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The cellular structure of the female reproductive system within the Heteroderinae and Meloidogyninae (Nematoda)

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Summary – Gonads from living young females, representing 23 different species, were extracted to study the cellular structure of the female genital structure within the Meloidogyninae and Heteroderinae. All genera studied can be characterised by their cellular spermatheca morphology. Within *Meloidogyne* a spherical spermatheca is found with lobe-like protruding cells, most species having 16 to 18 spermatheca cells with interlaced cell boundaries while *M. microtyla* and *M. ichinohei* have more spermatheca cells with different cell boundaries. *Heterodera* and *Globodera* reveal a comparable gonad structure. The spermatheca cells of *Heterodera* are columnar and arranged in a restricted number of rows, whereas in *Globodera* the spermatheca cells are squarish to rounded, depending on the species. The gonad morphology of *Afenestrata koreana* is clearly different from what would be expected based on the related genera *Globodera* and *Heterodera*. The apparently simplest genital system was found in *Meloidodera floridensis* where the uterus has a limited number of cells. In the other genera studied a large and variable cell number was found.

Keywords – *Afenestrata*, female genital system, *Globodera*, *Heterodera*, *Meloidodera*, *Meloidogyne*, morphology, SEM, variable cell number.

Analysis of the cellular structure of the female reproductive system for Secernentea was mainly executed by Geraert (1972, 1976, 1978) and Geraert *et al.* (1980a, b) who also pointed out the importance of the female reproductive system in nematode systematics (Geraert, 1981, 1983). More isolated studies of the reproductive system of Tylenchida were undertaken by several authors (Wu, 1958, 1967; Seinhorst, 1968; Bert & Geraert, 2000). Chizhov and Swiliam (1986) and Chizhov and Berezina (1988a, b) studied several nematode species, especially including Heteroderinae and Meloidogyninae. The first *Meloidogyne* spermatheca drawing was published by Nagakura (1930). Triantaphyllou and Hirschmann (1962) made a detailed illustration of the reproductive system of *Heterodera glycines* and Triantaphyllou (1962, 1987) focused on some *Meloidogyne* species. The terminology of the reproductive system used here is based on Geraert (1983) who in turn followed the interpretation of Chitwood and Chitwood (1950). The genital system consists of an ovary (= gonad) and gonoduct. The oviduct is the constricted region between the ovary and spermatheca or uterus. The rest of the gonoduct is called the uterus and uterine sac. The terms tricolumella (Hirschmann & Triantaphyllou, 1968), quadricolumella (Wu, 1958) and polycolumella (Chizhov & Swiliam, 1986) describe the spatial arrangements of the uterus cells. Diversity and evolution of Heteroderinae and Meloidogyninae are of special interest because world-wide they include some of the most destructive plant-parasitic nematodes. In their comprehensive study, based on comparative detailed morphology, of the phylogeny of the Heteroderinae, Baldwin and Schouest (1990) commented on the cellular structure of the female reproductive tract as being potentially useful for interpreting phylogeny. However, they excluded existing information from their matrix pending examination of more excised reproductive tracts. More recently, Zunke and Eisenback (1998) remarked that additional investigations are needed, particularly on the fine structure of the reproductive system, to improve our understanding of the phylogenetic relationships among the numerous members of this group. In this paper, we examine the cellular structure of several species and genera within the Heteroderinae and Meloidogyninae in order to shed new light on such questions.

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Material and methods

A list of the species examined (and their authorities) is presented in Table 1. To study the cellular structure of the female reproductive system, gonads were first extruded. Four to six young females of every population were put in a drop of water on the glass slide and then cut with an eye-knife, leading to expulsion of gut and gonad. After removal of most of the non-reproductive tissue, the preparation, covered by a coverslip, was studied at once under the light microscope. In order to have an idea about the variability of the studied structure, this procedure was repeated until at least 20 preparations could be observed for each population. The dissection process must have an influence on the size and shape of the cells due to a changing osmolarity. However, repeated dissections demonstrated that such influences are relatively constant within a species. When preparations are stained with acetic orcein (2% aqueous solution of orcein in acetic acid) or acid fuchsin (aqueous solution), the nuclei of the cells are more clearly visible. This can be very helpful for smaller nematodes, but for members of Heteroderinae and Meloidogyninaeno additional information is obtained from such staining.

Observations on the internal structures can best be made on freshly dissected specimens, which usually plasmolyse and decompose within 1 h. With the purpose of permanently recording the morphology of the reproductive system, we have also produced some short video clips that mimic multifocal observation through the light microscope. This was done with the aid of a Video Capture and Editing (VCE) method (De Ley & Bert, in press) and the results can be viewed on http://www.vce.be.tf.

For scanning electron microscopic (SEM) observation, excised reproductive systems from 50 specimens were transferred and fixed in freshly prepared 4% formaldehyde (from paraformaldehyde) in phosphate buffered saline (PBS) pH 7 at room temperature. The reproductive systems were fixed overnight and subsequently dehydrated in 25, 50, 75 and 95% ethanol at 2-hourly intervals, followed by an overnight dehydration in absolute ethanol and final removal of the remaining water using silica gel. After critical-point drying using CO_2 (Wergin, 1981) as the drying liquid and sputter coating with 20 nm gold, they were examined with a JEOL JSM-840 at 15 kV.

The cellular structure of the ovary is excluded from this study, because it proved difficult to observe the cellular architecture using only light microscopy. Furthermore, there is no reported evidence for any taxonomic value of this cellular structure. Because of the elongation of the uterus in nearly all Heteroderinae and Meloidogyninae, only the part following the spermatheca was illustrated in those cases where the uterus was demonstrably homogeneous in structure.

Results

A summary of the results with emphasis on the divergent characters is presented in Table 2.

Meloidogyne Göldi, 1892

The oviduct-spermathecaregion of all the species studied is characteristic and typical of the genus *Meloidogyne*. The oviduct comprises two rows of four thick cells. The spermatheca is spherical and formed by a variable number of lobe-like cells. The uterus consists of three long rows of cells (= a tricolumella). In *M. incognita* 3×65 to 3×95 cells have been counted.

The spermathecae of *M. arenaria*, *M. ardenensis, M. artiellia*, *M. chitwoodi*, *M. exigua*, *M. graminicola*, *M. hapla*, *M. hispanica*, *M. incognita*, *M. javanica, M. morocciensis* and *M. trifoliophila* are similar, comprising 16 to 18 lobe-like cells with interlaced cell boundaries (Fig. 1A). The spermatheca of *M. fallax* is slightly different in that in 35% of the specimens four or five spermatheca cells are clustered together to form separate lobes (Fig. 1B). *Meloidogyne microtyla* has a spermatheca with 18 to 26 cells and the cell boundaries are only slightly interlaced (Fig. 1C). The spermatheca of *M. ichinohei* is clearly different from the remaining *Meloidogyne* species and has 18 to 30 cells forming irregular lobes (Fig. 1D). Sperm was only observed in the spermatheca of *M. artiellia* and *M. fallax*. SEM of the reproductive system of *M. incognita* (Fig. 2) confirms the light microscopic observations. The two rows of four large oviduct cells can be observed as protruding cells. The membranes of the last two oviduct cells seem to be fused in some specimens and appear more like spermatheca cells (Fig. 2B). The lobe-like cells of the spermatheca are pronounced, but the interlaced cell boundaries, as seen by light microscopy (Fig. 1), cannot be seen with SEM. SEM also fails to show the external cell boundaries of the uterus.

Globodera Skarbilovich, 1959

The oviduct is made up of two rows of four cells. The spermatheca of an excised gonad forms the corner

Table 1. *List of species studied, identification method, examination method(s) and source.*

Species	Identification method	Examination method	Source
Meloidogyne arenaria (Neal, 1889) Chitwood, 1949	Light microscopy (LM) & isozymes	LM & Video Capture and Editing (VCE)	Gerrit Karssen Plant Protection Service, Wageningen, The Netherlands
M. ardenensis Santos, 1968	Idem	LM	Idem
M. artiellia Franklin, 1961	Idem	LM & VCE	Idem
M. chitwoodi Golden, O'Bannon, Santo & Finley, 1980	Idem	LM & VCE	Idem
M. exigua Göldi, 1892	Idem	LM	Idem
M. fallax Karssen, 1996	Idem	LM & VCE	Idem
M. graminicola Golden & Birchfield, 1965	Idem	LM	Idem
M. hapla Chitwood, 1949, race A	Idem	LM & VCE	Idem
M. hispanica Hirschmann, 1986	Idem	LM & VCE	Idem
M. ichinohei Araki, 1992	Idem	LM	Idem
M. incognita (Kofoid & White, 1919) Chitwood, 1949	Idem	LM & TEM	Idem
M. javanica (Treub, 1885) Chitwood, 1949	Idem	LM & VCE	Idem
M. morocciensis Rammah & Hirschmann, 1990	Idem	LM	Idem
M. microtyla Mulvey, Townshend & Potter, 1975	Idem	LM & VCE	Idem
M. trifoliophila Bernard & Eisenback, 1997	Idem	LM & VCE	Idem
Globodera pallida (Stone, 1973) Behrens, 1975	Protein electrophoresis	LM & VCE	Agricultural Research Centre, Department Crop Protection, Ghent, Belgium
G. rostochiensis (Wollenweber, 1923) Skarbilovich, 1959	Idem	LM	Idem
G. tabacum (Lownsbery & Lownsbery, 1954) Behrens, 1975	LM & Protein electrophoresis	LM & VCE	Gerrit Karssen Plant Protection Service, Wageningen, The Netherlands
Heterodera avenae Wollenweber, 1924	LM	LM	Botanical garden Ghent University, Belgium
H. fici Kirjanova, 1954	LM	LM	Gerrit Karssen Plant Protection Service, Wageningen, The Netherlands
H. schachtii Schmidt, 1871	LM	LM	Department of Genetics, Ghent University, Belgium
Afenestrata koreana Vovlas, Lamberti & Choo, 1992	LM	LM	Renato Inserra FDACS, Gainesville, FL, USA
Meloidodera floridensis Chitwood, Hannon & Esser, 1956	LM	LM & VCE	Manuel Mundo-Ocampo University of California, Riverside, CA, USA

Table 2. *Summary of the results with emphasis on the divergent characters.*

Meloidogyne	spermatheca: spherical, formed by lobe-like cells uterus: long tricolumella	*M. arenaria*, *M. ardenensis*, *M. artiellia*, *M. chitwoodi*, *M. exigua*, *M. graminicola*, *M. hapla*, *M. hispanica*, *M. incognita*, *M. javanica*, *M. morocciensis*, *M. trifoliophila* and *M. fallax* spermatheca: 16 cells with interlaced cell boundaries *M. fallax* spermatheca cells often form separate lobes *M. microtyla* spermatheca: 18 to 26 cells, the cell boundaries are only slightly interlaced *M. ichinohei* spermatheca: 18 to 30 cells forming irregular lobes, no interlaced cell boundaries
Globodera	spermatheca: variously shaped cells, forms corner of a right angle between oviduct and uterus uterus: polycolumella	*G. pallida* spermatheca: mostly 12 cells, two big round cells adjacent to oviduct *G. rostochiensis* spermatheca: mostly 14 equally sized cells *G. tabacum* spermatheca: mostly 12 cells, four cells adjacent to oviduct squarish to round and followed by flattened cells
Heterodera	spermatheca: ten to 16 columnar cells basically arranged in two rows, bend at junction of the spermatheca and uterus uterus: tricolumella (with in some cases additional rows)	*H. avenae* and *H. fici* uterus: tricolumella *H. schachtii* uterus: additional rows of cells along the tricolumella
Afenestrata koreana	spermatheca: oval shaped, consists of 20 to 26 variably arranged cells uterus: tricolumella	
Meloidodera floridensis	spermatheca: partly offset, composed of 12 variably shaped small cells with unclear cell boundaries, uterus forms a 90 degree angle with oviduct uterus: short tricolumella	

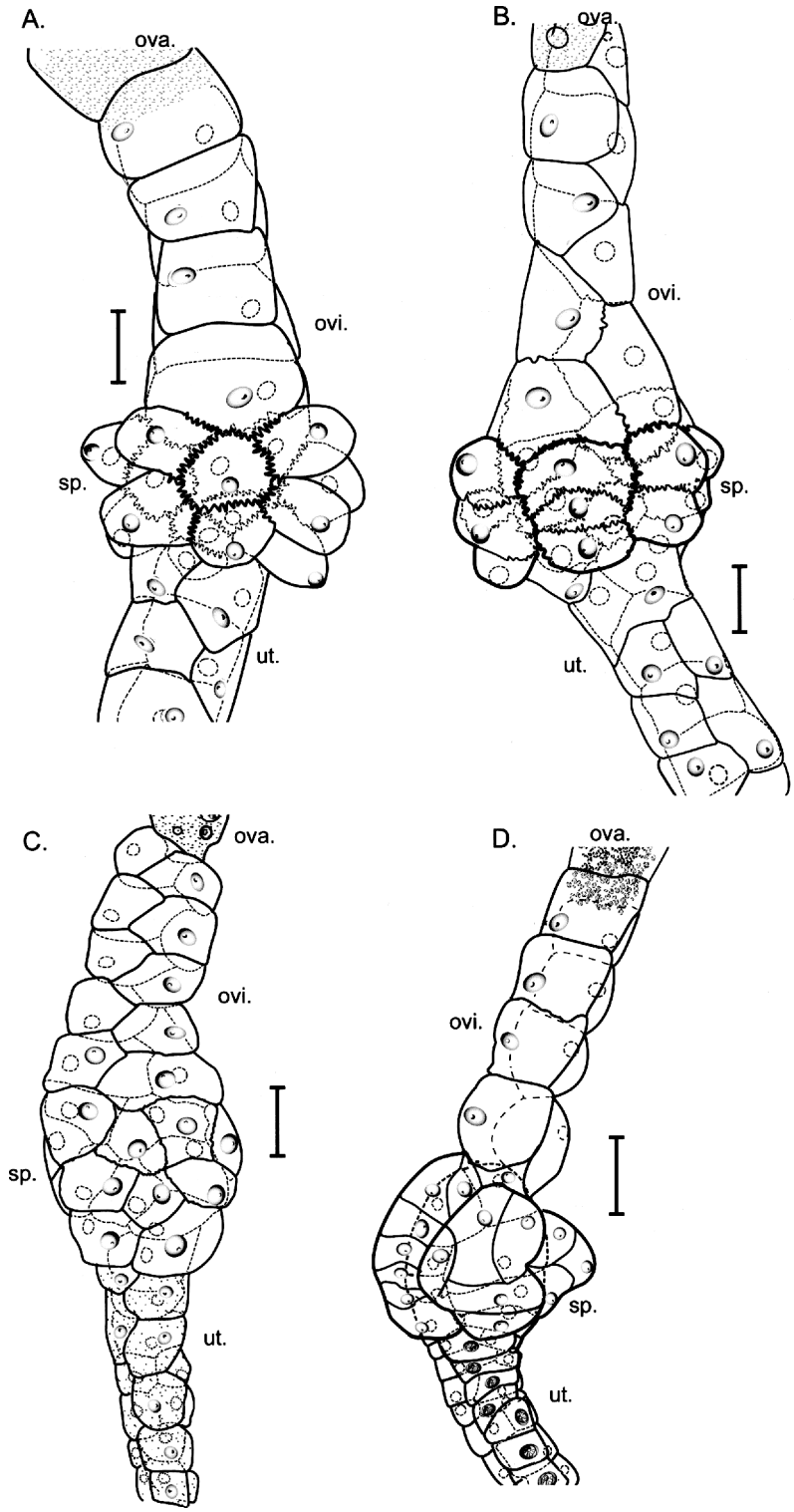

Fig. 1. *Oviduct-spermatheca region of* Meloidogyne *spp. A:* Meloidogyne hispanica*; B:* M. fallax *C:* M. microtyla*; D:* M. ichinohei*. End of ovary (ova.), oviduct (ovi.), spermatheca (sp.), beginning of uterus (ut.). (Scale bars = 30 μm.)

of a right angle between oviduct and uterus. Thirty eight of 45 females from the three studied *G. pallida* populations have 12 cells comprising the spermatheca. Two rounded cells, larger than the other spermatheca cells, are located adjacent to the oviduct. Sperm was observed in the vicinity of these cells. The remaining cells are flattened to slightly rounded (Fig. 3B). The spermatheca of *G. rostochiensis* has a slightly different cellular architecture with 12 to 16 (mostly 14) cells of more or less equal in size with a rounded to squarish shape. Sperm was always observed dispersed uniformly in the spermatheca (Fig. 3A). The spermatheca of *Globodera tabacum* consists of 12 cells, the first four cells being squarish to round and followed by flattened cells arranged in two rows. The highest concentration of sperm was found in that part of the spermatheca closest to the oviduct. The uterus of the studied *Globodera* species consists of two or three short rows (mostly four cells long) at the connection with the spermatheca, but increases to multiple (six to ten) rows (an irregular polycolumella).

Heterodera Schmidt, 1871

Heterodera species (Fig. 3C, D) exhibit a reproductive system similar to that of *Globodera*. The oviduct consists of two rows of four large oval cells. The spermatheca of *H. avenae*, *H. fici* and *H. schachtii* is composed of ten to 16 high columnar cells, 12 cells being observed in most cases. The cells are basically arranged in two rows, but in the middle of the spermatheca three cells are positioned at the same level. A characteristic bend occurs at the junction of the spermatheca and uterus. The uterus of *H. avenae* and *H. fici* consists of an elongated tricolumella. *Heterodera schachtii* has additional rows of cells along the tricolumella and, closer to the vulva, up to five cell rows occur. Two nuclei were observed in some of these cells.

Afenestrata koreana

Two rows of four cells make up the oviduct. The spermatheca is more or less oval shaped and consists of 20 to 26 variably arranged cells (Fig. 4A). Counting the exact number of cells was difficult because cell boundaries were not always clearly visible. The transition to the uterus is vague and no clear bend occurs at the junction of spermatheca and uterus as in *Globodera* or *Heterodera*. The uterus includes an elongated tricolumella.

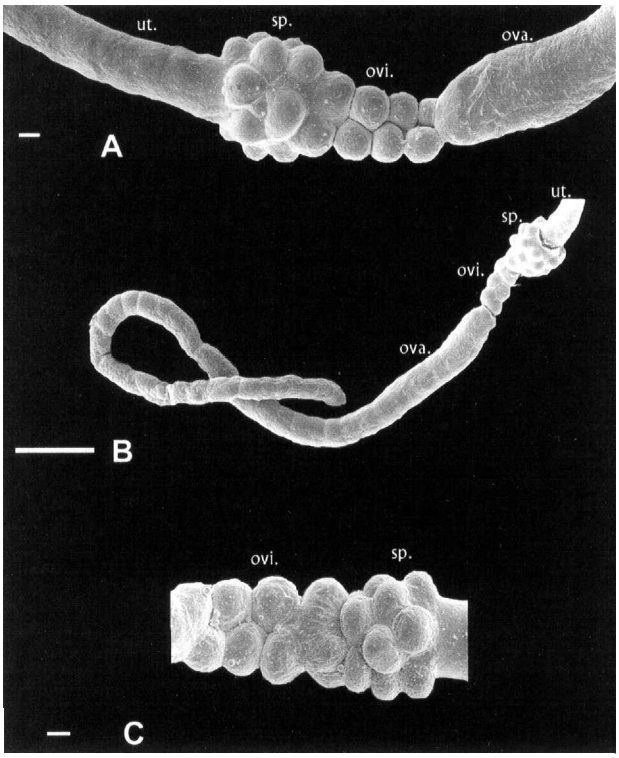

Fig. 2. *SEM of female reproductive system of* Meloidogyne incognita*: end of ovary (ova.), oviduct (ovi.), spermatheca (sp.) and beginning of uterus (ut.). (Scale bars: A, C = 10 μm, B = 100 μm.)*

Meloidodera floridensis

As is typical within Tylenchida, the oviduct of *M. floridensis* consists of two rows of four cells each. The spermatheca is partly offset and composed of 12, variably shaped, small cells, the boundaries of which are unclear. After excision, the uterus forms a 90° angle with the oviduct. The tricolumella forming the uterus is short and only six to seven cells long. The eggs are deposited in a membranous, outstretched uterine sac following the tricolumella (Fig. 4B).

Discussion

The oviduct

The morphology of the oviduct has been described as a remarkably stable structure within the Tylenchida (Geraert, 1983). Our expanded light microscopic studies do not deviate from this as, without exception, the oviduct comprises two rows of four cells. However, with SEM the oviduct of *M. incognita* did not appear to be a

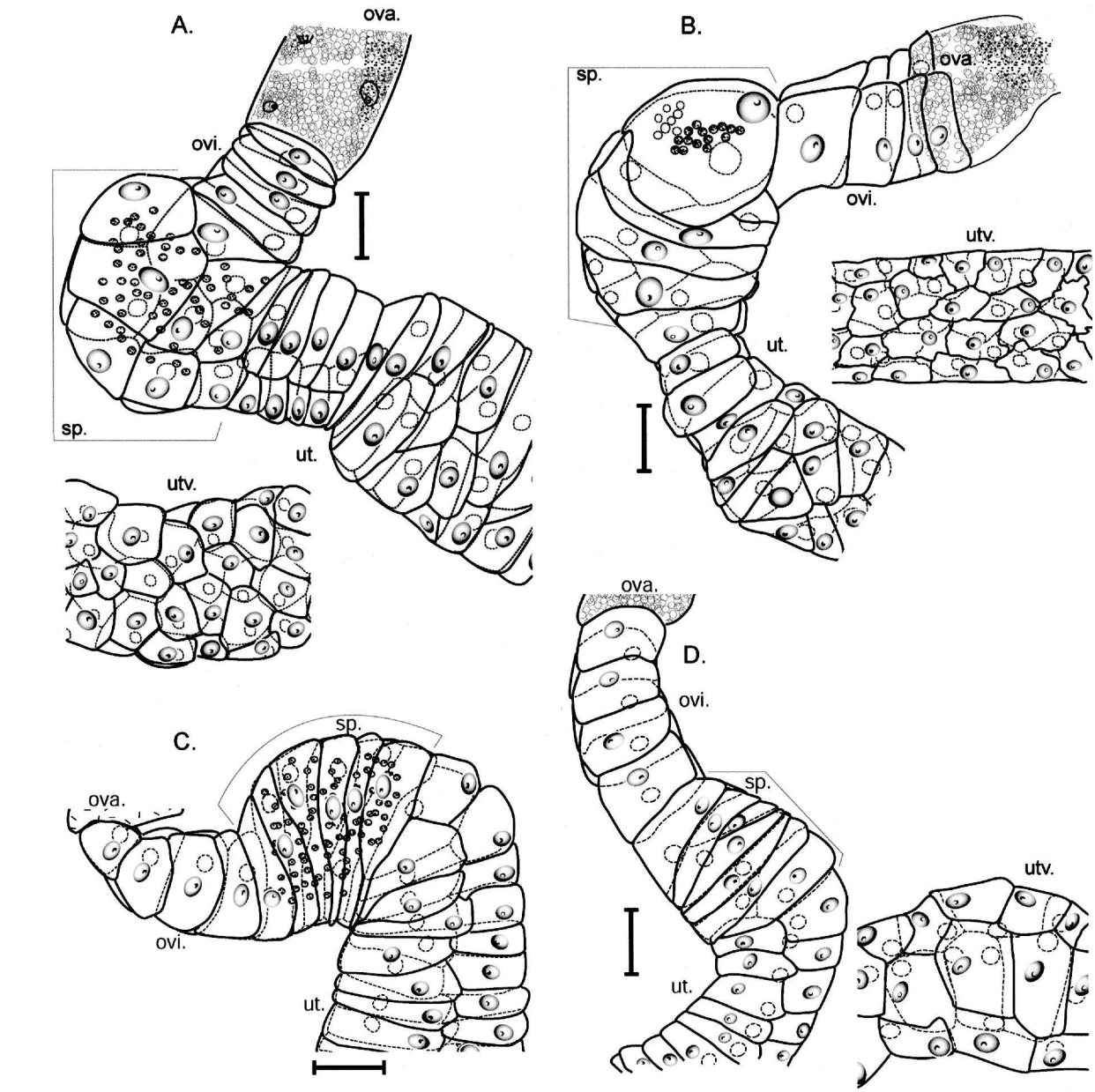

Fig. 3. *Female reproductive system of Heteroderinae. A:* Globodera rostochiensis*; B:* G. pallida *C:* Heterodera avenae*; D:* H. schachtii. *End of ovary (ova.), oviduct (ovi.), spermatheca (sp.), beginning of uterus (ut.) and uterus closer to the vulva (utv.). (Scale bars = 30 μm.)*

clear, morphologically discrete, unit. Other studies (TEM, immuno-histochemical) are required in order to determine exactly, on the basis of function, the separate gonoduct components.

THE SPERMATHECA

The spermatheca has been shown to have particular value as a taxonomic character. All studied genera can be characterised by a combination of spermatheca morphology and cell number.

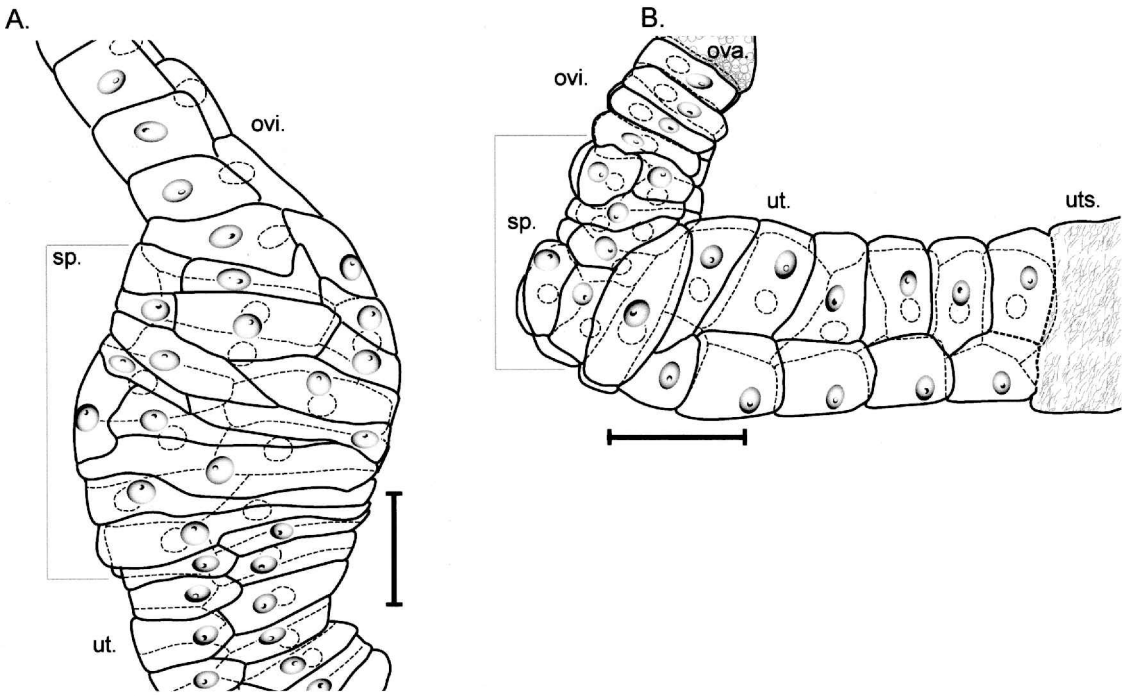

Fig. 4. *Female reproduction systems. A:* Afenestrata koreana*; B:* Meloidodera floridensis*. End of ovary (ova.), oviduct (ovi.), spermatheca (sp.), uterus (ut.) and uterine sac (uts.). (Scale bars = 30 μm.)*

Meloidogyne

Since spermatheca morphology and the number of cells varies among species of *Meloidogyne*, one consideration is whether this variability is convergent or if it directly reflects evolution within the genus. Conversely, these differences in spermatheca may be convergent functional adaptations linked to particular modes of reproduction that also vary within the genus. According to Chizhov (1981), obligatory mitotic parthenogenetic species, such as *M. incognita*, have a spermatheca with 24 small cells. Conversely, facultative meiotic parthenogenetic species, such as some *M. hapla,* have a spermatheca with only 16 large cells. This does not correspond with our results or with results from the literature as described below.

We found a spermatheca composed of approximately 16 cells in both obligate, as well as facultative, meiotic parthenogenetic species. These results are congruent with those of Geraert (1978), who found a maximum of 16 spermatheca cells for *M. incognita*, *M. arenaria* and *M. javanica.* Only a maximum of 18 large cells can be inferred from Triantaphyllou's (1962) drawing of the spermatheca of *M. javanica.* Triantaphyllou (1987), who studied *M. spartinae* (Rau & Fassuliotis, 1965) Whitehead, 1965, an amphimictic species with approximately 34 cells, used Chizhov's (1981) results to suggest that obligate mitotic parthenogenetic species with many spermatheca cells probably evolved from amphimictic species. These amphimictic species also have a large number of spermatheca cells, the additional cells presumably being required to accommodate many spermatozoa. Our results do not support this view. Nevertheless, the hypothesis that strictly amphimictic species have a larger number of spermatheca cells can be acknowledged given the fact that *M. microtyla*, an amphimictic species, displays a large number of cells. However, in other nematode genera, such as *Pratylenchus*, a correlation with the number of spermatheca cells and the mode of reproduction has never been found (Bert, unpubl.). The presence of males may influence spermatheca shape, the spermatheca cells of a *M. fallax* population with abundant males being clustered together and forming lobes compared with the morphologically similar *M. chitwoodi.*

Triantaphyllou (1985) inferred the phylogeny of root-knot nematodes from cytogenetic data and hypothesised that strictly amphimictic species are ancestral compared to the parthenogenetic ones. If this is true it would suggest a root-knot spermatheca with a higher number of cells is

the more primitive condition relative to those with fewer cells.

In a recent comprehensive phylogenetic analysis of *Meloidogyne* SSU rDNA (Tandigan De Ley *et al.*, in press), the amphimictic species *M. microtyla* is, however, positioned in a clade together with *M. hapla*. In this study, *M. ichinohei*, a species with a morphologically divergent spermatheca (18 to 30 cells forming four lobes), was invariably placed with maximal support as a sister taxon to all other species of *Meloidogyne*.

Although we have demonstrated intraspecific spermatheca variability within the genus *Meloidogyne*, the spermatheca is always spherical and formed by a variable number of thick, lobe-like cells, thus making it distinctive from any other known nematode genus. Members of the genus *Meloidogyne* are strongly unified by this feature (Triantaphyllou, 1987) and this feature can be added to the long list of reasons for not considering root-knot nematodes and cyst nematodes as closely related taxa (Baldwin, 1992; Geraert, 1997). This is also congruent with molecular findings as *Meloidogyne* species were found to be remarkably divergent from each other in 18S sequence and genetically distant from other genera in Tylenchida (Szalanski *et al.*, 1997). Our results do not allow us to speculate about the relationship with other genera. Pourjam *et al.* (2000) working with a *Zygotylenchus* population from Iran, described a spermatheca apparently consisting of numerous cells, *i.e.*, reminiscent of *Meloidogyne*. However, their observation was based on fixed nematodes mounted in glycerine. After dissection of living specimens of *Zygotylenchus* it appeared that some sperm cells were remarkably swollen (Bert, unpubl.) and they were probably interpreted as spermatheca wall cells by Pourjam *et al.* (2000).

Globodera

The different spermatheca morphology of *G. pallida* and *G. rostochiensis* is remarkable. The two potato cyst nematode species are known to exhibit exceptionally similar morphologies and were, until 1970, considered to be pathotypes of the species *Heterodera rostochiensis* Wollenweber, 1923. Both sibling species, however, were shown to diverge at the molecular level (Folkertsma *et al.,* 1994), protein profile (Bakker & Bouwman-Smits, 1988) and behaviour (Mugniéry, 1979; Den Nijs, 1992).

The seven populations in this study were investigated to determine whether the spermatheca morphology could be used as a differentiating character. Unfortunately, the inter- and intrapopulation variation of the spermatheca is too high to be practically useful on its own for species diagnosis. Nevertheless, spermatheca morphology can supplement other tools to morphologically separate the two cyst nematodes.

Heterodera

The *Heterodera* reproductive structures as described in the literature are comparable with our results. *Heterodera glycines* Ichinohe, 1952 was illustrated by Triantaphyllou and Hirschmann (1962), and *H. avenae* and *H. schachtii* were described by Chizhov and Swiliam (1986). It can be concluded that the genus *Heterodera* exhibits a uniform and characteristic spermatheca.

Afenestrata koreana

In most recent phylogenetic analyses, *Afenestrata* is suggested as a sister taxon of *Heterodera* (Wouts, 1985; Baldwin & Schouest, 1990; Baldwin, 1992) and *A. orientalis* Kazachenko, 1989 is even placed within *Heterodera* based on ribosomal DNA sequences (Subbotin *et al.*, 2001). However, in this study the spermatheca morphology of *Afenestrata* was shown to be clearly different from that of *Heterodera*, whereas *Heterodera* and *Globodera* appear to have similar spermathecas. Recently, Inserra *et al.* (1999) found clear differences between *A. koreana* and *Heterodera* in cyst morphology, physiology and other features. For instance, the syncytium induced by *A. koreana* differed from that of *Heterodera* spp. because it lacked ingrowths of the cell walls. All these new data suggest that the position of the *Afenestrata* species within the subfamily Heteroderinae is still far from resolved.

Meloidodera floridensis

Meloidodera is generally recognised as expressing mostly conserved characters for the subfamily. A spermatheca with 12 cells and a short tricolumella is reminiscent of that described for some Belonolaimidae, Pratylenchidae and Rotylenchidae (Geraert, 1981; Bert & Geraert, 2000). The morphology of the female reproductive system, based on outgroup comparisons, also suggests that this character is plesiomorphic.

THE UTERUS

Geraert (1986) pointed out the importance of the cellular arrangement of the uterus, in particular the tricolumella/quadricolumella difference, for the classification of the Tylenchida. Although a tricolumella is clearly the basic scheme in Heteroderinae and Meloidogyninæ, in some *Globodera* and *Heterodera* multiple rows of cells are found which enlarge the uterus to a polycolumella.

A tricolumella is found without exception in the families Dolichodoridae, Belonolaimidae, Pratylenchidae and Rotylenchidae and in the subfamilies Heteroderinae and Meloidogyninae (Geraert, 1981; Chizhov & Berezina, 1988b; Bert, unpubl.). These taxa were collectively given a subordinal rank by Chizhov and Berezina (1988b) and the tricolumella was used as one of the synapomorphies, an action supported by Siddiqi (2000).

According to the literature and research on about 70 species of Tylenchida not belonging to the Heteroderinae and Meloidogyninae (Bert, unpubl.), the female genital apparatus is composed of a small and nearly constant number of cells. Geraert (1981), based on observations on several nematode orders, but excluding Heteroderinae and Meloidogyninae, concluded that the genital system had an invariable number of cells. However, the Heteroderinae and Meloidogyninae (except *Meloidodera* with conserved characters) have a large and variable number of cells in the uterus. Furthermore, in some uterus cells of *Heterodera schachtii* more than one nucleus was found, giving the impression of an ongoing cell division. These differences from other Nematoda may be determined by differences in functional requirements such as the mode of reproduction associated with a sedentary way of life.

The number of uterus cells in *Meloidodera* is limited and invariable and the eggs are found in a membranous structure which is not formed by separate cells. This can be regarded as a primitive way to accommodate the large egg numbers characteristic of the extremely fecund Heteroderinae and Meloidogyninae.

Evolution of the uterus from one with a small number of cells to one with a large number must have arisen at least twice; once in the genus *Meloidogyne* and once in the Heteroderinae.

The cellular enlargement of the uterus differs among the genera studied. Specifically, *Meloidogyne*, *Afenestrata* and *Heterodera avenae* (*Bidera*) have a regular elongated tricolumella, *Heterodera schachtii* has an elongated tricolumella which enlarges to a variable polycolumella towards the vulva while *Globodera* has an elongated polycolumella almost immediately proximal to the spermatheca.

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