et al.*, 1974; Walsh and Shepherd, 1983; Cares and Baldwin, 1995).

14.5 Females

Being sedentary and gradually enlarging from the infective feeding stage (J2) onwards, mature females are generally swollen/rounded with a narrow neck (Fig. 14.1). These white or cream-coloured enlarged females are typically found on the surface of host roots (Fig. 14.6E). Details of shape include various taxon-specific modifications; for example, a posterior cone may be present and prominent, reduced in prominence or absent (Fig. 14.8A–C). To some extent shape may be a function of body wall, including differential cuticle thickness, elasticity or composition in an individual body region, and/or the persistence, arrangement and points of attachment of vaginal muscles in the terminal region. Heteroderids with a prominent cone, such as *Heterodera schachtii* and *H. trifolii* are said to be lemon-shaped, whereas those that lack a cone, such as

Globodera and *Punctodera* are said to be globose; intermediates with small cones include, for example, *H. carotae*; *Cactodera* also typically have a small cone (Fig. 14.8A–C). Details of shape and size may be influenced by environmental factors including the host, nutrition, age and crowding; these variables may confound diagnostics, leading some to suggest that interspecific comparisons are best made across the largest individuals of a population (Hirschmann, 1956; Thorne, 1961).

In contrast to the body wall of vermiform juveniles and males, that of females is especially modified by increased thickness and complexity of the cuticle (Fig. 14.8G). Furthermore, body wall somatic musculature in these sedentary stages is mostly lost, being primarily limited to the somewhat-mobile head region (Shepherd and Clark, 1978). External to the cuticle, in some cases, as in certain species of *Heterodera*, presence of a 'white, flakey' subcrystalline layer is considered to be somewhat diagnostic (Liebscher, 1892; Fuchs, 1911; Kirjanova, 1969) (Fig. 14.8H). The origin of the layer has been controversial even including the question of whether or not it is of nematode or fungal origin (Brown *et al.*, 1971; Zunke, 1986; Endo and Wyss, 1992; Endo, 1993; Zunke and Eisenback, 1998).

The cuticle of mature white females retains the cortex, including typical outer and inner components. Underlying the cortex is what appears to be a homologue of the basal striated region that is recognized in J2 and males, but in females the layer is interrupted by regions that lack striation and these gaps in striations may facilitate expanding girth (Fig. 14.8G). Posterior to the basal region, the cuticle of mature females is primarily thickened by a deep homogeneous layer designated C and this pattern of cortex, basal and C layers is taxon-specific, characterizing, for example, females of all species examined of *Heterodera*. By contrast, species of *Globodera*, *Cactodera* and *Punctodera* all have an additional layer internal to the C layer. This layer, designated D, is composed of helical layers of fibres such that in transverse section they appear to form a herringbone pattern (Shepherd *et al.*, 1972; Cliff and Baldwin, 1985). These taxon-specific variations in cuticular layering of mature females are informative for interpreting phylogenetic relationships among heteroderids, including relationships between cyst- and non-cyst-forming taxa (Baldwin and Schouest, 1990). Notably these patterns first described by TEM can also be interpreted from light microscope sections and particularly so by staining with toluidine blue (Shepherd and Clark, 1978; Baldwin, 1983).

Cuticular surface patterns of mature females range from striations to variations of irregular patterns often designated by ambiguous and inconsistently applied descriptors, including rugose, zigzag, reticulate, striated, punctate or lace-like (Fig. 14.8D–F). These patterns show some species-specific distinctions of diagnostic value, but they also may be subject to intraspecific variability and even within an individual they may differ by position on the body and age of the female. For example, posterior to the head region they may be transverse ridges that at midbody are zigzag and further posteriorly, whorled ridges (Zunke and Eisenback, 1998). Cuticular surface patterns of the lip region of females include a somewhat irregular oblong to rectangular elevated labial disc (Fig. 14.5H). Surrounding and posterior to the disc the lips are fused to form a circular plate. Immediately posterior to the lips transverse rows of protuberances have been reported in females of *Globodera* but these were not observed in other genera of Heteroderinae (Othman *et al.*, 1988).

Surface patterns are modified relative to the rest of the body in the terminal region of females. In a region surrounding the vulva the cuticle is thin, composed of a loose mesh of fibres that eventually ruptures in the cyst-forming fenestrae. Cuticular patterns external to the thin region are distinctive among taxa (Fig. 14.9A–C) (Green, 1975; Mulvey and Golden, 1983). In globose females, such as *Globodera*, the area surrounding the vulva may become sunken and is aptly named the vulval basin demarcated by a thickened rim. A discrete region peripheral to the rim may be characterized by a distinctive pattern, in some cases including tubercles (Green, 1971) (Fig. 14.9C). By contrast, in the tip of the cone of mature lemon-shaped females the basin is distinguished by a region on each side of the vulva slit that in younger females typically includes a fine surface pattern of rivulets that are finer in texture relative to the overall cuticular pattern (Fig. 14.9A). As the lemon-shaped female matures the region immediately surrounding the vulval slit remains elevated as a vulval bridge, thus bisecting the sunken basin (Fig. 14.9B). The width and breadth of this

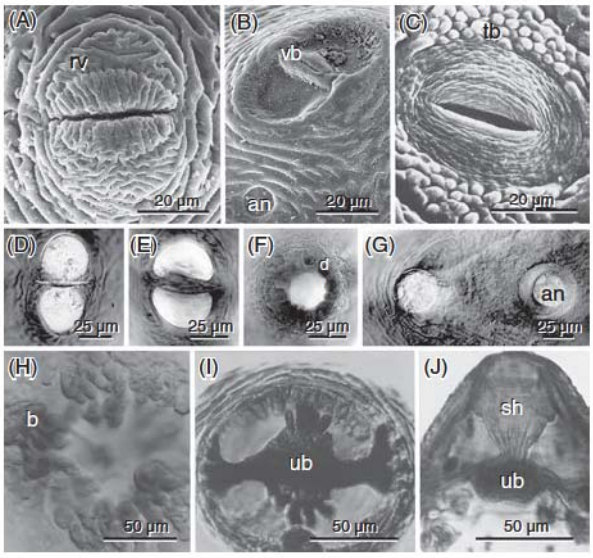

Fig. 14.9. Structures of the terminal region of cyst nematodes. (A–C) Scanning electron micrograph end views of terminal region of females. (A) Mature *Heterodera glycines* showing developing semifenestrae including rivulets (rv). (B) Young *H. glycines* showing developing vulval bridge (vb) and anus (an). (C) *Globodera tabacum* with vulval region surrounded by tubercles (tb). (D–G) Light micrographs of fenestrae of terminal region of cysts. (D) Bifenestrate *H. humuli*. (E) Ambifenestrate *H.trifolii*. (F) Circumfenestrate *Cactodera weissi* showing vulval denticles (d). (G) Circumfenestrate with separate anal fenestrate, *Punctodera punctata*. (H–J) Light micrographs of structures within terminal region. (H) Bullae (b) of *H. iri.* (I) Underbridge (ub) of *H. leucilyma*. (J) Dorsoventral view of *H. trifolii* showing vulval sheath (sh) and underbridge (ub). (A, B after Subbotin *et al.* (2010); C–J after Mulvey and Golden (1983); C–I courtesy of USDA Nematode Laboratory and *Journal of Nematology*.)

bridge, together with the length of the vulval slit are of diagnostic value. In both round and lemon-shaped females the distance of the vulva from the anus opening and the cuticular pattern between the vulva and the anus are diagnostic for some taxa.

The digestive system of females is not known to vary among species, with the exception of the length of the often-curved stylet, the size and shape of knobs and posteriorly the position of the dorsal gland orifice (Fig. 14.6A–D). The pharynx, compared to J2 and males, has a large median bulb and gland lobe, but fine structural detail of this region is not available, in part because of technical difficulties in processing globose females for TEM. The intestine is also not well understood although there is evidence that it functions primarily as a storage organ; consistent with this possibility, Raski (1950) observed that the rectum of the adult female is not well developed. The anus occurs on the cuticle surface dorsal to the vulva (Figs 14.9A, G and 14.10).

The didelphic coiled reproductive tracts of heteroderid females extend from the terminal vulva where anteriorly each gonad has a short germinal zone and proximally an extended growth zone followed posteriorly by a short narrow oviduct; in amphimictic species there is a pronounced sperm-filled spermatheca and this joins, at a sharp bend, with the uterus (Triantaphyllou and Hirschmann, 1962). The reproductive tract terminates posteriorly as a vulval slit and internal to the slit is a complex morphology including the cuticle-lined vagina. The vagina is lined by dilator muscles that, in taxa with cones, attach to the body wall. In *Heterodera* species that have been examined, these include 48 muscles as four levels of six on each side (Fig. 14.10), but in other taxa, such as *Cactodera*, the musculature is greatly reduced (Cordero and Baldwin, 1991; Cordero *et al.*, 1991). Proximally the lining of the vagina is enclosed by a thick sphincter muscle and in some species immediately distal to this muscle in the mature female the cuticle

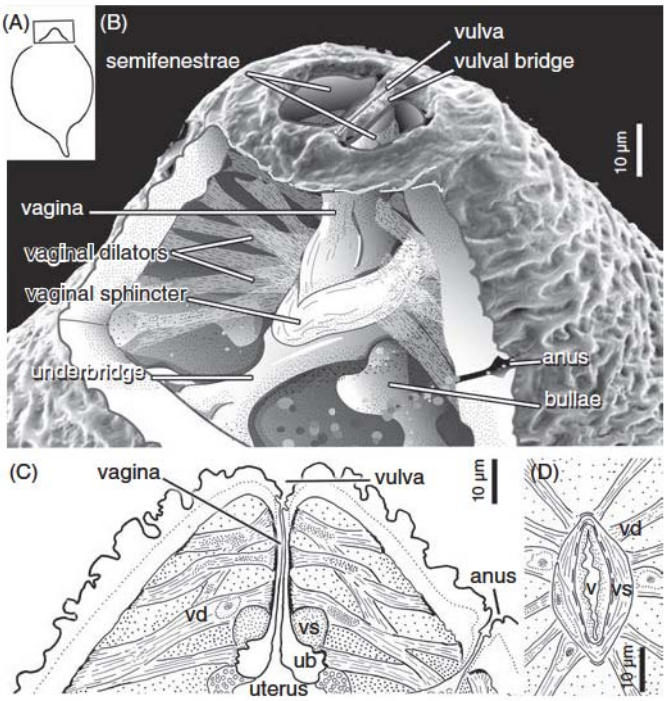

Fig. 14.10. Diagrammatic representation of basic structures of terminal region of cyst nematodes. (A) Overall profile showing cone. Box indicates terminal region as enlarged in B and C. (B) Ventrolateral 3D with cut away showing internal structures of female and/or cyst. (C) Right lateral view through cone; vd = vaginal dilator, vs = vaginal sphincter, ub = underbridge. (D) Cross-section through vagina (v) at level of vulval sphincter. (A, B after Subbotin *et al*. (2010); C, D after Cordero and Baldwin (1991) courtesy of *Journal of Nematology*.)

lining thickens (Fig. 14.10). There is some evidence that development of vaginal musculature is associated with the capacity of females to lay eggs in a matrix *vs* retaining all eggs into the cyst stage; that is, species vary with respect to retention of eggs within the body of the female and cyst. Some species retain all the eggs through maturation of the female into the cyst, whereas others produce an eggsac depositing at least some eggs externally in a gelatinous matrix (Fig. 14.6F). The source of the gelatinous matrix, while uncertain, is most likely the uterus and even taxa that retain eggs may nevertheless produce a small amount of gelatinous exudate to which males may be attracted (Hesling, 1978; Turner and Subbotin, 2013).

The nervous system and excretory system of females are not well understood. Yet, with SEM, depending on preparation techniques, amphid and some inner labial sensory openings are generally observed in the head region of females and the excretory pore is typically present ventrally at about the level of the gland lobe (Fig. 14.6A). In particular, insight into female sensory systems and differences relative to corresponding organs in migratory stages could provide important specific insight into function.

14.6 Cysts

The distinguishing feature of cyst nematodes is that mature egg-filled females, upon death, are transformed into persistent tanned 'vessels' that retain and provide protection for viable eggs. Tanning is the result of polyphenoloxidase (Ellenby, 1946; Awan and Hominick, 1982) and following death of the female tanning transitions to final levels of pigmentation ranging in intensity from light tan to nearly black (Fig. 14.11). Some cysts, prior to reaching their

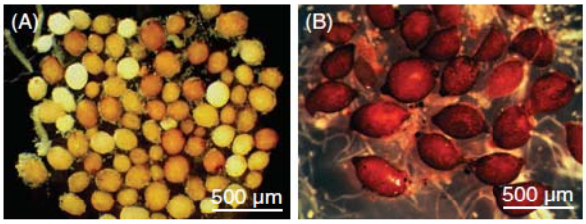

Fig. 14.11. Cysts showing variation in colour. (A) *Heterodera zeae*. (B) *H. glycines*.

darkest colour, pass through a phase described as golden and the presence or absence of this yellowish stage is considered to be diagnostic. Franklin (1951) suggested that the yellow colour of some *Globodera* cysts might be attributed to changes in the state of internal contents of the cyst, prior to later full pigmentation of the persistent cuticle. Regardless of pigment, the whitish subcrystalline surface, described in females of certain taxa, may persist in the cyst phase.

Cuticular layering of the young cyst is at first recognizable as consistent with that of white females, although Cordero and Baldwin (1990) note that in some young cysts additional layers of the cuticle may appear internally towards the epidermis. Whether cuticular layers described in white females remain distinguishable in mature cysts is not known. However, cuticular transformations, including with respect to layers, during cyst development have been described for the terminal region of some cysts (Cordero and Baldwin, 1990, 1991; Cordero *et al*., 1991). Cuticular surface patterns are typically not modified from those of mature females except, as noted, fenestrae open in the terminal region corresponding in females, to a discrete area defined by a loose mesh of fibres (Figs 14.9A–G and 14.10B). This may be expressed as semifenestrae, where two openings are separated by a retained cuticular 'vulval bridge' that includes a persistent vulval slit (Figs 14.9B, D, E and 14.10B). Semifenestrae may be expressed as ambifenestrae, where each opening is semicircular separated by a narrow vulval bridge, or as bifenestrae, where the pair of semifenestrae are round and separated by a relatively stout vulval bridge (Wouts and Baldwin, 1998). In circumfenestrae individuals a vulval slit and cuticular vulval bridge is not retained and there is a singular circular fenestral opening. In certain circumfenestrae taxa, specifically in *Punctodera*, there is an additional fenestrae area corresponding to the anal region (Fig. 14.9D–G). Types of fenestrae are taxon-specific. In addition to *Punctodera*, circumfenestrate cysts characterize *Globodera* and *Cactodera*; species within these genera may be further characterized by variations in size and shape of this opening, as well as by the distance of the fenestrae to the anus (e.g. see the ratio of Granek, 1955, and as further defined by Hesling, 1973). By contrast, semifenestrae, either ambifenestrae or bifenestrae, characterize most lemon-shaped cysts of *Heterodera* and the specific size and shape of these openings may further characterize species. In general ambifenestrae are associated with a persistent relatively long vulval slit and an associated bridge, in contrast to bifenestrae in which the pair of openings is separated by a persistent shorter vulval slit. Notably some cyst taxa do not form fenestrae as is reported to be the case for *Heterodera (Afenestrata) africana*.

As the cyst matures, cuticular transformations of the body wall are particularly apparent in the terminal region and many of these are important in diagnostics. Bullae, apparently noted by Franklin (1939) but described and named by Cooper (1955), are irregularly shaped, sometimes branched, large darkened cuticular deposits that extend inward from the body wall cuticle. Variation in shape, size, position and pattern of bullae may have diagnostic value (Figs 14.9H and 14.10B). For example, *H. schachtii* is characterized by the presence of bullae, whereas bullae are said to be absent in the closely related *H. cruciferae*. Where present within *Heterodera*, bullae of the *Schachtii* group (see Chapter 16, this volume) tend to be scattered and positioned near the underbridge (see below) (Hesling, 1978), whereas those of the *H. avenae* complex tend to be particularly prominent, more specific in structure, and positioned closer to the cone terminus. Bullae are common among species of *Punctodera* in contrast to *Globodera* and *Cactodera*. However, many taxa lacking bullae may have comparable tooth-like pigmented structures such as those termed 'vulval denticles' (Fig. 14.9F). Unlike bullae of

cuticular origin, vulval denticles and perhaps also vulval bodies (Wilson, 1968; Mulvey, 1973, 1974) and 'Mulvey's bridge', are understood to be remnants of vulval musculature (Mulvey, 1959; Golden and Raski, 1977; Cordero, 1989; Cordero and Baldwin, 1991; Cordero *et al.*, 1991).

In cyst development, internally there is a transformation of the vaginal region. In some *Heterodera* species, the cuticle thickens in the proximal region of the vagina and this area becomes anchored to the lateral cone wall by a pair of branched or forked projections that comprise the cyst underbridge (Figs 14.9I and 14.10B, C). The underbridge is so-called because it is oriented internally and aligned parallel to the superficial vulval bridge. The presence, shape and orientation of the underbridge are diagnostically significant. In some cases persistent remnants of the vagina transverse between the bridge and underbridge and these may be referred to as a 'sheaf' (Golden, 1986) (Fig. 14.9J).

14.7 Techniques

14.7.1 Light microscopy (LM)

There is a rapidly expanding array of tools applicable for understanding heteroderid structure, yet traditional LM remains essential to the discovery and understanding of morphology. Even at the relatively low magnifications of a dissecting microscope, in an aqueous solution with transmitted light an initial assessment can be made of key features of heteroderid J2 and males. These include an estimate of overall length, relative length of the tail, hyaline region and stylet. Similarly, the dissecting microscope, particularly equipped with reflected light, is a primary tool for revealing cyst shape and colour. These initial low power assessments, especially combined with knowledge of the host, provide a simple but powerful starting point to morphological understanding and diagnostics. Clearly, the dissecting microscope is also the starting point for many other morphological techniques, including preparing LM slides as well as processing material for SEM and TEM.

The dissecting microscope is essential but limited by its relatively low magnification. Assessment of key aspects of heteroderid morphology also requires a compound light microscope equipped with high resolution oil immersion lens and condenser and field diaphragm suitable for focusing/optimizing transmitted bright field or differential interference contrast (DIC) optics. Techniques for preparing permanent microscope slides of heteroderid J2 and males follow standard approaches for tylenchids and other small nematodes (Thorne, 1961; Goodey, 1963; Golden, 1986; Eisenback and Zunke, 1998). Specimens are typically killed and 'relaxed' (straightened) by gentle heat, followed by aldehyde fixation and then dehydrated through increasing concentrations of ethanol, which is finally replaced by anhydrous glycerin. Specimens mounted in glycerin are sealed under a cover glass supported so as to not crush specimens. Notably, for some types of evaluation, temporary slides are suitable and these can be prepared by mounting living, heat-killed or anaesthetized specimens directly in water or on a thin layer of water agar (Sulston and Hodgkin, 1988).

Some light microscope methods have been developed to interpret unique aspects of heteroderid females and cysts. Although whole individuals can be mounted on slides, provided adequate support of cover glasses, the thickness of the specimens may not allow for adequate transmitted light, and particularly so for viewing surface patterns. Such cases typically require dissecting areas of cuticle that can be separately mounted on slides for viewing. For certain studies there is value in dissecting female heads for separate mounting and viewing of features such as the stylet, DGO position and excretory pore. Previously, some investigators dissected and mounted head regions for *en face* viewing, but this approach is now used less often in lieu of broader availability of SEM of lip patterns. One of the most important cyst nematode-specific procedures for LM is that of dissecting the terminal region for detailed interpretation of morphology (Golden, 1986; Mulvey, 1973; Hesling, 1978). For some structures, including bullae, the underbridge and position of the anus, lateral side mounts are particularly informative. More difficult, but essential for viewing posterior cuticular patterns, vulval slits, the bridge and fenestrae, are terminal mounts (Riggs, 1990; Subbotin *et al.*, 2010). Such patterns are prepared under a dissecting microscope by cutting off the terminal region with a fine knife or scalpel (Fig. 14.12).

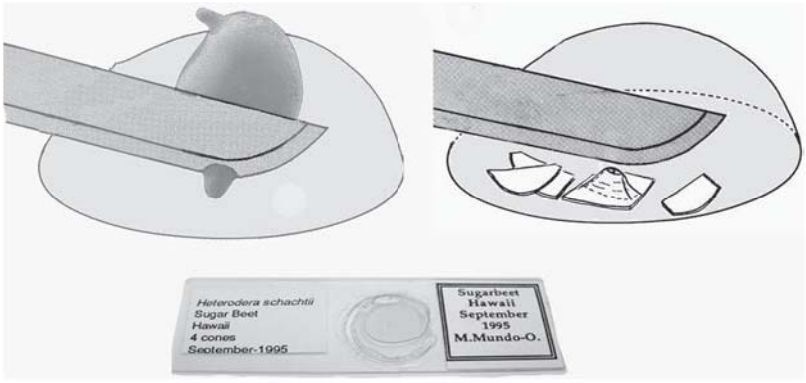

Fig. 14.12. Diagrammatic representation of the procedure for excising, trimming and mounting the terminal region of a female or cyst.

Using fine needles or an eyelash mounted on a handle the end can then be cleaned of debris, oriented end up and, if needed, the periphery can be further trimmed. To provide support, mounting can be in Canada balsam (Goodey, 1963), a glycerin agar block (Correia and Abrantes, 1997) or in glycerin jelly (Thorne, 1961; Subbotin *et al.*, 2010).

Particularly crucial to heteroderid morphology, especially with regard to cone patterns, are applications of LM that allow recording of enhanced depth of focus including multiple-focus photomicroscopy (Eisenback, 1988). Similarly, through-focus videos enable high definition and high magnification digital recording through fresh or preserved specimens of all stages, and digitally archiving and enhancing access to records of morphological details (De Ley and Bert, 2002). Beyond depth of focus, video microscopy is also employed as a tool for understanding cyst nematode morphology and function including time lapse or 4D recording of development and pathogenesis (Wyss and Zunke, 1986a, b; Cordero and Baldwin, 1990, 1991). Additional enhancements of LM applicable to cyst nematodes are achieved with specialized staining, florescence and confocal techniques. For example, fluorescent phalloidin has been shown to be an efficient complement to TEM in interpreting dilator muscles of the cyst cone (Cordero and Baldwin, 1991) and fluorescent antibodies and confocal microscopy have been used to evaluate specific cyst nematode gland activity (Willats *et al.*, 1995). Fluorescent antibodies and confocal microscopy, used to visualize cell boundaries, also show promise for applications to understanding heteroderid morphology (Burr and Baldwin, 2016).

14.7.2 Scanning electron microscopy (SEM)

SEM is crucial to interpreting surface details of cyst nematodes including many of diagnostic importance (Eisenback, 1991). Often morphological understanding gained with SEM, can subsequently 'open our eyes' to understand, anticipate and then interpret those same features using routine LM. Key among details best resolved by SEM are lip patterns, especially of J2. Also SEM is an important supplement to LM in resolving aspects of J2 with respect to numbers, areolation and anterior/posterior terminus of lateral lines. Sensory openings often best resolved by SEM with respect to shape and position include those of the inner labial papilla, amphids and phasmids of J2 and males; in some cases (as when phasmid openings are positioned within the fold of a lateral line) these may be difficult to observe using LM. In males, presence/absence of phasmids vary among taxa and this diversity is best examined by SEM. Details of the morphology of the male tail and particularly diagnostic aspects of the spicule tip also can best be resolved with SEM. In females and cysts, beyond the lip region, SEM is of particular importance in resolving more general details of surface patterns that may have diagnostic importance. In the terminal region patterns of the basin, vulval bridge, vulva, anus and surrounding cuticle patterns

are ideally resolved by SEM, not obscured, relative to LM, by underlying focal planes. Beyond surface structures, SEM of cutaways of cyst cones may be useful for interpreting underlying structures such as the underbridge and bullae. SEM also has special importance in observing, on eggs, the presence of punctations/tubercles that characterize some species of *Cactodera*.

Preparation of specimens for SEM typically requires cleaning, fixation, dehydration, mounting, treating with a conductive coating and imaging. The first step of cleaning is often best accomplished prior to fixation and by repeated rinsing in clean water; it may be helpful to use an eyelash mounted on a supporting handle to brush away any specs of debris. In the Baldwin laboratory at UCR (University of California, Riverside, USA) on occasion we have used gentle sonication in water to promote clean specimens but usually this is not necessary. Mature air-dried cysts may be directly suitable for mounting, coating and imaging but living material requires fixation and particular care during dehydration to minimize distortion. Fixation for SEM usually is by aldehydes. The approach at UCR is to place the living specimens in 12 ml of filtered tap water in a 25-ml vial. The vial with specimens is then partly immersed in a hot water bath (60°C), relaxing vermiform stages, and to this is added 10 ml of 10% buffered (pH 7.0) formalin solution, or alternatively 10% buffered glutaraldehyde solution. Although fixation overnight is adequate, it has been observed that longer periods enhance results. For some particularly fragile specimens prone to collapsing, post-fixation with osmium tetroxide is advantageous, resulting in improved rigidity of delicate features and slightly increased conductivity. Specimens are placed in 2% buffered osmium textroxide solution overnight and subsequently repeatedly rinsed in buffer.

The first step in dehydration is processing specimens through a series of aqueous ethanol solutions ranging from 10–100%. To avoid collapsed specimens it is crucial that the series culminates in absolute ethanol, and a consideration is that opened bottles of 100% ethanol are hydroscopic and may quickly become slightly diluted. In this regard a freshly opened bottle of ethanol is recommended for the final steps of dehydration. Dehydrated specimens are then dried in a critical point dryer. This instrumentation allows replacing ethanol (or in some procedures, acetone) with liquid CO_2 (or freon) and then regulating temperature and pressure to dry material while minimizing the impact of liquid surface tension that would otherwise damage tissue.

Using a dissecting microscope, critical point dried specimens are mounted on SEM stubs. At UCR the preference is aluminium stubs using double sticking copper (conductive) tape (Fig. 14.13A). Males or J2 are typically mounted on wrinkles in the tape so that heads, for example, can project beyond the tape for optimal viewing. Excised cones can be oriented directly on the tape, and often whole females or cysts can be oriented for optimal viewing of the terminal region. Critical point dried specimens generally have sufficient charge to allow picking and orienting them on tape using an eyelash mounted on a holder. Prior to viewing, specimens are coated with an ultrathin (25 nm thick) coating of conductive surface such as gold-palladium. This is accomplished using sputter coater instrumentation.

Alternatives to critical point drying of cyst nematodes include direct SEM viewing of specimens that are infiltrated with dehydrated glycerin by a methodology typically used for preparing permanent slides (Sher and Bell, 1975). Advantages of this method are its simplicity, its accommodation to using archived specimens from permanent slides and that it minimizes risk of specimens' surface artefacts associated with other forms of drying. Disadvantages include vulnerability of the specimens to overheating and collapse in elevated temperatures under the beam as well as some potential for contaminating the SEM column with glycerin. To minimize these effects, specimens infiltrated with glycerin must typically be viewed at very low accelerating voltages of 5–15 KV, but such low voltage also may reduce resolution. Additional potential alternatives to critical point drying may include applications of low vacuum SEM instrumentation (Sammons and Marquis, 1997); this allows viewing specimens with reduced preparation including the option of omitting a conductive coating. At UCR a range of procedures using SEM instrumentation designed for such low vacuum have been tested, including on cyst nematodes, but results (particularly resolution) remain unsatisfactory.

One of the challenges of processing specimens for both SEM and TEM is that of transferring nematodes in solutions without loss of

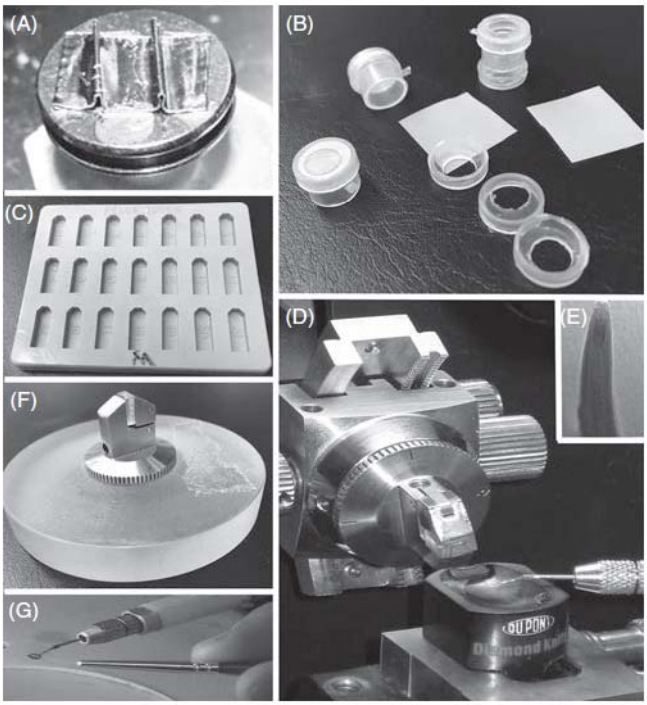

Fig. 14.13. Procedures for preparing specimens for scanning (SEM) and transmission (TEM) microscopy. (A) SEM stub with folded foil tape upon which specimens are mounted. (B) Containers modified from Beem® capsules for processing specimens. The base is excised from the capsule and each end is covered with a lid from which the centre is removed using a hole punch. Each lid holds in place a fine nylon mesh fabric. (C) Moulds designed to polymerize specimens in epoxy for subsequent TEM sectioning. (D) An epoxy block with specimens mounted on an ultramicrotome with a diamond knife for producing ribbons of sections. Sections are being removed using a loop. (E) Osmium tetroxide-fixed specimen embedded in a layer of epoxy. (F) A lucent block developed to secure the specimen holder for trimming an epoxy block. (G) Using a mounted eyelash to manipulate a ribbon of sections on a loop. (A, D and E after Subbotin *et al*. (2010).)

specimens and without exposure of the researcher to toxic materials including fixatives. With this in mind a number of chambers have been proposed (McClure and Stowell, 1978; Bumbarger *et al*., 2006). In our experience, the most helpful container for this purpose is an adaptation of the BEEM® or Eppendorf capsule container modified with very fine (<10 µm mesh) nylon mesh cloth to facilitate exchange of liquids (Fig. 14.13B) (Eisenback, 1991).

14.7.3 Transmission electron microscopy (TEM)

TEM is invaluable for elucidating details of nematode morphology. As noted for SEM, often once TEM is used to clarify detailed structures those details can sometimes subsequently be interpreted from LM. For example, cuticular layers of females first clearly resolved by TEM could also be visualized using LM sections (Shepherd *et al*., 1972). In the 1970s, heteroderid J2 and males were among the first tylenchids in which details of body wall, sensory and digestive system were described together with host parasite relationships (Baldwin and Hirschmann, 1975a, b, 1976; Baldwin *et al*., 1977; Endo, 1980, 1983, 1984, 1985, 1991). Later TEM was applied to females and cysts with respect to the body wall and terminal region as well as to the male tail and sperm diversity. TEM has been central to addressing questions of origin, function, diversity and homologies of classical features of heteroderids; often these features, otherwise, based on the limited resolution of LM, are subject to

misinterpretation. Examples include the taxon-specific diversity in cuticle-layering of females and of nurse cells as well as, for example, understanding the nature of bullae (Baldwin, 1983; Cliff and Baldwin, 1985; Cordero and Baldwin, 1991; Cordero *et al.*, 1991; Mundo-Ocampo and Baldwin, 1984, 1992). Although approaches may vary with different applications and goals, TEM procedures typically include fixation, dehydration, infiltration, embedding/polymerization, trimming, sectioning, staining, coating and imaging (Carta, 1991).

Excellent fixation is essential for successful TEM, yet typically success of this first step cannot be fully evaluated until the final step of imaging. A challenge with preparing heteroderids is that fixatives may penetrate slowly and this often results in inadequate preservation, in part probably associated with catabolism. Early attempts to address poor preservation often included prolonged fixation times, but this may have resulted in artefacts including those associated with leaching of cytoplasm. Leaching, particularly of cytoplasm, may result in images with impressive contrast but nevertheless loss of information. Where fixation is too slow to prevent catabolism, results can include artefacts of broken discontinuous cell membranes and this confounds understanding cell topology. In some cases cutting specimens in buffer or fixative can improve fixation but this can be technically difficult (especially safely handling specimens in fixatives) and it can result in distorted topologies and leaching. The approach that provides the best preservation to date is that of rapidly freezing specimens in liquid nitrogen under high pressure followed by chemical fixation by substitution within an autofreeze substitution apparatus (Bumbarger *et al.*, 2006). Classic chemical preservation typically first includes a buffered solution of glutaraldehyde, paraformaldehyde or a mixture (e.g. Karnovsky, 1965) followed by post-fixation in osmium tetroxide. In some applications, where the emphasis is on preservation of membranes, aldehydes may be omitted in favour of only using osmium tetroxide. At UCR a standard procedure, including for heteroderids, is fixation in 3.5% glutaraldehyde followed by post-fixation in 2% osmium tetroxide; pH is regulated at 7.2 with 0.2 M phosphate buffer.

Thin sectioning requires fixed specimens to be infiltrated with epoxy that is then polymerized to provide a suitable substrate. Since most epoxies are not water soluble, infiltration typically requires complete dehydration in an intermediate solvent miscible with the epoxy of choice; usually this requires dehydration by a series of aqueous buffered solutions to 100% ethanol or acetone. As for SEM, it is crucial that hydroscopic properties of solutions be considered to ensure complete dehydration. With dehydration, complete infiltration is accomplished by slowly introducing epoxy into solvent and subsequently allowing all solvent to evaporate resulting in 100% liquid (but highly viscose) infiltrated epoxy. Often 72 h are needed to meet the principle of complete infiltration, although timing is variable in relation to conditions including the epoxy of choice as well as properties of the tissue. Epoxys commonly used for TEM of heteroderids include Epon 812 and mixtures of Epon with Araldite 6005, Durcupan and Spurr.

Following infiltration and prior to polymerization of epoxy, specimens must be oriented to optimize their position for subsequent trimming. Depending on the tissue and goals the typical approach at UCR is orientate and embed the specimens using block moulds optimized in shape for trimming (Fig. 14.13C). The dark colour of osmium-treated specimen aids visualization for orientation (Fig. 14.13E). Specimens can be placed and oriented directly in the moulds filled with fresh epoxy, but a disadvantage is that specimens sink to a suboptimal position at the bottom of the mould. A solution is to first embed the specimens in a small block of 2% water agar prior to infiltration. This block containing the specimen can then be oriented so that the specimen is at an intermediate level from the bottom of the mould. An alternative to an agar block is to first embed and polymerize specimens in a thin plate-like layer of epoxy using a flat slide-shaped mould (Kolotuev *et al.*, 2010). Once removed from the mould, using a compound microscope, specimens can be observed within the thin layer to make an initial assessment of quality of preservation, and to prepare a LM photo record of the specimen (Fig. 14.13E). Individual specimens can then be cut from this layer, oriented and re-embedded within a block mould (Fig. 14.13C, F). Polymerization is typically accomplished with heat; optimal temperatures for this process vary with the epoxy formulation. After polymerization the epoxy block is trimmed

to a trapezoid shape, ideally only slightly larger than the specimen. Trimming can be accomplished with a single edge razor blade, shaving thin layers under a dissecting microscope (Fig. 14.13F); the trimming can be further refined using an old diamond knife with the epoxy block mounted on a microtome. Although some have had success with glass knives, successful microtome sectioning generally requires a flawless-edged diamond knife where the goal is to achieve a ribbon of sections at about 70 nm thickness. These float from the knife edge into a boat filled with filtered distilled water and from this ribbons of sections are transferred onto a grid (a slot grid for serial sections) coated with a commercially available electron-lucent plastic film. Transfer is done by using a fine loop in which the ribbon is supported by a film spanning the loop and created by water surface tension from the knife's boat (Fig. 14.13D, E)

Sections of fixed specimens inherently are of very low contrast in TEM but this is addressed by staining with heavy metals that differentially impede penetration of electrons in subcellular components. Typically these stains are heavy metals through treatments with uranyl acetate and lead citrate. Procedures for uranyl acetate include staining sections directly on grids and/or (additionally or alternatively) the stain may be introduced at the time of fixation (Bumbarger *et al*., 2007, 2009). At UCR uranyl acetate staining on grids is typically done by floating or submerging sections in a saturated solution in ethanol, followed by rinsing in ethanol. Staining with lead is particularly challenging because of the risk of introducing residues including products of interaction of the stain with atmospheric carbon dioxide. Such residues can be minimized by staining below room temperature, by using one of various commercial tools designed for submerging grids to minimize exposure to air and by careful rinsing following staining. Following staining and prior to TEM viewing and recording images, sections and the supporting plastic film are rendered less fragile and more stable under the electron beam by first treating them with a thin electron-transparent coat of carbon. This is done by transferring a current through carbon rods under high vacuum using a vacuum evaporator.

Although not yet applied to heteroderids, digital images from serial sections have been demonstrated to be useful for creating 3D reconstructions of nematodes (Bumbarger *et al*., 2006, 2007, 2009; Ragsdale *et al*. 2008, 2009; Subbotin *et al*., 2010). These approaches may be particularly promising for interpreting morphological transformations, including of phylogenetically informative structures such as the posterior region of heteroderid females/cysts. After many years of technical stagnation TEM is undergoing a transformation towards automated sectioning, simultaneous sectioning of multiple specimens and high throughput imaging (D.J. Bumbarger, 2016, pers. comm.). These transformations result in improved cost and labour efficiency to support the application of 3D reconstruction and phylogenetic comparisons of heteroderids.

14.8 Minimal Standards for Species Descriptions

For heteroderids, as for other taxa, the question of standards for species descriptions is integral to the question of *what is a species*. Distilled from ongoing discussion of species concepts, is that a species, as an *independent evolutionary trajectory*, cannot be delineated separately from a phylogenetic context (Adams, 1998; Nadler, 2002; De Queiroz, 2007). The 'ideal' of *what is a species* is further confounded because what is perceived as a present 'independent lineage' might be challenged by future reticulation with other putative lineages. Consequently, species descriptions are *testable hypotheses* and the strength of such hypotheses depends on the degree to which those tests continue to be supportive of lineage independence. An important question, relevant to cyst nematodes, is *what is the minimum strength of support for a species hypothesis recommended as a basis for description, naming and publication*. Minimally, description as a new cyst species should briefly introduce the author's statement of species concept, what operational parameters are being used to test species status, and what are the limitations of the methods used for assessing that status. Relevant to establishing independence and uniqueness of the candidate species, there must be clear evidence that there has been a thorough review of extant species (literature and in some cases direct examination of type specimens). Precise collecting site(s),

preferably presented as GPS coordinates, and host(s) should be specified.

The classical ideas of 'typological species' have been largely rejected (Adams, 1998), and there is a growing role of reverse taxonomy[3] and molecular markers in species discovery (see section 14.9). Nevertheless, morphological distinctiveness is often the first test used to predict species status of cyst nematodes. Since a goal is comparison with extant species, descriptions must consider features and morphometrics for J2, males, females and cysts as they are used in comparable extant descriptions, and to do so with adequate representation (e.g. numbers of individuals) to allow for statistical assessment of intra- and interspecific variation. It may be possible to demonstrate that certain morphological features are particularly convincing as indicators of species independence. For example, distinguishing morphological characters that track evolution versus those that are not informative (e.g. stochastic or mosaic) might be based on mapping such features on existing molecular-based phylogenetic trees of Heteroderinae. Minimally, morphology should be represented by drawings and/or informative photographs that target diagnostic features. SEM and through-focus videos (see section 14.9) are optional but potentially powerfully informative additions (De Ley and Bert, 2002) with videos being referenced as supplementary material accessible through websites. Morphometrics are typically best presented in tables.

Delineating species solely by morphology may be problematic as suggested by lineages that are phenotypically plastic (including at the intraspecific level) as well as by cryptic species, where independence is reflected by parameters (i.e. molecular) other than morphology. The best tests of independence and species status include examination of the new candidate in the context of extant heteroderid species through constructing phylogenies based on multiple genes. However, minimally it is practical to consider ribosomal DNA molecular markers (e.g. ITS and 28S) that are widely reported and comparable among existing cyst species. When relying on such markers to support species status it is also important to acknowledge the limitations relative to assessing phylogenetic monophyly and the potential for misinterpretation, for example, as a result of paralogy due to gene duplications of ribosomal DNA as a multicopy gene (Nyaku *et al.* 2013; Pereira and Baldwin, 2016).

In addition to morphological and molecular characters, reproductive isolation is often proposed as a test of species status and indeed in this regard it is advantageous that many cyst species are cross-fertilizing. However, there are widely discussed arguments for rejecting reproductive isolation as a criterion for defining species (Mallet, 2008, 2010). Lineages essentially independent under natural conditions may retain the capacity to cross under rare or artificial conditions and, notably, fertile crosses have been reported across cyst genera (Miller, 1983).

Consistent with the International Code on Zoological Nomenclature (Anonymous, 1999) minimal standards of species description must include reporting deposit of type specimens in one or more curated and assessable collections; for cyst nematodes this should include J2, males, females and cysts (including mounts of the terminal region) appropriately prepared on microscope slides. While not minimal requirements, there is certainly strong justification for supplementing conservation of types with through-focus videos and with sources of molecular data for types (see section 14.9).

Whereas meeting minimal standards for description of cyst nematodes should be possible under nearly every circumstance, we note arguments for exceptions. For example, it may be especially valuable to phylogenetic studies to report limited information on unique cyst specimens collected through rare opportunities in exotic localities. Reports of such new linages may have broad significance, and arguments for species status may be considered even under circumstances where criteria that are normally expected cannot be met (numbers of individuals are limited, certain stages are missing, sequencing efforts are not successful). The quest for worldwide discovery of cyst nematode diversity and broad phylogenetic representation of cyst nematodes must engage global efforts.

[3] The approach of *reverse taxonomy* is prospecting for new species with molecular markers that can then be linked to other parameters including morphology (De Ley *et al.*, 2005; Markmann and Tautz, 2005).

To this end participation must not be unnecessarily restrictive and standards must be applied with a degree of flexibility in order to drive the process with broad-based access, communication and collaboration (Eyualem-Abebe *et al.*, 2006).

14.9 Conclusions and Future Prospects

Morphology of cyst nematodes has long held a central place for understanding biology, diversity, evolution, physiology, pathogenicity and management/regulation. Technological advances are transforming ways in which new growing morphological understanding interfaces with complementary avenues of investigation. These include applications of morphology in the context of genomics towards understanding the regulation of phenotypic expression, to pinpoint gene expression at specific morphological sites as in understanding regulation of diapause, the role of specific neurons in host/mate-seeking or the role of particular glands in hatching or establishing feeding sites.

Molecular phylogenetics and diagnostics have been at the forefront of advances that intersect with classical and new approaches in morphology. Morphological phylogenetics, while informative when applied under rigorous models (Baldwin and Schouest, 1990; Baldwin, 1992), is often confounded by misunderstood homology such as similar phenotypes based, not on shared evolution, but on convergence. Misinterpretations of relationships, including of cyst nematodes, may also stem from phenotypic plasticity, cryptic species and characters poorly understood because they are at the limits of LM resolution. Molecular phylogenetics (see Chapter 16, this volume) is becoming increasingly sophisticated in providing an independent character set against which to test classical morphology-based hypotheses of phylogeny and, in so doing, sometimes challenging classical classification systems. In the search for congruence between morphology and molecular character sets, there is a challenge for an increasingly sophisticated understanding of morphology. For example, the classical proposal of a unique shared ancestry between cyst and root-knot nematodes based on features linked to sedentary parasitism have been challenged and revised (Baldwin, 1992; Baldwin *et al.*, 2004). Such revisions provide new insight into morphological evolution. For example, absence of fenestra was previously considered definitive to a cyst genus, *Afenestrata*, until molecular phylogeny helped to demonstrate that this distinctive feature and genus was embedded within the clade that defines *Heterodera* (Baldwin and Bell, 1985; Mundo-Ocampo *et al.*, 2008). Revisions enlightened by molecular phylogeny also provide a basis for more predictable and meaningful applications of model systems. For example, investigating specific pathogenesis of cyst nematodes will more likely benefit from comparisons to the phylogenetic context of hoplolaimids than to convergent and phylogenetically distant root-knot nematodes.

We have noted that in diagnostics increasingly morphology is complementary to the use of molecular markers, and this is underscored by the very broad and growing representation of cyst species on GenBank. Linkage with molecular markers has been well verified for widespread agricultural pests, but for some taxa the process of verification continues including addressing, as noted in section 14.8, issues of intra- and interspecific variability. Are the samples to which the marker is linked co-specific with the types (e.g. as often best verified by topotypes) designated for the species? Do problems with the initial species designation (e.g. lack of monophyly) confound use of markers? There is growing recognition of an interdependence of morphological and molecular technology in addressing these challenges.

Whereas diagnostics of cyst nematodes is often considered in relation to agricultural regulation and management, it is also relevant to biodiversity, species discovery and bioprospecting, including of cyst nematodes from non-agricultural sites. Such discoveries are essential to broad taxon representation in developing a sound phylogenetic framework and classification for the Heteroderinae. Previously, species discovery was driven primarily by morphological novelties. For cyst nematodes this typically meant recognizing heteroderid J2 in a soil sample and then resampling the site to search for females, cysts and possible host associations. The added steps to the process of discovery probably have resulted in under-representation of Heteroderinae

in species discovery from non-agricultural sites. However, we have noted that increasingly sequences (*versus* morphology) are becoming the most efficient driver for discovering species diversity. Through reverse taxonomy, once sequence-delineated species are discovered, these 'species hypotheses' are then tested with additional data including morphological methods.

With broader access to increasingly powerful computers, software and internet, digital through-focus videos (DeLey and Bert, 2002) will play a powerful role in advancing understanding of cyst nematodes. These video records can be applied not only to vermiform specimens but also to other vouchers (e.g. cones) specific to cyst nematodes. Here morphological and molecular technologies are again linked where imaging provides a morphological voucher of a live or freshly mounted individual that is then linked to DNA product/sequences of that same individual (De Ley *et al.*, 2005). Digital through-focus imaging can exceed quality of slide-preserved vouchers and particularly so with software that allows knitting into a single movie 100× components from head to tail of a specimen. This can be accomplished with an automated software-controlled microscope stage. With growing internet capabilities, a desktop computer-controlled stage in one location can also engage global expertise for worldwide remote morphological examination of specimens.

The role of classical morphology and morphology-based species has driven priorities of taxonomic repositories (museums) and voucher specimens and for heteroderids this has meant the preservation and deposit of types, vouchers and other slides including J2, males, cysts, females and cone/terminal mounts. However, advances in the ways we discover species must also drive new ways in which we conserve information. Modern taxonomic museums, in addition to curating slides and wet collections, must also conserve and make accessible LM digital virtual specimens and SEM. Beyond morphology there is a growing need for collections to conserve sources of DNA (DNA extraction, frozen and/or dried specimens) as vouchers linked to morphology and with global access through relational databases linked across collections.

14.10 References

Adams, B.J. (1998) Species concepts and the evolutionary paradigm in modern nematology. *Journal of Nematology* 30, 1–21.

Anonymous (1999) *International Code of Zoological Nomenclature*, 4th edn. International Trust for Zoological Nomenclature, Natural History Museum, London.

Awan, F.A. and Hominick, W.H. (1982) Observation on tanning of the potato cyst nematode, *Globodera rostochiensis*. *Parasitology* 85, 61–71. DOI: 10.1017/S0031182000054159

Baldwin, J.G. (1983) Fine structure of body wall cuticle of females of *Meloidodera charis, Atalodera lonicerae* and *Sarisodera hydrophila* (Heteroderidae). *Journal of Nematology* 15, 370–381.

Baldwin, J.G. (1985) Fine structure of the phasmid of second-stage juveniles of *Heterodera schachtii* (Tylenchida: Nematoda). *Canadian Journal of Zoology* 63, 534–542.

Baldwin, J.G. (1992) Evolution of cyst and non-cyst forming Heteroderinae. *Annual Review of Phytopathology* 30, 271–290. DOI: 10.1146/annurev.py.30.090192.001415

Baldwin, J.G. and Bell, A.H. (1985) *Cactodera eremica* n. sp., and *Afenestrata africana* (Luc *et al.*, 1973) n. gen., n. comb. and an emended diagnosis of *Sarisodera* Wouts & Sher, 1971 (Heteroderidae). *Journal of Nematology* 17, 187–201.

Baldwin, J.G. and Hirschmann, H. (1975a) Fine structure of cephalic sense organs in *Heterodera glycines* males. *Journal of Nematology* 7, 40–53.

Baldwin, J.G. and Hirschmann, H. (1975b) Body wall fine structure of the anterior region of *Meloidogyne incognita* and *Heterodera glycines* males. *Journal of Nematology* 7, 175–193.

Baldwin, J.G. and Hirschmann, H. (1976) Comparative fine structure of the stomatal region of males of *Meloidogyne incognita* and *Heterodera glycines*. *Journal of Nematology* 8, 1–17.

Baldwin, J.G. and Mundo-Ocampo, M. (1991) Heteroderinae, cyst- and non-cyst forming nematodes. In: Nickel, W.R. (ed.) *Manual of Agricultural Nematology*. Marcel Dekker Inc., New York, pp. 275–363.

Baldwin, J.G. and Perry, R.N. (2004) Nematode morphology, sensory structure and function. In: Chen, Z.X., Chen, S.Y. and Dickson, D.W. (eds) *Nematology Advances and Perspectives Volume 1 (Nematode Morphology, Physiology and Ecology)*. CAB International, Wallingford, UK, pp. 175–257.

Baldwin, J.G. and Schouest, L.P. Jr (1990) Comparative detailed morphology of Heteroderinae Filip'ev & Schuurmans Stekhoven, 1941, *sensu* Luc *et al*. (1988) phylogenetic systematics and revised classification. *Systematic Parasitology* 15, 81–106.

Baldwin, J.G., Hirschmann, H. and Triantaphyllou, A.C. (1977) Comparative fine structure of the esophagus of males of *Heterodera glycines* and *Meloidogyne incognita*. *Nematologica* 23, 239–252. DOI: 10.1163/187529277X00598

Baldwin, J.G., Nadler, S.A. and Adams, B.J. (2004) Evolution of plant parasitism among nematodes. *Annual Review of Phytopathology* 42, 83–105. DOI: 10.1146/annurev.phyto.42.012204.130804

Bernard, E.C., Handoo, Z.A., Powers, T.O., Donald, P.A. and Heinz, R.D. (2010) *Vittatidera zeaphila* (Nematoda: Heteroderidae). A new genus and species of cyst nematode parasitic on corn (*Zea mays*). *Journal of Nematology* 42, 139–150.

Borgonie, G., Claeys, M., De Waele, D. and Coomans, A. (1995) Ultrastructure of the intestine of the bacteriophagous nematodes *Caenorhabditis elegans*, *Panagrolaimus superbus* and *Acrobeloides maximus* (Nematoda: Rhabditida). *Fundamental and Applied Nematology* 16, 47–56.

Brown, G., Callow, R.K., Green, C.D., Jones, F.G.W., Rayner, J.J., Shepherd, A.M. and Williams, T.D. (1971) The structure, composition and origin of the sub-crystalline layer in some species of the genus *Heterodera*. *Nematologica* 17, 591–599. DOI: 10.1163/187529271X00305

Bumbarger, D.J., Crum, J., Ellisman, M.H. and Baldwin, J.G. (2006) Three-dimensional reconstruction of the nose epidermal cells in the microbial feeding nematode, *Acrobeles complexus* (Nematoda: Rhabditida). *Journal of Morphology* 267, 1257–1272.

Bumbarger, D.J., Crum, J., Ellisman, M.H. and Baldwin, J.G. (2007) Three-dimensional fine structural reconstruction of the nose sensory structures of *Acrobeles complexus* compared to *Caenorhabdtis elegans* (Nematoda: Rhabditida). *Journal of Morphology* 268, 649–663.

Bumbarger, D., Wijeratne, S., Carter, C., Crum, J., Ellisman, M. and Baldwin, J.G. (2009) Three-dimensional reconstruction of the amphid sensilla in the microbial feeding nematode, *Acrobeles complexus* (Nematoda: Rhabditida). *Journal of Comparative Neurology* 512, 271–181. DOI: 10.1002/cne.21882

Burr, A.J. and Baldwin, J.G. (2016) The nematode stoma: homology of cell architecture with improved understanding by confocal microscopy of labeled cell boundaries. *Journal of Morphology* 277, 1168–1186.

Cares, J.E. and Baldwin, J.G. (1995) Comparative fine structure of sperm in *Heterodera schachtii* and *Punctodera chalcoensis,* with phylogenetic implications for Heteroderinae (Nematoda). *Canadian Journal of Zoology* 73, 309–320.

Carta, L.K. (1991) Preparation of nematodes for transmission electron microscopy. In: Nickel, W.R. (ed.) *Manual of Agricultural Nematology*. Marcel Dekker Inc., New York, pp. 97–106.

Carta, L.K. and Baldwin, J.G. (1990) Phylogenetic implications of phasmid absence in males of three genera in Heteroderinae. *Journal of Nematology* 22, 386–394.

Clark, S.A., Shepherd, A.M. and Kempton, A. (1973) Spicule structure in some *Heterodera* spp. *Nematologica* 19, 242–247. DOI: 10.1163/187529273X00367

Cliff, G. and Baldwin, J.G. (1985) Fine structure of body wall cuticle of females of eight genera of Heteroderidae. *Journal of Nematology* 17, 286–296.

Cooper, B.A. (1955) A preliminary key to British species of *Heterodera* for use in soil examination. In: Kevan, D.K.McE. (ed.) *Soil Zoology*. Butterworths, London, pp. 269–280.

Cordero, D.A. (1989) Comparative morphology and development of the cone of *Heterodera schachtii* and *Cactodera cacti*. PhD thesis, University of California, Riverside, California.

Cordero, D.A. and Baldwin, J.G. (1990) Effect of age on body wall cuticle morphology of *Heterodera schachtii* Schmidt females. *Journal of Nematology* 22, 356–361.

Cordero, D.A. and Baldwin, J.G. (1991) Fine structure of the posterior cone of *Heterodera schachtii* Schmidt (Heteroderinae) with emphasis on musculature and fenestration. *Journal of Nematology* 23, 110–121.

Cordero, D.A., Baldwin, J.G. and Mundo-Ocampo, M. (1991) Fine structure of the posterior cone of females of *Cactodera cacti* Filip'ev and Schuurmans Stekhoven (Nemata: Heteroderinae). *Revue de Nématologie* 14, 455–465.

Correia, J.S. and Abrantes, I.M. (1997) An improved technique for mounting *Heterodera* cysts for light microscopy. *Nematologica* 43, 507–509. DOI: 10.1163/005125997X00101

De Ley, P. and Bert, W. (2002) Video capture and editing as a tool for the storage, distribution and illustration of morphological characters of nematodes. *Journal of Nematology* 34, 296–302.

De Ley, P. and Blaxter, M.L. (2002) Systematic position and phylogeny. In: Lee, D.L. (ed.) *The Biology of Nematodes*. Taylor and Francis, New York, pp. 1–30.

De Ley, P., De Ley, I.T., Morris, K. *et al*. (2005) An integrated approach to fast and informative morphological vouchering of nematodes for applications in molecular barcoding. *Philosophical Transactions of the Royal Society* B 360, 1945–1958.

De Queiroz, K. (2007) Species concepts and species delimitation. *Systematic Biology* 56, 879–886.

Doncaster, C.C. and Shepherd, A.M. (1967) The behaviour of second-stage *Heterodera rostochiensis* larvae leading to their emergence from the egg. *Nematologica* 13, 476–478. DOI: 10.1163/187529267X00797

Eisenback, J.D. (1988) Multiple focus and exposure photomicroscopy of nematodes for increased depth of field. *Journal of Nematology* 20, 333–334.

Eisenback, J.D. (1991) Preparation of nematodes for scanning electron microscopy. In: Nickel, W.R. (ed.) *Manual of Agricultural Nematology*. Marcel Dekker Inc., New York, pp. 87–96.

Eisenback, J.D. and Zunke, U. (1998) Extraction, culturing and microscopy. In: Sharma, S.B. (ed.) *The Cyst Nematodes*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 141–155.

Ellenby, C. (1946) Nature of the cyst wall of the potato-root eelworm, *Heterodera rostochiensis* Wollenweber, and its permeability to water. *Nature* 157, 302–303.

Endo, B.Y. (1979) The ultrastructure and distribution of an intracellular bacterium-like microorganism in tissues of larvae of the soybean cyst nematode, *Heterodera glycines*. *Journal of Ultrastructure Research* 67, 1–14.

Endo, B.Y. (1980) Ultrastructure of the anterior neurosensory organs of the larvae of the soybean cyst nematode, *Heterodera glycines*. *Journal of Ultrastructural Research* 72, 349–366.

Endo, B.Y. (1983) Ultrastructure of the stomatal region of the juvenile stage of the soybean cyst nematode, *Heterodera glycines*. *Proceedings of the Helminthological Society of Washington* 50, 43–61.

Endo, B.Y. (1984) Ultrastructure of the esophagus of larvae of the soybean cyst nematode, *Heterodera glycines*. *Proceedings of the Helminthological Society of Washington* 51, 1–24.

Endo, B.Y. (1985) Ultrastructure of the head region of molting second-stage juveniles of *Heterodera glycines* with emphasis on stylet formation. *Journal of Nematology* 17, 112–123.

Endo, B.Y. (1988) Ultrastructure of the intestine of second and third juvenile stages of the soybean cyst nematode, *Heterodera glycines*. *Proceedings of the Helminthological Society of Washington* 55, 117–131.

Endo, B.Y. (1991) Ultrastructure of initial responses of susceptible and resistant soybean roots to infection by *Heterodera glycines*. *Revue de Nématologie* 14, 73–91.

Endo, B.Y. (1993) Ultrastructure of cuticular exudates and related cuticular changes on juveniles in *Heterodera glycines*. *Journal of the Helminthological Society of Washington* 60, 76–88.

Endo, B.Y. and Wyss, U. (1992) Ultrastructure of cuticular exudations in parasitic juvenile *Heterodera schachtii,* as related to cuticle structure. *Protoplasma* 166, 67–70.

Eyualem-Abebe, E., Baldwin, J.G., Adams, B. *et al*. (2006) A position paper on the electronic publication of nematode taxonomic manuscripts. *Journal of Nematology* 38, 305–311.

Franklin, M.T. (1939) On the structure of the cyst wall of *Heterodera schachtii* (Schmidt). *Journal of Helminthology* 17, 127–134.

Franklin, M.T. (1951) The cyst-forming species of *Heterodera*. Commonwealth Agricultural Bureaux, Farnham Royal, Farnham, UK.

Fuchs, O. (1911) Beitrage zur Biologie der Rubennematoden. *Zeitschrift für das landwirschaftliche Versuchswesen in Deutsch Österreich* 14, 923–949.

Golden, A.M. (1986) Morphology and identification of cyst nematodes. In: Lamberti, F. and Taylor, C.E. (eds) *Cyst Nematodes*. Plenum Press, New York, pp. 23–79.

Golden, A.M. and Raski, D.J. (1977) *Heterodera thornei* n. sp. (Nematoda: Heteroderidae) and a review of related species. *Journal of Nematology* 9, 93–112.

Goodey, J.B. (1963) *Laboratory Methods for Work with Plant and Soil Nematodes*. Technical Bulletin No. 2. Her Majesty's Stationery Office, Ministry of Agriculture, Fisheries and Food, London.

Granek, I. (1955) Additional morphological differences between the cysts of *Heterodera rostochiensis* and *Heterodera tabacum*. *Plant Disease Reporter* 39, 716–718.

Green, C.D. (1971) The morphology of the terminal area of the round-cyst nematodes S. G. *Heterodera rostochiensis* and allied species. *Nematologica* 17, 34–46. DOI: 10.1163/187529271X00396

Green, C.D. (1975) The vulval cone and associated structures of some cyst nematodes (genus *Heterodera*). *Nematologica* 21, 134–144. DOI: 10.1163/187529275X00491

Hesling, J.J. (1973) The estimation of Granek's ratio in round-cyst *Heteroderas*. *Nematologica* 19, 119–120. DOI: 10.1163/187529273X00213

Hesling, J.J. (1978) Cyst nematodes: morphology and identification of *Heterodera , Globodera* and *Punctodera*. In: Southey, J.F. (ed.) *Plant Nematology*. Her Majesty's Stationery Office, London, pp. 125–155.

Hirschmann, H. (1956) Comparative morphological studies on the soybean cyst nematode, *Heterodera glycines* and the clover cyst nematode, *H. trifolii* (Nematoda: Heteroderidae). *Proceedings of the Helminthological Society of Washington* 23, 140–151.

Hirschmann, H. and Triantaphyllou, A.C. (1979) Morphological comparison of members of the *Heterodera trifolii* species complex. *Nematologica* 25, 458–481. DOI: 10.1163/187529279X00613

Karnovsky, M.J. (1965) A formaldehyde-glutardehyde fixative of high osmolarity for use in electron microscopy. *Journal of Cell Biology* 27,137–138A.

Kang, H., Eun, G., Ha, J., Kim, Y., Park, N. and Kim, D. (2016) New cyst nematode, *Heterodera sojae* n. sp. (Nematoda: Heteroderidae) from soybean in Korea. *Journal of Nematology* 48, 280–289.

Kirjanova, E.S. (1969) [On the structure of the subcrystalline layer of the nematode genus *Heterodera* (Nematoda: Heteroderidae) with a description of two new species.] *Parazitologiya* 3, 81–91.

Kolotuev, I., Schwab, Y. and Labouesse, M. (2010) A precise and rapid mapping protocol for correlative light and electron microscopy of small invertebrate organisms. *Biology of the Cell* 102, 121–132.

Liebscher, G. (1892) Beobachtungen über das Aufreten eines Nematoden an Erbseb, *Journal für Landwirtschaft* 40, 357–368.

Luc, M., Weischer, B., Stone, A.R. and Baldwin, J.G. (1986) On the definition of heteroderid cyst. *Revue de Nématologie* 9, 418–421.

Mallet, J. (2008) A century of evolution: Ernst Mayr (1904–2005) Mayr's view of Darwin: was Darwin wrong about speciation. *Biological Journal of the Linnean Society* 95, 3–16.

Mallet, J. (2010) Why was Darwin's view of species rejected by twentieth century biologists? *Biology and Philosophy* 25, 497–527.

Markmann, M. and Tautz, D. (2005) Reverse taxonomy: an approach towards determining the diversity of meiobenthic organisms based on ribosomal RNA signature sequences. *Philosophical Transactions of the Royal Society B* 360, 1917–1924.

McClure, M.A. and Stowell, L.J. (1978) A simple method of processing nematodes for electron microscopy. *Journal of Nematology* 10, 376–377.

Miller, L.I. (1983) Diversity of selected taxa of *Globodera* and *Heterodera* and their interspecific and intergeneric hybrids. In: Stone, A.R. Pratt, H.M. and Khali, L.F. (eds) *Concepts in Nematode Systematics*. The Systematics Association Special Volume No. 22. Academic Press, New York, pp. 207–220.

Mulvey, R.H. (1959) Investigations on the clover cyst nematode, *Heterodera trifolii* (Nematoda: Heteroderidae). *Nematologica* 4, 147–156. DOI: 10.1163/187529259X00138

Mulvey, R.H. (1973) Morphology of the terminal areas of white females and cysts of the genus *Heterodera* (s.g. *Globodera*). *Journal of Nematology* 5, 303–311.

Mulvey, R.H. (1974) Cone top morphology of white females and cysts of the genus *Heterodera* (subgenus *Heterodera*), a cyst-forming nematode. *Canadian Journal of Zoology* 52, 77–81.

Mulvey, R.H. and Golden, A.M. (1983) An illustrated key to the cyst-forming genera and species of Heteroderidae in the Western Hemisphere with species morphometerics and distribution. *Journal of Nematology* 15, 1–29.

Mundo-Ocampo, M. and Baldwin, J.G. (1984) Comparison of host response of *Cryphodera utahensis* with other Heteroderidae and a discussion of phylogeny. *Proceedings of the Helminthological Society of Washington* 51, 25–31.

Mundo-Ocampo, M. and Baldwin, J.G. (1992) Comparison of host response of *Ekphymatodera thomasoni* with other Heteroderinae. *Fundamental and Applied Nematology* 15, 63–70.

Mundo-Ocampo, M., Troccoli, A., Subbotin, S.A., Del Cid, J., Baldwin, J.G. and Inserra, R.N. (2008) Synonymy *of Afenestrata* with *Heterodera* supported by phylogenetics with molecular and morphological characterization of *H. koreana* comb. n. and *H. orientalis* comb. n. (Tylenchida: Heteroderidae). *Nematology* 10, 611–632. DOI: 10.1163/156854108785787190

Nadler, S.A. (2002) Species delimitation and nematode biodiversity: phylogenies rule. *Nematology* 4, 615–625.

Nyaku, S.T., Sripathi, V.R., Kantety, R.V., Gu. U.Q., Lawrence, K. and Sharma, G.C. (2013) Characterization of the two intra-individual sequence variants in the 18S rRNA gene in the plant-parasitic nematode, *Rotylenchulus reniformis*. *PLOS ONE* 8, e60891. DOI: 10.1371/journal.pone.0060891

Othman, A.A., Baldwin, J.G. and Mundo-Ocampo, M. (1988) Comparative morphology of *Globodera, Cactodera,* and *Punctodera* spp. (Heteroderidae) with scanning electron microscopy. *Revue de Nématologie* 11, 53–63.

Pereira, T.J. and Baldwin, J.G. (2016) Contrasting evolutionary patterns of 28S and ITS rRNA genes reveal high genomic variation in *Cephalenchus* (Nematoda): implications for species delimitation. *Molecular Phylogenetics and Evolution* 98, 244–260. DOI: 10.1016/j.ympev.2016.02.016

Ragsdale, E.J., Crum, J., Ellisman, M.H. and Baldwin, J.G. (2008) Three-dimensional reconstruction of the stomatostylet and anterior epidermis in the nematode *Aphelenchus avenae*: Nematoda: Aphelenchidae) with implications for the evolution of plant parasitism. *Journal of Morphology* 269, 1181–1196. DOI: 10.1002/jmor.10651.

Ragsdale, E.J., Ngo, P., Crum, J., Ellisman, M.H. and Baldwin, J.G. (2009) Comparative, three-dimensional anterior sensory reconstruction of *Aphelenchus avenae* (Nematoda: Tylenchomorpha). *Journal of Comparative Neurology* 517, 616–632. DOI: 10.1002/cne.22170

Raski, D.J. (1950) The life history and morphology of the sugar-beet nematode, *Heterodera schachtii* Schmidt. *Phytopathology* 40, 135–152.

Riggs, R.D. (1990) Making perineal patterns of root-knot nematodes and vulval cones of cyst nematodes. In: Zuckerman, B.M., Mai, W.F. and Krusberg, L.R. (eds) *Plant Nematology Laboratory Manual*, revised edn. The University of Massachusetts Agricultural Experiment Station, Amherst, Massachusetts, pp. 103–106.

Sammons, R. and Marquis, P. (1997) Application of the low vacuum scanning electron microscope to the study of biomaterials and mammalian cells. *Biomaterials* 18, 81–86.

Shepherd, A.M. and Clark, S.A. (1978) Cuticle structure and 'cement' formation at the anterior end of female cyst-nematodes of the genera *Heterodera* and *Globodera* (Heteroderidae: Tylenchida). *Nematologica* 24, 201–208. DOI: 10.1163/187529278X00407

Shepherd, A.M., Clark, S.A. and Dart, P.J. (1972) Cuticle structure in the genus *Heterodera*. *Nematologica* 18, 1–17. DOI: 10.1163/187529272X00197

Shepherd, A.M., Clark, S.A. and Kempton, A. (1974) Spermatogenesis and sperm ultrastructure in some cyst nematodes, *Heterodera* spp. *Nematologica* 19, 551–560. DOI: 10.1163/187529273X00574

Sher, S.A. and Bell, A.H. (1975) Scanning electron micrographs of the anterior region of some species of Tylenchoidea (Tylenchida: Nematoda). *Journal of Nematology* 7, 69–83.

Stone, A.R. (1975) Head morphology of second-stage juveniles of some Heteroderidae (Nematoda: Tylenchoidea). *Nematologica* 21, 81–88. DOI: 10.1163/187529275X00374

Sturhan, D. (2016) On the presence or absence of phasmids in males of Heteroderidae (Tylenchida). *Nematology* 18, 23–27. DOI: 10.1163/15685411-00002939

Subbotin, S.A., Mundo-Ocampo, M. and Baldwin, J.G. (2010) *Systematics of cyst nematodes (Nematoda: Heteroderinae)*. Nematology Monographs and Perspectives, 8A. (Series eds: Hunt, D.J. and Perry, R.N.). Leiden, The Netherlands, Brill.

Sulston, J. and Hodgkin, J. (1988) *Methods*. In: Wood, W.B. (ed.) *The Nematode* Caenorhabditis elegans. Cold Springs Harbor Press, New York, pp. 587–606.

Thorne, G. (1961) *Principals of Nematology*. McGraw Hill, New York.

Triantaphyllou, A.C. and Hirschmann, H. (1962) Oogenesis and mode of reproduction in the soybean cyst nematode, *Heterodera glycines*. *Nematologica* 7, 235–241. DOI: 10.1163/187529262X00224

Triantaphyllou, A.C. and Hirschmann, H. (1980) Cytogenetics and morphology in relation to evolution and speciation of plant-parasitic nematodes. *Annual Review of Phytopathology* 18, 333–359.

Turner, S.J. and Subbotin, S.A. (2013) Cyst nematodes. In: Perry, R.N. and Moens, M. (eds) *Plant Nematology*. CAB International, Wallingford, UK, pp. 91–122.

Walsh, J.A. and Shepherd, A.M. (1983) A further observation on sperm structure in a *Heterodera* sp. *Revue de Nématologie* 6, 148–150.

Willats, W.G., Atkinson, H.J. and Perry, R.N. (1995) The immunofluorescent localization of subventral pharyngeal gland epitopes of preparasitic juveniles of *Heterodera glycines* using laser scanning confocal microscopy. *Journal of Nematology* 27, 135–142.

Wilson, E.M. (1968) Vulval bodies in certain species of *Heterodera*. *Nature* 217, 879.

Wouts, W.M. and Baldwin, J.G. (1998) Taxonomy and identification. In: Sharma, S.B. (ed.) *The Cyst Nematodes*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 83–122.

Wouts, W.M. and Weischer, B. (1977) Eine Klassifizierung von fünfzehn in Westeuropa häufigen Arten der Heteroderinae auf Grund von Larvenmerkmalen. *Nematologica* 23, 289–310. DOI: 10.1163/187529277X00039

Wyss, U. and Zunke, U. (1986a) The potential of high resolution video-enhanced contrast microscopy in nematological research. *Revue de Nématologie* 9, 91–94.

Wyss, U. and Zunke, U. (1986b) Observations on the behaviour of second-stage juveniles of *Heterodera schachtii* inside host roots. *Revue de Nématologie* 9, 153–165.

Zunke, U. (1986) Zur Bildung der subkristallinen Schicht bei *Heterodera schachtii* unter aseptischen Bedingungen. *Nematologica* 31, 117–120. DOI: 10.1163/187529285X00157

Zunke, U. and Eisenback, J.D. (1998) Morphology and ultrastructure. In: Sharma, S.B. (ed.) *The Cyst Nematodes*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 31–56.