Morphology, diversity, taxonomy and phylogeny of Tylenchidae (Nematoda, Tylenchomorpha)

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Acknowledgments

First and foremost I would like to express my deepest gratitude to my promoter, Professor Wim Bert, who has been my promoter since my master thesis, for his excellent guidance, caring, patience, and providing me with an excellent atmosphere for doing research. I also would like to thank my jury members for their critical comments and suggestions: Prof. Tom Moens, Prof. Annemieke Verbeken, Prof. Etienne Geraert, Prof. Olivier De Clerck and Dr. Matthew Back.

Every result described in this thesis was accomplished with the help and support of my colleagues: Marjolein Couvreur, Myriam Claeys, Alcides Sanchez-Monge, Dieter Slos, Hanne Steel, Yao Kolombia, Toon Janssen, Giselle Herren and Daniel Apolonio, fellow by my collaborators: Prof. Wilfrida Decraemer, Dr. Tiago Pereira and Dr. Irma De Ley.

This thesis could not have been completed without the great support and encouragement that I have received from many Chinese professors over the years. I wish to offer my heartfelt thanks to Prof. Hongmei Li who recommended me to study in Ghent. I would like to thank Prof. Guokun Liu, Prof. Wenkun Huang and Prof. Kan Zhuo, I sequentially met them in Ghent and spent really nice time together.

I am grateful for the Chinese Scholarship Council (CSC) who provides funding that allowed me to pursue my PhD research. I would like to thank my master scholarship VLIR-UOS, who offered me the opportunity to come to Belgium and study at Ghent University. I would like to thank the coordinators for the Nematology master course: Nicole Smol and Inge Dehennin. My graduate experiences benefited greatly from the course.

I would like to thank Huaihan Cai, Yiwu Fang and my other Chinese friends in Ghent, without you my life in Ghent would limited to work.

I finish with my family, where the most basic source of my life energy resides: I have a nice family, unique in many ways. They have cherished with me every great moment and supported me whenever I needed it. I would like to express my gratitude to my girlfriend Meng Wang, who I will marry with at the end of this year. Her support and encouragement has been unconditional all these years.

I would like to apologize for names that were not mentioned here, but please know that I appreciated every single help you may have provided. Thank you!

Samenvatting

Nematoden van de familie Tylenchidae komen met een grote densiteit en diversiteit in de bodem voor. Ecologisch zijn ze belangrijke bodemfaunaen ze kunnen tot 30% van de nematoden in een bepaald bodemmonster vormen. In tegenstelling tot de meeste andere Tylenchomorpha, omvatten Tylenchidae geen economisch belangrijke plantenparasieten. Ze worden ook gekenmerkt door primitieve kenmerken zoals bijvoorbeeld een beperkt ontwikkeld stylet, een ongedifferentieerde, niet gespierde farynx en een filiforme staart. Hun kleine lichaamsgrootte en onduidelijk morfologische eigenschappen bemoeilijken het opstellen van een systematisch kader. Als gevolg hiervan blijft de afbakening van taxa in deze groep slecht gedocumenteerd en zeer onzeker. Bovendien blijft de kennis van hun voedselbronnen beperkt.Nochtans is dit belangrijk voor trofische analysenen bodemkwaliteitsevaluatie, zeker gezien hun numerieke belang.

In deze studie werden verschillende vertegenwoordigers van Tylenchidae geselecteerd met nadruk op het genus *Malenchus*, geselecteerd uit circa 120 monsters van 90 locaties wereldwijd. De gedetailleerde morfologie werd bestudeerd met behulp van lichtmicroscopie, scan- en transmissie-elektronenmicroscopie. Moleculaire data werden verkregen door sequentiebepaling van 18S en 28S rRNA genen en dit resulteerde in 92 nieuwe sequenties. Vervolgens werden fylogenetische analyses uitgevoerd gebaseerd op diverse methoden. Tenslotte werden de uitgebreide morfologische gegevens geëvalueerd in een fylogenetisch kader en dit bracht de evolutionaire complexiteit van deze structureel minimalistische groep van nematoden naar voor.

Twintig bekende verschillende genera van de familie Tylenchidae werden voor het eerst in China waargenomen en gekenmerkt door morfologische en morfometrische gegevens. Twee nieuwe soorten, *Malenchus sexlineatus* sp. n. en *Malenchus cylindricus* sp. n. werden ontdekt uit respectievelijk de Filippijnen en België, en deze werden beschreven op basis van morfologische en moleculaire data. Een nieuw genus, *Labrys chinensis*gen. n., sp. n., werd beschreven met behulp van een integratieve aanpak: een combinatie van morfologie (lichtmicroscopie, elektronenmicroscopie en 3D-reconstructie), moleculaire fylogenie en populatiegenetica.

Het genus *Malenchus* is geherdefinieerd op basis van een combinatie van nieuw materiaal, type-materiaal en literatuurgegevens. Wij hebben inter- en intraspecifieke variaties vergeleken

en daaruit taxonomische informatieve eigenschappen bepaald. Gewijzigde definities van *Malenchus* en het nauw verwante genus *Ottolenchus* werden weergegeven op basis van een combinatie van morfologie en recente moleculaire data, en hun fylogenetische posities werden geanalyseerd ten opzichte van andere Tylenchidae. Daarnaast werden verschillende schimmels en mossen getest als mogelijke voedselbron van *Malenchus*.

Fylogenetische resultaten tonen aan dat het genus *Filenchus* polyfyletisch is in zowel de 18S- als 28S-rRNA-fylogenie, terwijl *Malenchus* polyfyletisch en monofyletisch blijkt, gebaseerd op respectievelijk 28S rRNA en 18S rRNA. Een ultrastructurele studie toont aan dat specifieke aspecten van laterale lijnen, cuticula-lagen en de foveavan de amfiden verrassend congruent zijn met de verkregen moleculaire fylogenieën, terwijl klassieke kenmerken zoals lichaamsannuleringen evolutionair bijzonder variabel zijn. De studie onthult ook de ontoereikendheid van D2 / D3 domein in 28S rRNA als een fylogenetische merker voor vroeg divergerende Tylenchomorpha (= tylenchiden met vermoedelijk voorouderlijke kenmerken).

Ook werd een vertegenwoordiger van Sphaerularioidea onderzocht, een taxon dat nauw verwant is aan Tylenchidae. De schimmel-etende vrouwtjes werden bekomen van het oude vruchtenlichaam van het elfenbankje *Trametes*sp. groeiend op verrottend hout. *Abursanema quadrilineatum* n. sp. werd beschreven met behulp van lichtmicroscopie, scanning elektronenmicroscopie, transmissie-elektronmicroscopie en moleculaire data gebaseerd op 18S en 28S rRNA. De secundaire structuren van het D2 en D3 domein van 28S rRNA werden gemodelleerd voor de nieuwe soort en een algemene structuur voor desuperfamilie Sphaerularioidea werd gemodelleerd om een vergelijkende analyse mogelijk te maken. De ultrastructuur van de cuticula, spermacellen en oocyten werd onderzocht en de cuticula-lagen werden gedefinieerd. Naaldvormige kristallen werden teruggevonden in de darm en spermatheca van het vrouwelijke schimmel-etende stadium. Verschillende chemische tests en de ultrastructurele studie konden echter geen uitsluitsel geven over de functie ende structuur van deze kristallen.

Daarnaast werden 3D modellerings- en printtechnologieën uitgewerkt en opgenomen in de beschrijving van *Labrys chinensis* gen. n., sp. n. en *Malenchus* spp. als aanvulling op beelden en tekeningen en in het bijzonder om complexe 3D-structuren te illustreren. Ook de typische kop-regio van mononchiden en verschillende genera van Tylenchidae werden in 3D geprint en gebruikt voor onderwijsdoeleinden. Tenslotte werden de prestaties van verschillende 3D-printmaterialen vergeleken en getest, waarbij resin hars naar voor wordt geschoven als de meest geschikte optie voor het zoölogische veld.

Trefwoorden: *Filenchus*, *Malenchus*, *Lelenchus*, *Tenunemellus*, *Miculenchus*, Scanning-elektronmicroscopie, transmissie-elektronmicroscopie, nieuwe soorten, nieuw genus, 3D-modellering, 3D-printen.

Summary

Nematodes of the family Tylenchidae are abundant and diverse. Ecologically, they are important soil fauna which may constitute up to 30% of the nematodes in any given soil sample. In contrast to most other Tylenchomorpha, Tylenchidae do not comprise economically important plant-parasites and are also characterized by ancestral traits, for example a weak stylet, an undifferentiated non-muscular pharyngeal corpus and a filiform tail. Their small body size prevented us from deriving a consistent systematic framework. As a result, the delimitation of taxa in this group remains poorly documented and highly uncertain. Furthermore, knowledge of their food resources remains limited, albeit, given their numeric importance, this subject is important for trophic guild analysis or soil quality evaluation.

In this study several representatives of Tylenchidae (*c.a.* 90 locations worldwide representing 120 samples) were selected with focus on the genus *Malenchus*. Detailed morphology was recovered using light microscopy, scanning- and transmission- electron microscopy. Molecular data were obtained by sequencing 18S and 28S rRNA genes, resulting in 92 new sequences, and phylogenetic analyses were conducted with multiple approaches. Comprehensive morphological data are evaluated in the context of a molecular framework, thus highlighting the phylogenetic and evolutionary complexity of this structurally minimalistic group.

Twenty known species belong to different genera of Tylenchidae were first recorded from China. Two new species, *Malenchus sexlineatus* n. sp. and *Malenchus cylindricus* sp. n. discovered from the Philippines and Belgium respectively, were described based on morphological and molecular data. A new genus *Labrys chinensis* gen. n., sp. n. was described using an integrative approach: morphology, molecular phylogeny and population genetics. 20 known species belonging to different genera of Tylenchidae were for the first time recorded from China and characterised by morphological and mophometric data.

The genus *Malenchus* has been redefined based on a combination of new material, type material and literature data. We have compared inter- or intraspecific variations and extracted taxonomically informative traits. Amended definitions of *Malenchus* as well as the closely related genus *Ottolenchus* were given based on a combination of morphology and recent molecular data, and their phylogenetic positions were analysed in a context of Tylenchidae.

Furthermore, we tested different fungi and moss as a food resource of *Malenchus* and their feeding behavior is also discussed.

Phylogenetic results show that the genus *Filenchus* is polyphyletic in both the 18S and 28S rRNA phylogeny, while *Malenchus* is polyphyletic and monophyletic in the 28S rRNA and the 18S rRNA, respectively. An ultrastructural study demonstrates that specific aspects of lateral cuticular incisures, cuticular layering and the amphideal fovea are surprisingly congruent with the obtained molecular phylogenies, while classical characteristics such as cuticle annulations are evolutionary highly plastic and mosaic in distribution. The study also reveals the inadequacy of D2/D3 domain in 28S rRNA as a phylogenetic marker for early diverging (=tylenchs with supposedly ancestral characters) Tylenchomorpha.

Also a representative of Sphaerularoidea was investigated, a taxon that is closely related to Tylenchidae. The mycophagous females were recovered from the old fruiting body of bracket fungus *Trametes* sp. growing on decaying wood. *Abursanema quadrilineatum* n. sp. was described morphologically from light microscopy, scanning electron microscopy and transmission electron microscopy and molecularly based on 18S and 28S rRNA. The secondary structures of the D2 and D3 domain of 28S rRNA were predicted for the new species and a general model for the superfamily Sphaerularioidea was built for comparative analysis. The ultrastructure of the cuticle, sperm cells and oocytes was examined and cuticle layers were defined. Needle-shaped crystals were recovered in the intestines and spermatheca of mycophagous females. However, chemical tests and the ultrastructural study could not reveal the identity and structure of these crystals.

In addition, 3D modeling and printing technologies were incorporated in the description of *Labrys chinensis* gen. n., sp. n. and *Malenchus* spp. as a complement to pictures and drawings to illustrate complex 3D structures. Also the typical cephalic region of mononchids and several different genera of Tylenchidae were printed and used for education. Hereby, we also tested the performance of different printing materials and forwarded resin as the most suitable option for the zoological field.

Keywords: *Filenchus*, *Malenchus*, *Lelenchus*, *Tenunemellus*, *Miculenchus*, Scanning electron microscopy, transmission electron microscopy, new species, new genus, 3D modeling, 3D printing.

Chapter I

General introduction

Background

Tylenchidae is one of the most important soil inhabiting nematode family (Andrássy, 1981), and species belonging to Tylenchidae may constitute up to 30% of the nematode individuals in a soil sample (Yeates & Bird, 1994; Ferris & Bongers, 2006). As early diverging Tylenchomorpha (=tylenchs with supposedly ancestral characters), they do not comprise economically important plant-parasites and are characterized by ancestral characters (Luc *et al.*, 1987; Siddiqi, 2000; Bert *et al.*, 2008). Knowledge of their food resources remains limited, albeit, given their numeric importance, this subject is important for trophic guild analysis or soil quality evaluation. Furthermore, their small body size and a lack of clearly homologous characters prevented us from deriving a consistent systematic framework. As a result, the delimitation of taxa in this group remains poorly documented and highly uncertain (Bongers & Bongers, 1998; Yeates, 2003; Ferris & Bongers, 2006).

Classification

Nematodes of the suborder Tylenchina *sensu* De Ley and Blaxter (2002) include four infraorders: Panagrolaimorpha, Cephalobomorpha, Drilonematomorpha and Tylenchomorpha (Fig. 1). They are an ecologically and morphologically diverse array of species that range from soil dwelling bacteriovores to highly specialized plant-parasites. Tylenchomorpha is the most intensively investigated infraorder within the Tylenchina, and five superfamilies are included: Aphelenchoidea; Criconematoidea, Sphaerularioidea, Tylenchoidea and Myenchoidea (De Ley & Blaxter, 2002). The Tylenchomorpha without Aphelenchoidea are popularly called tylenchs or tylenchids and Aphelenchoidea as aphelenchs or aphelenchids (without hierarchical position of the taxa, in order to avoid confusion by different taxonomic system), the latter representing Aphelenchoidea while the former refer to other superfamilies (Fig. 1). Aphelenchoidea contains plant-parasitic and fungivorous nematodes. Criconematoidea and Tylenchoidea (Hoplolaimidae, Meloidogynidae, Pratylenchidae, Belonolaimidae and Tylenchidae) comprise the largest and economically most important group of plant-parasitic nematode; Sphaerularioidea have complex fungi-insect interactions or are parasites of aerial parts of plants. Myenchoidea comprise of parasites of leeches or frogs and may represent a separate origin of parasitism (Siddiqi, 2000).

Within Tylenchoidea, The family Tylenchidae was proposed by Örley (1880). It contains tylenchs characterized by relatively short body and long tail (conoid to filiform shape), not

overlapping pharynx and short, delicate stylet. The Female reproductive system in Tylenchidae *sensu* Geraert (2008) is predominantly monodelphic, but also rarely didelphic (*Atetylenchus*, *Antarctenchus*, *Psilenchus*). Bursa is adanal, small, rarely absent. It is the only family where amphidial apertures can be seen on the lateral side of the head.

The taxonomy in Tylenchidae is problematic: most species combine a low observational resolution with high intraspecific variability in measurements, and DNA sequences of most species are not available. As a result, there is no consensus regarding their classification from species level up to family level (Brzeski, 1998; Siddiqi, 2000; Andrássy, 2007; Geraert, 2008).

The main dispute of Tylenchidae classifications are the placements of four didelphic genera: *Atylenchus*, *Antarctenchus*, *Atetylenchus*, *Psilenchus*. They are either considered to belong to Tylenchidae (*Atylenchus* and *Antarctenchus* were included in subfamily Atylenchinae while *Atetylenchus* and *Psilenchus* belongs to Boleodorinae) (Geraert & Raski, 1987, Geraert, 2008) or outside of Tylenchidae as two separated families (*Atylenchus* and *Eutylenchus* as family Atylenchidae characterized by cephalic setae while *Antarctenchus*, *Atetylenchus* and *Psilenchus* constitute family Psilenchidae) (Siddiqi, 2000; Andrássy, 2007).

Table 1 The placement of family Tylenchidae according to the most authorative classifications.

Rank	Maggenti, Luc, Raski, Fortuner & Geraert, 1988	Siddiqi, 2000	De Ley and Blaxter, 2002*
Order	Tylenchida	Tylenchida	Rhabditida
Suborder	Tylenchina	Tylenchina	Tylenchina
Infraorder	-	Tylenchata	Tylenchomorpha
Superfamily	Tylenchoidea	Tylenchoidea	Tylenchoidea

* The placement of Tylenchidae follows De Ley and Blaxter (2002) in this thesis.

Table. 1 Comparison of the taxonomic content of Tylenchidae according to four widely used classifications.

Authors	Maggenti, Luc, Raski, Fortuner, & Geraert, 1988	Siddiqi, 2000	Andrássy, 2007	Geraert, 2008*
Genera	33 genera	25 genera	29 genera	42 genera

Subfamilies	Atylenchinae, Boleodorinae, Tylenchinae, Ecphyadorinae, Tylodorinae	Boleodorinae, Duosulciinae,Tanzaniinae, Thadinae, Tylenchinae	Boleodorinae, Duosulciinae, Thadinae,Tylenchinae, Tylodorinae	Atylenchinae, Boleodorinae, Ecphyadorinae, Tylenchinae, Tylodorinae

* The classification system of Geraert (2008) is used at family and genus level in this thesis.

Ecphyadophorinae and Tylodorinae *sensu* Geraert (2008) are divergent from the typical Tylenchidae species and their placements are also controversial. Species belonging to Ecphyadophorinae have extreme slender bodies, they were considered as a subfamily in family Tylenchidae, consisting of nine genera (*Chilenchus*, *Ecphyadophora*, *Ecphyadophoroidea*, *Epicharinema*, *Lelenchus*, *Mitranema*, *Tenunemellus*, *Tremonema* and *Ultratenella*) (Raski, Koshy & Sosamma, 1982; Geraert 2008) or considered a separate family with eight genera (similar with above except of *Epicharinema*) (Siddiqi, 2000; Andrássy, 2007). The Tylodorinae consists of five (*Arboritynchus*, *Campbellenchus*, *Cephalenchus*, *Eutylenchus* and *Tylodorus*) (Geraert, 2008) or six genera (*Arboritynchus*, *Campbellenchus*, *Cephalenchus*, *Pleurotylenchus*, *Gracilancea* and *Tylodorus*) (Andrássy, 2007).The general morphology of Tylodorinae is similar to some Dolichodoridae (e.g. *Dolichodorus*, *Macrotrophurus* and *Belonolaimus*) but Dolichodoridae are didelphic and have a distinct phasmid in the tail region. It belongs to Tylenchidae by the locations of the prophasmids (outside the lateral fields and lack of a phasmid) and filiform tail, but differs from other subfamilies by having a strong stylet measuring about as long as or longer than the procorpus. Currently,

In this study we follow the taxonomic system of Geraert (2008) and five subfamilies are recognized with family Tylenchidae: Tylenchinae, Ecphyadophorinae, Tylodorinae; Atylenchinae and Boleodorinae. Aside from 42 valid genera listed by Geraert (2008), two new genera were recently described (Yaghoubi *et al.*, 2016; Qing & Bert, 2017) and thus a total of 44 genera are included in Tylenchidae:

Order Rhabditida

Suborder Tylenchina

Infraorder Tylenchomorpha

Family Tylenchidae

Subfamiliy Atylenchinae

Genus *Aglenchus* Andrássy, 1954

Genus *Antarctenchus* Spaull, 1972

Genus *Atylenchus* Cobb, 1913

Genus *Coslenchus* Siddiqi, 1978

Genus *Pleurotylenchus* Szczygiel, 1969

Subfamily Boleodorinae

Genus *Atetylenchus* Khan, 1973

Genus *Basiria* Siddiqi, 1959

Genus *Boleodorus* Thorne, 1941

Genus *Discopersicus* Yaghoubi, Pourjam, Alvarez-Ortega, Liebanas, Atighi and Pedram, 2016

Genus *Neopsilenchus* Thorne & Malek, 1968

Genus *Neothada* Khan, 1973

Genus *Psilenchus* de Man, 1921

Genus *Ridgellus* Siddiqi, 2000

Genus *Thada* Thorne, 1941

Subfamily Ecphyadophorinae

Genus *Chilenchus* Siddiqi, 2000

Genus *Ecphyadophora*, de Man, 1921

Genus *Ecphyadophoroides*, Corbett, 1964

Genus *Epicharinema* Raski, Maggenti, Koshy & Sosamma, 1982

Genus *Lelenchus* Andrássy, 1954

Genus *Mitranema* Siddiqi, 1986

Genus *Tenunemellus* Siddiqi, 1986

Genus *Tremonema* Siddiqi, 1994

Genus *Ultratenella*, Siddiqi, 1994

Subfamily Tylenchinae

Genus *Allotylenchus* Andrássy, 1984

Genus *Cervoannulatus* Bajaj, 1997

Genus *Cucullitylenchus* Huang & Raski, 1986

Genus *Discotylenchus* Siddiqi, 1980

Genus *Filenchus* Andrássy, 1954

Genus *Fraglenchus* Siddiqi, 2000

Genus *Gracilancea* Siddiqi, 1976

Genus *Irantylenchus* Kheiri, 1970

Genus *Labrys* Qing & Bert, 2017

Genus *Malenchus* Andrássy, 1968

Genus *Miculenchus* Andrássy, 1959

Genus *Polenchus* Andrássy, 1980

Genus *Sakia* Khan, 1964

Genus *Silenchus* Andrássy, 2001

Genus *Tanzanius* Siddiqi, 1991

Genus *Tylenchus* Bastian, 1865

Subfamily Tylodorinae

Genus *Arboritynchus* Reay, 1991

Genus *Campbellenchus* Wouts, 1977

Genus *Cephalenchus* Goodey, 1962

Genus *Eutylenchus* Cobb, 1913

Genus *Tylodorus* Meagher, 1964

Ecology

Allocation of the feeding habitats in Tylenchidae is one of the most important discussion points amongst nematologists (Bongers & Bongers, 1998). Normally they are treated as root hair feeders (Bongers & Bongers, 1998) or algal, moss and fungal feeders (Siddiqi, 2000; Okada, 2002). Although they may also be parasites of lower and higher plants (Siddiqi 1986, 2000; Andrássy, 2007), they do not cause economic losses to crops. The available studies show contrasting information about their feeding behaviors: *Malenchus bryophilus* (as *Tylenchus bryophilus* in Khera & Zuckerman (1963)), *Aglenchus* (as *T. Agricola*) (Khera & Zuckerman, 1963), *Coslenchus costatus* (Wood, 1973, Andrássy, 1976), *Cephalenchus emarginatus* (Hooper, 1974; Sutherland, 1967; Gowen, 1971) and *Tylodorus fisheri* (Reay, 1991) have been described to feed on roots of higher plant; *Ottolenchus cabi* is associated with a lichen (*Cladonia glauca*) (Siddiqi & Hawksworth, 1982); *M. pachycephalus* probably feed on moss (Qing & Bert, 2017); and *Filenchus* spp. can be grown in multiple fungi species (Okada, 2002, 2003, 2005). Therefore, feeding behavior in Tylenchidae is genus or even species specific.

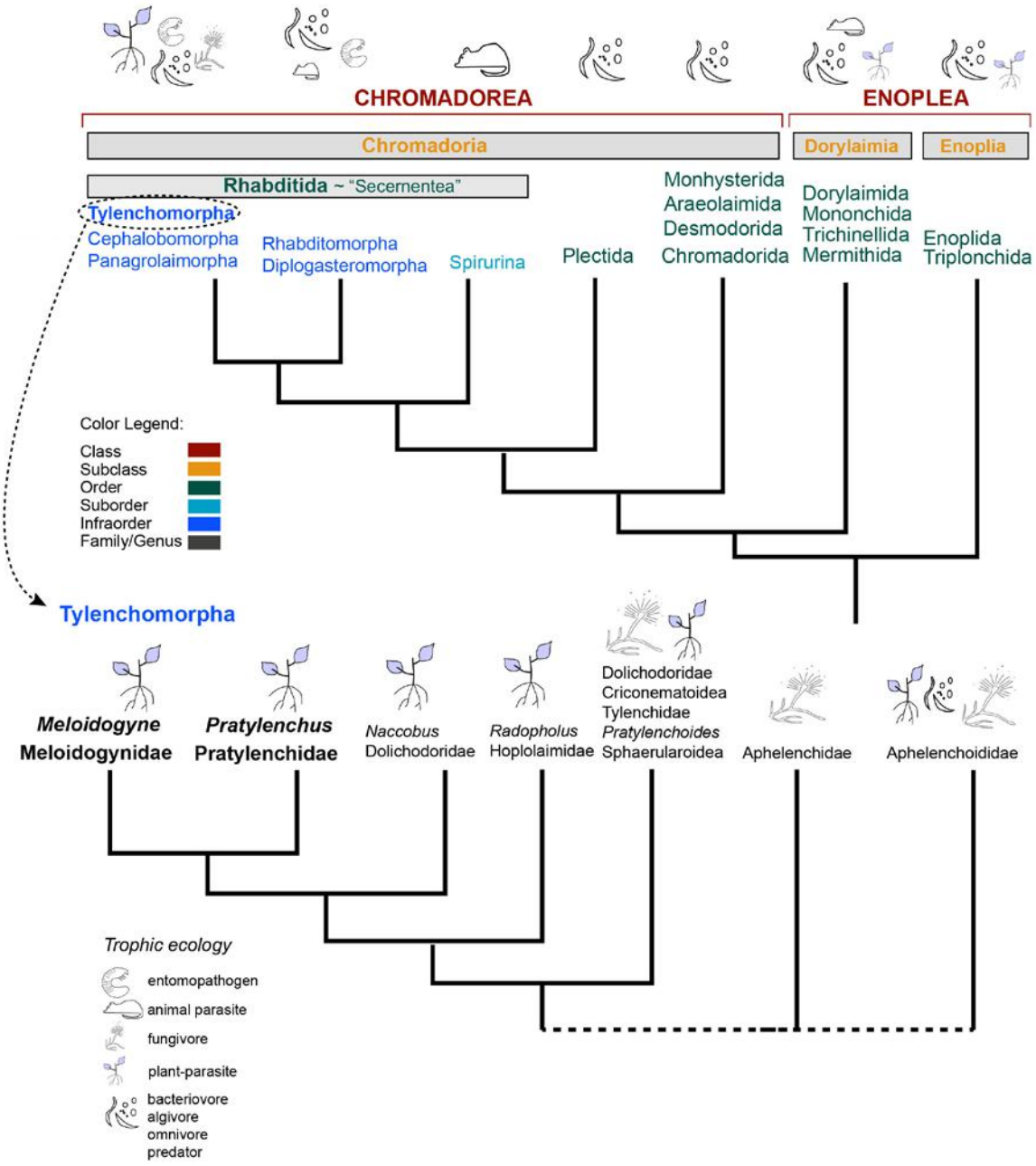

Fig. 1 The Phylogeny and evolution of Nematoda and showing the position and composition of Tylenchomproha.

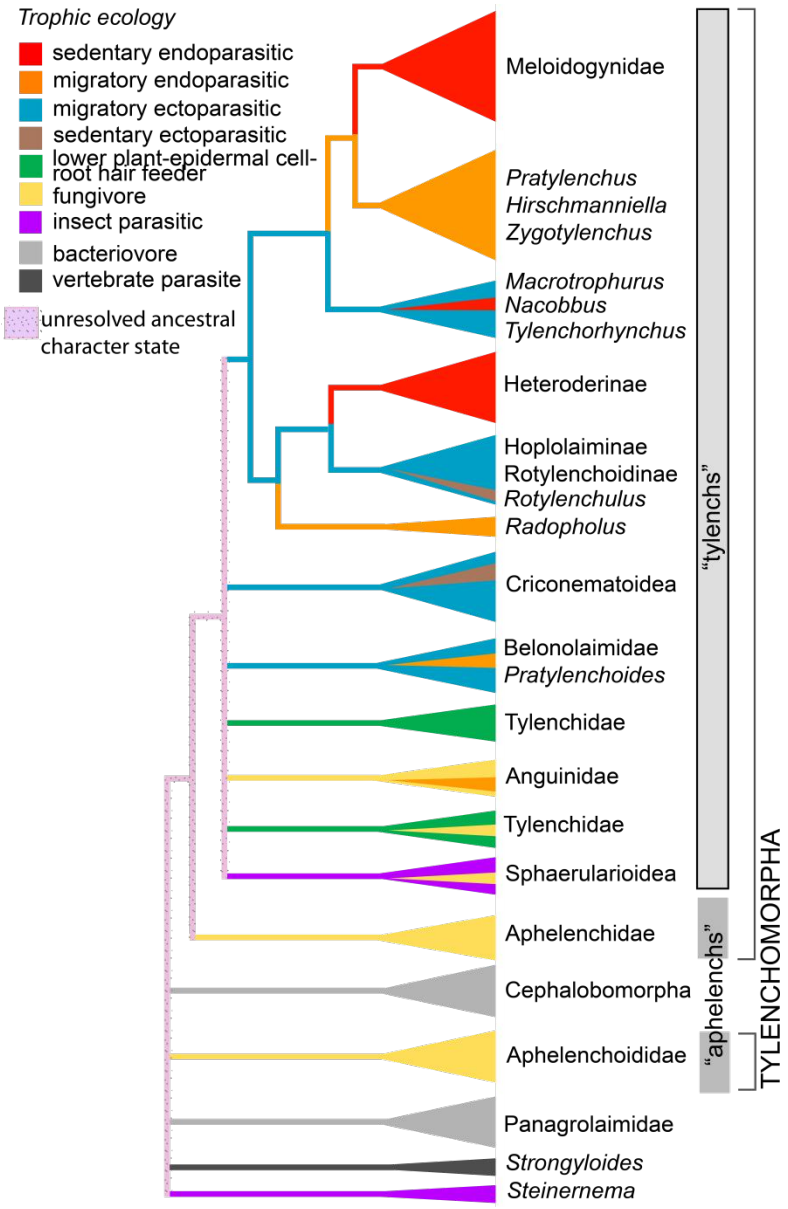

Fig. 2 The Ancestral state reconstruction of feeding strategy of Tylenchomorpha superposed on a ribosomal DNA-based phylogenetic backbone (Bert *et al.*, 2011).

Phylogeny and evolution

Tylenchidae show many supposedly primitive morphological characters (*e.g.* weak stylet and median bulb, basal bulb with full complement of nonglandular cells, monodelphy, elongate tails, uterus cells that are arranged in 4 rows = quadricolumella.) (Siddiqi, 2000; Baldwin *et al.*, 2001; Bert *et al.*, 2008), supposedly primitive feeding habitats (algal and moss feeding) (Siddiqi, 1986, 2000) and the embryology of Tylenchidae (including *Psilenchus*) is similar to that of the Cephalobidae: an asynchronous division order and a partially linear blastomere arrangement *vs* a synchronous division order and a completely linear blastomere

arrangement in Meloidogynidae, Pratylenchidae, Belonolaimidae, Hoplolaimidae and Criconematoidea) (Dolinski *et al.*, 2001). Consequently, tylenchids nematodes were divided into early diverging tylenchs (=tylenchs with supposedly ancestral characters) groups including Tylenchidae, Anguinidae and Sphaerularioidea and more derived groups (=tylenchs with supposedly derived characters) that include the remaining tylenchid taxa (*e.g.* Siddiqi, 2000). Current molecular phylogeny inferred from small subunit ribosomal DNA shows different pictures: either congruent with classical views (Bert *et al.*, 2008) or Tylenchidae as sister to Criconematoidea within other derived nematodes (Holterman *et al.*, 2008; van Megen *et al.*, 2009). Recently, a phylogeny based on the concatenated data of SSU and large subunit (LSU) ribosomal DNA phylogeny suggest that the early diverging tylenchs (Tylenchidae, Anguinidae and Sphaerularioidea) are well separated from tylenchs with more derived traits, with exception of *Malenchus pressulus* which is placed as sister to Criconematina (Fig. 3) (Pereira *et al.*, 2016). However, the support values for the backbone in these studies are very lower and deep subdivision at the early diverging Tylenchomorpha remains unresolved (Figs. 1, 2).

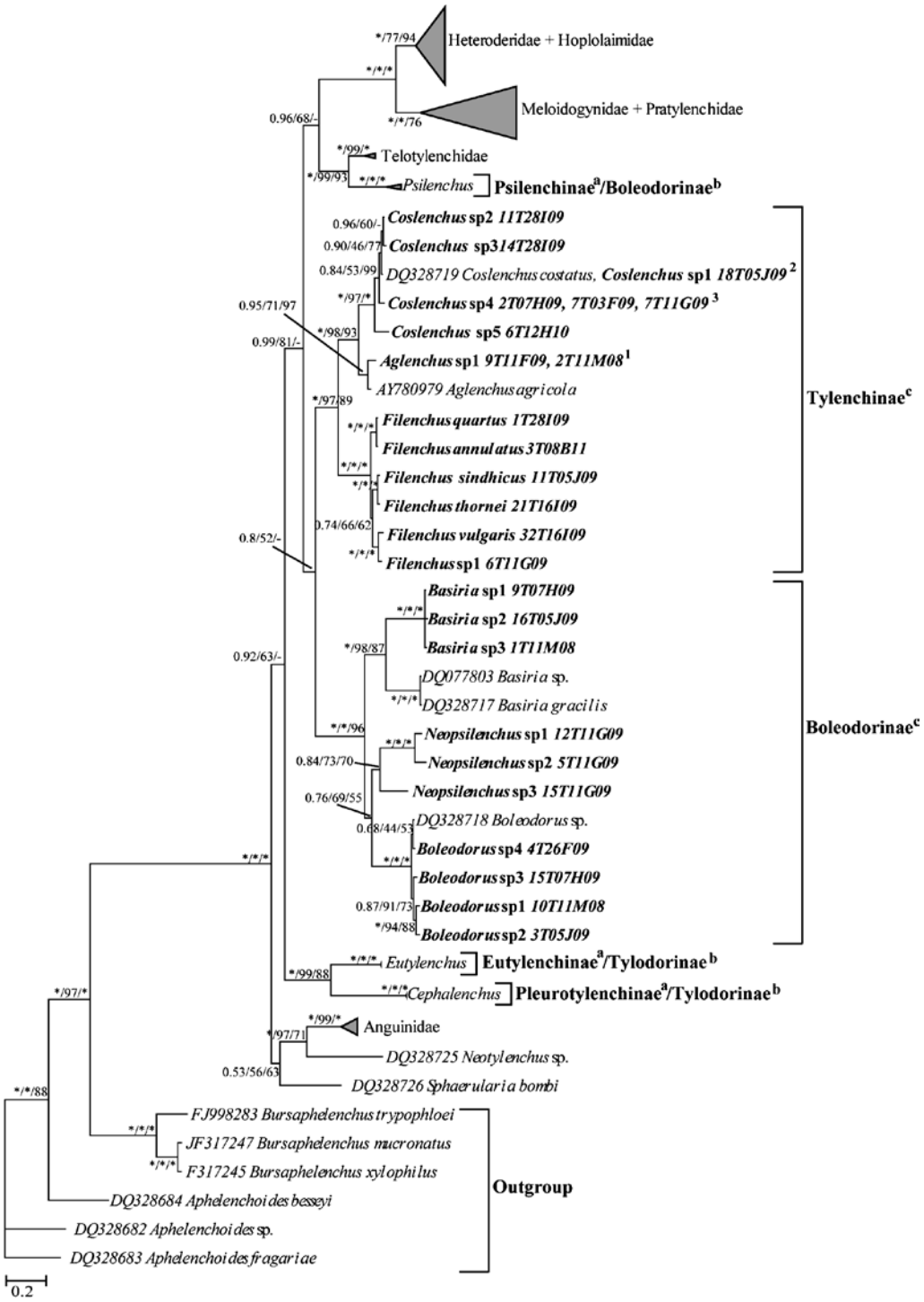

Fig. 3 Phylogenetic analysis of the family Tylenchidae. Bayesian 50% majority rule consensus tree inferred from sequences of the D2-D3 domains of the 28S rRNA gene. Branch support values are given in the following order: BI, ML, and MP. An asterisk (*) in any position denotes maximum branch support for that method; – indicates no branch support in MP (Atighi *et al.*, 2013).

Within these tylenchs, the relationship of Tylenchidae, Anguinidae and Sphaerularioidea is subject to discussion. Morphology-based phylogenetic concepts suggested Tylenchidae to

be either more closely related to Anguinidae distantly related to Sphaerularioidea (*e.g.* Maggenti *et al.*, 1987; Brzeski, 1998; Siddiqi, 2000, Andrássy, 2007), sister to Sphaerularioidea+Anguinidae (*e.g.* Siddiqi, 1986; Ryss, 1993) or a broader concept of the Tylenchidae that includes Anguinidae and at least part of the Sphaerularioidea (Raski and Maggenti, 1983). However, molecular phylogeny rejects monophyly for three groups and support values to establish the relations between these groups are generally low (Bert *et al.*, 2008; Holterman *et al.*, 2009; van Megen *et al.*, 2009) (Fig. 2) .

Also within Tylenchidae, the phylogenetic resolution is problematic. Hence, at this moment a more definitive framework cannot be established and we summarized some of available knowledge on Tylenchidae phylogeny:

1. Tylenchidae is heterogeneous group, all available 18S and 28S rRNA genes based analyses suggested it is polyphyletic (*e.g.* Holterman *et al.*, 2006; Subbotin *et al.*, 2006; Bert *et al.*, 2008; van Megen *et al.*, 2009).

2. Boleodorinae is polyphyletic. However, except for two didelphic genera (*Psilenchus* and *Atetylenchus*), other genera (represented by *Boleodorus*, *Basiria*, *Neopsilenchus*, *Neothada*) form a well-supported monophyletic clade (e.g. Yaghoubi *et al.*, 2015). In fact, genus *Psilenchus* is the subject of longstanding discussions if it is either a early diverging genus (=with supposedly ancestral characters, *e.g.* Luc *et al.*, 1987) or a genus with derived position (=with supposedly derived characters, *e.g.* Siddiqi, 2000) and has been removed from Tylenchidae in several studies (Siddiqi, 2000, Andrássy, 2007).

3. Atylenchinae represented by *Aglenchus* and *Coslenchus* is monophyletic (*e.g.* Atighi *et al.*, 2013).

4. Tylenchinae is the most heterogeneous subfamily in Tylenchidae. *Malenchus* has divergent position with other Tylenchinae and several genera are polyphyletic (*e.g. Filenchus*, *Tylenchus*) (Bert *et al.*, 2008; Atighi *et al.*, 2013).

5. Little is known for Ecphyadophorinae, at this subfamily is considered as a heterogeneous group (Siddiqi, 2000; Geraert, 2008).

6. Tylodorinae is represented by only two genera (*Eutylenchus* and *Cephalenchus*) and is monophyletic, but in a divergent clade which is not related to other Tylenchidae (Pereira *et al.*, 2016) (Fig. 3).

General morphology

The general terminology for the main parts used in the thesis is indicated in Fig. 4.

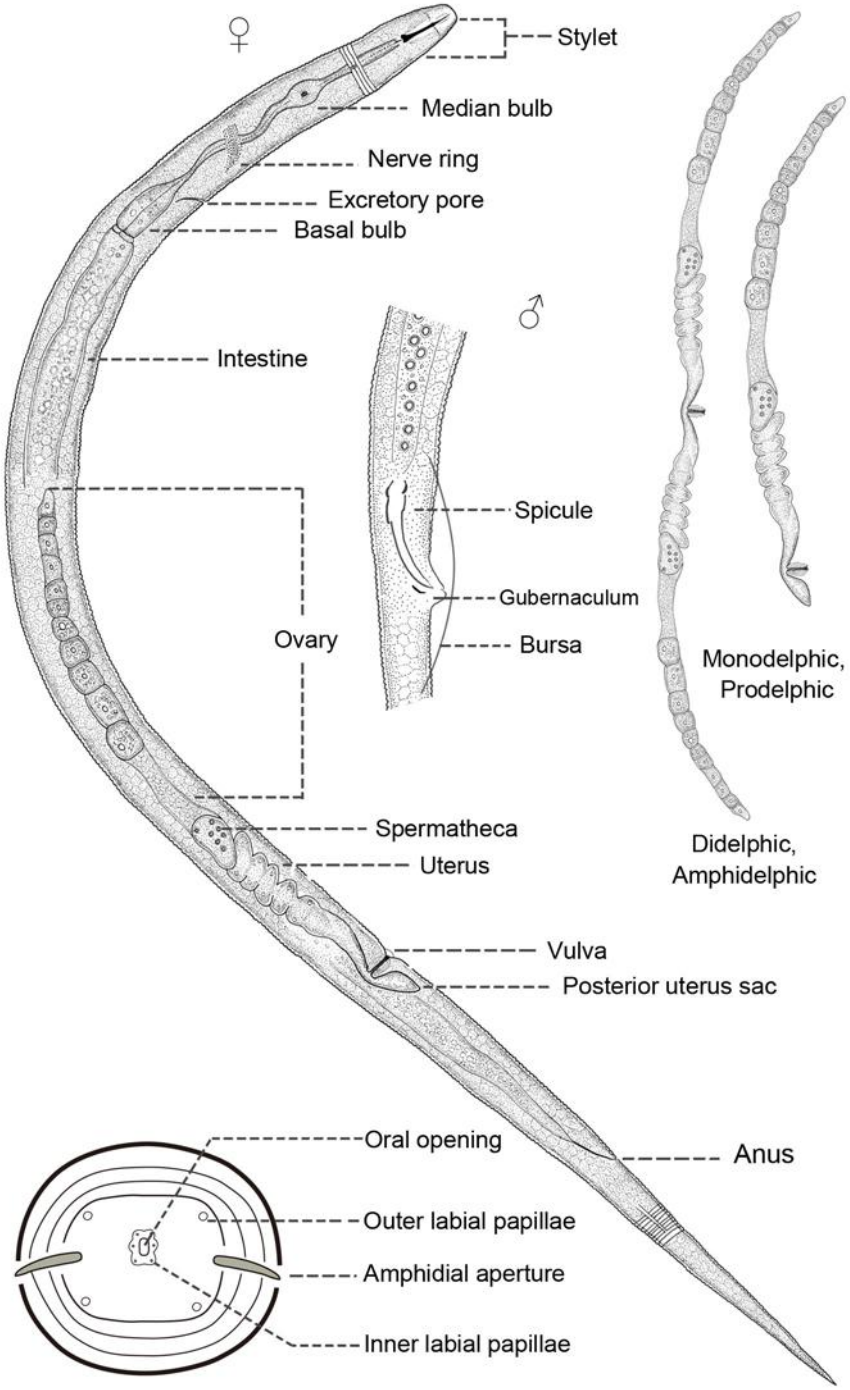

Fig.4. The general terminology for the main parts of Tylenchidae

Lip region

Lip regions in Tylenchidae are usually round, but laterally elongated (dorso-ventrally

flattened) in *Malenchus*, *Lelenchus*, *Ecphyadophoroides*, *Epicharinema* and *Tenunemellus*. Sensilla 6 inner labial papillae + 4 outer labial papillae, but the former is usually invisible. Amphidial aperture varies from pore to long slit. Amphidial foveas are considered taxonomically important at generic level (Qing *et al.*, 2017a), in most genera they are invisible, but can be pouch-like in *Malenchus*, *Lelenchus*, *Ecphyadophoroides* and *Tenunemellus*. Geraert and Raski (1987) classified the lip regions into seven patterns and highlighted its taxonomic importance. Such assignment was rejected by Siddiqi (2000) and Andrássy (2007) but concur with recent molecular based phylogeny (Qing and Bert, 2017). Although the fine structure of lip region can vary intraspecifically, its main patterns (amphidial aperture shape and location, labial plat shape, sensilla arrangement) are conserved. Currently eight patterns are recognized (Fig. 5).

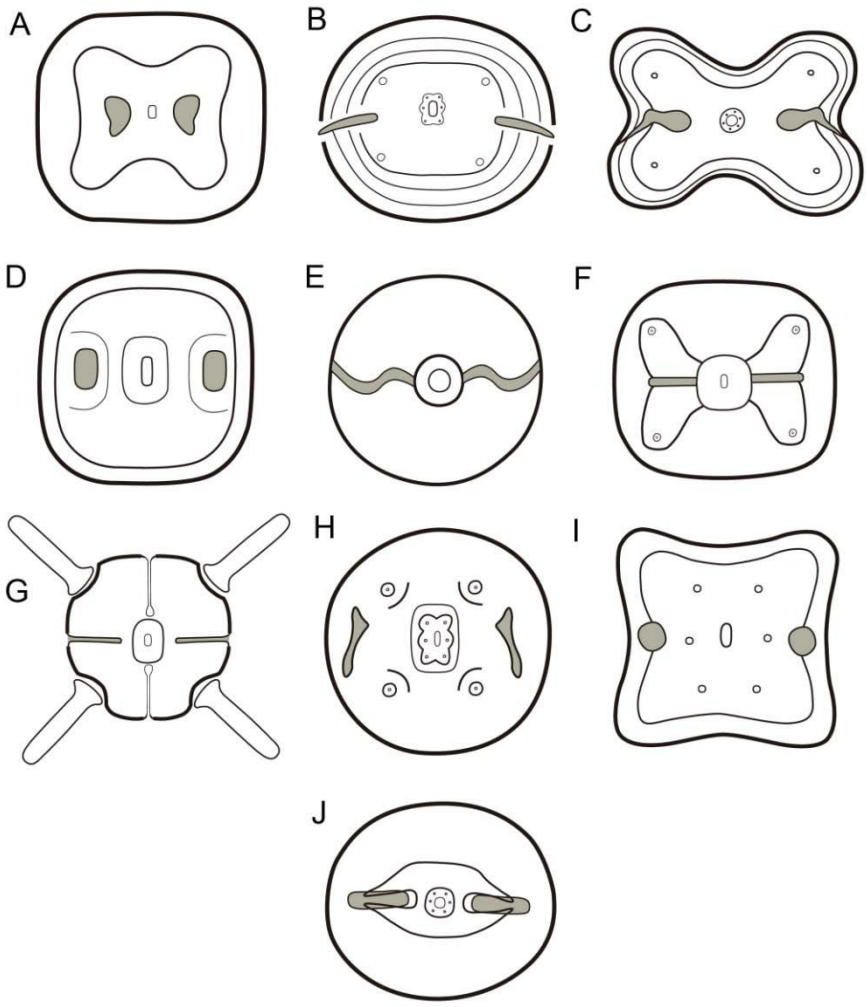

Fig. 5 Illustrations indicate lip region arrangement in different genera of Tylenchidae (A-J). A: front plate laterally elongated, undivided, carries al1 the sensillae. The amphidial apertures are entirely within the plate, typical for genus *Aglenchus* and *Coslenchus*; B: Amphidial apertures

are not confined to the oral plate but continue on the lateral side as longitudinal slits. The end-on view is round to quadrangular, typical for most species belonging to genus *Filenchus*, including type species *F. vulgaris*; C: Similar with II-a except for a dorso-ventrally flattened end-on view, typical for genus *Malenchus*; D: Slit-like amphidial apertures confined to the oral plate but the slits are dorso-ventrally directed, typical for few species in genus *Filenchus, e.g. F. misellus, F. ditissimus and F. neonanus*; E: Offset oral disc, the cephalic region is dorso-ventrally flattened. The amphidial aperture is very long and mostly sinuous, it starts at oral disc and continues longitudinally on the narrow lateral side of the cephalic region, typical for genus *Lelenchus, Tenunemellus, Epicharinema, Chilenchus, Ecphyadophoroides*; F: amphidial slits start immediately at the oral disc, laterally directed but are only found on the front end of the cephalic region. The amphidial apertures are surrounded by a plate that bears the four cephalic papillae, that plate is constricted dorso-ventrally to form lobes, typical for *Cephalenchus*; G: similar with V-a but labial plate is constricted to form a cleft and with seta, typical for *Eutylenchus*; H: Labial plate undivided, four prominent cephalic papillae dome-shaped, outside of anterior surface. Amphidial apertures start between or outside the four cephalic papillae and are simple oblique slits or have an inverted V-shape, typical for *Basiria* and *Boleodorus*; I: with very small pore-like amphidial apertures, typical for *Ecphyadophora*; J: Labial plate offset and constricted dorso-ventrally, forming four lobes, taping towards tip and detached from adjacent cuticle, typical for genus *Labrys.*

Cuticle and lateral region

The cuticle in Tylenchidae generally has six patterns: (1) cuticle only marked with transverse annuli. The width, thickness of the annuli and presence of grooves between two annuli vary among genera. It is the predominant pattern and present in most genera in Tylenchidae (Fig.6A). (2) Cuticle with deep, transverse zigzag striae. This character is unique for *Miculenchus* (Fig. 6B). (3) Cuticle coarsely annulated, with longitudinal ridges: the cuticle surface outside the lateral fields shows minute squares or rectangles. The number of these longitudinal ridges is either fixe at genus level (*e.g. Eutylenchus* has 10, excluding lateral ridges) or intragenerically vary (*e.g. Coslenchus* has 10-34 and *Neothada* has 12-20, excluding lateral ridges) and been used as species delimitation character (Fig. 6C). (4) Cuticle smooth in LM, but faintly annulated in SEM. It has been used as generic character for *Polenchus* (Fig. 6D). (5) Cuticle with pronounced annulation only in lip region, annuli extending twice as far posteriorly in the lateral as in the dorsal and ventral zones, but not past

base of stylet. The rest of the body marked with longitudinal ridges and deep grooves in-between. This pattern is only known for *Campbellenchus* (Fig. 6E). (6) The surface of cuticle has longitudinal striae but very faint (probably only in epicuticle) and is only visiable in SEM. This presents in some of *Malenchus* species (Fig. 6F).

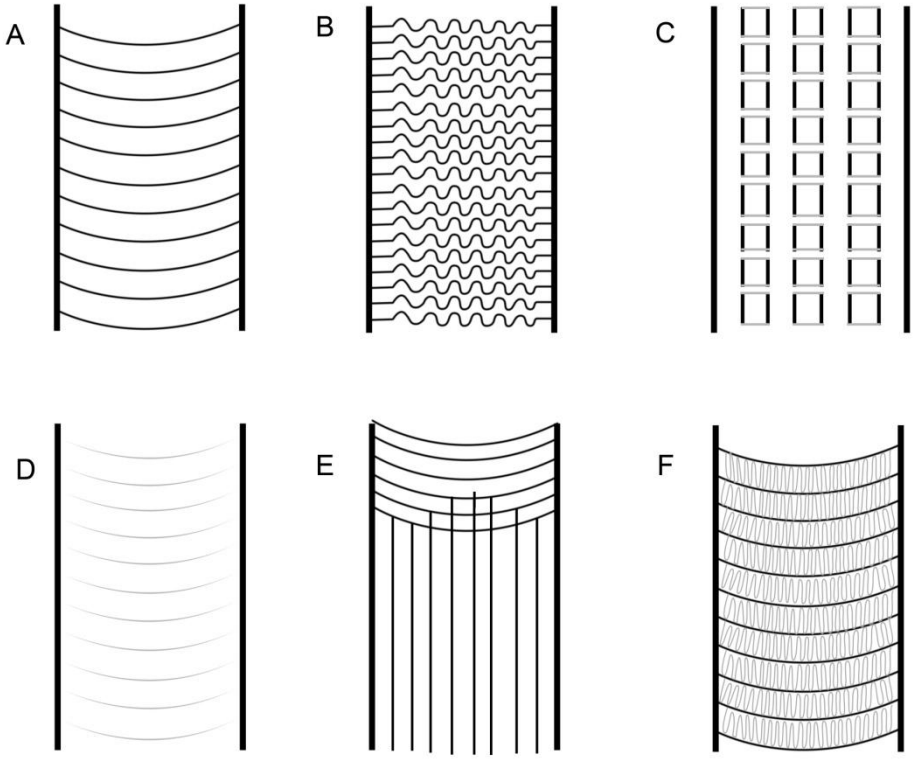

Fig. 6 The cuticle annulation patterns in Tylenchidae. A. cuticle only marked with transverse annuli: this is the most common pattern in Tylenchidae. B: the zigzag transverse annuli: only found in *Miculenchus.* C: cuticle with longitudinal ridges or grooves that divide the surface into minute squares or rectangular blocks: this pattern is presented in *Atylenchus*, *Coslenchus, Ecphyadophoroidea*, *Eutylenchus*, *Neothada*, *Pleurotylenchus, Ridgellus* and *Tanzanius*. D: cuticle appears smooth without annulation: this pattern is found in *Allotylenchus*, *Polenchus* and *Lelenchus.* E: the distinct transverse annuli only in lip region, other part of body marked by longitudinal ridges: this pattern is only known for *Campbellenchus*. F: cuticle marked with transverse annuli but surface has shallow longitudinal striae: this is represented in some of *Malenchus* species, e.g. *M. nanellus* and *M. parthenogeneticus*.

The lateral regions in Tylenchidae are very heterogeneous. Generally, there are five patterns: (1) Lateral region with four incisures, resulting from two elevated ridges separated by wide grooves (*e.g. Campbellenchus filicauda* and most species in *Aglenchus, Coslenchus,* Fig. 7 A, C, D) or three ridges separated by narrow grooves (*e.g. Filenchus vulgaris* and

Basiria hiberna, Fig. 7 G, N, F); the latter is the most common pattern. (2) Lateral region with two incisures, resulting from one broad (*e.g. Filenchus discrepans*, Fig. 7 M) or narrow (*Filenchus arcutus*, Fig. 7 O) ridge. (3) Lateral region invisible in LM: however, in SEM lateral region appears with shallow incisures (*e.g. Lelenchus leptosome* and many species belong to Ecphyadophorinae, see Fig. 7 H). (4) Lateral region with one offset ridge with several sub-ridges forming 14-22 incisures: in LM only one offset ridge with two incisures is visible: this pattern is typical for most species in *Malenchus* (Fig. 7 L). (5) Lateral region with five ridges forming six incisures: this pattern is present in most species of *Cephalenchus* (Fig. 7 K), some species in *Boleodorus* and in *M williamsi.* The number of incisures in Tylenchidae is largely convergent: a similar number can be found in different genera while it's common that one genus has a variable number of incisures (*e.g.* two vs four incisures in *Basiria*, four vs six incisures in *Cephalenchus*).

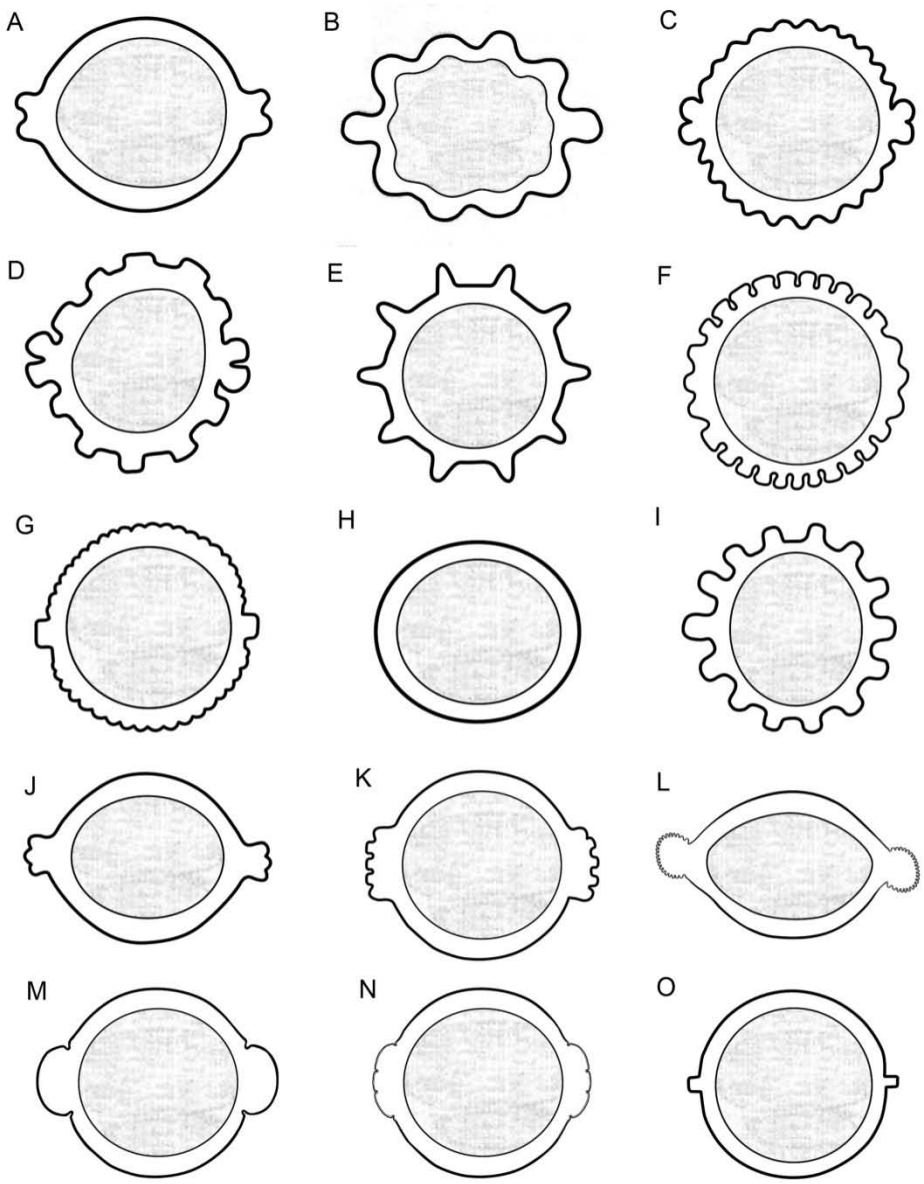

Fig. 7 Cross section of different genera in Tylenchidae. A: *Aglenchus agricola* B: *Atylenchus decalineatus*; C: *Coslenchus japonicas*. D: *Coslenchus oligogyrus*. E: *Pleurotylenchus minor*. F: *Campbellenchus filicauda*. G: *Tanzaniu coffeae*. H: *Lelenchus leptosome*. I: *Ridgellus elenae*. J: *Basiria hiberna*. K: *Cephalenchus hexalineatus*. L: *Malenchus pachycephalus*. M: *Filenchus discrepans*. N: *Filenchus vulgaris*. O: *Filenchus acutus*

Stylet

Stylets in Tylenchidae are generally thin and short, but can also be robust and long in few species (Fig. 8). The length of the stylet ranges from 4 μm (*Filenchus infirmus*) to 120 μm (*Tylodorus* spp.). It consists of three parts: cone, shaft and knob. The cone part is usually shorter than or equals shaft but can be longer in *Tylodorus*. In most species, the cone is

straight, connects to shaft with comparable width and tapers sharply anteriorly. However, some species in *Neopsilenchus* have a cylindrical, dorsally or ventrally bent cone (*e.g. N. magnidens*, *N.minor*, *N.affinis* and *N. similis*, Fig 8 L). The knobs vary greatly in absence/presence, size, shape, direction (Fig. 8): the most common knobs are round in shape, they are perpendicular to shaft (*e.g.* some of *Coslenchus Aglenchus* and *Filenchus*), directed backwards (*e.g. Malenchus*, most of *Tylenchus* and *Filenchus*) or anteriorly directed (e.g. some of *Aglenchus*). In some cases the stylet is cylindroid and knobs are completely absent, this is the case for *Psilenchus*, *Neopsilenchus*, *Chilenchus*, *Atetylenchus* and few species in *Basiria* (e.g. *B. gracilis*); more rarely, *Irantylenchus* and *Antarctenchus* have amalgamated or flange-like stylet knob and *Cephalenchus* and *Tanzanius* have a stylet with large, flatted/elongated, knobs.

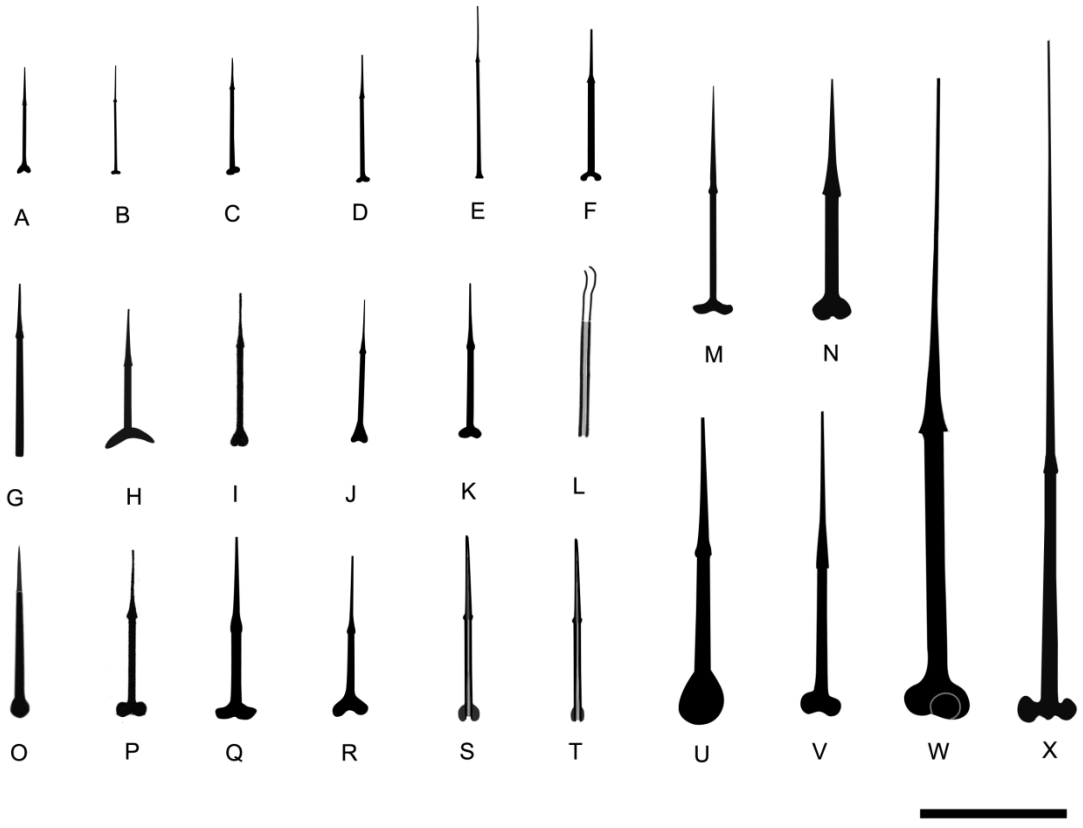

Fig. 8. Stylet in different genera of Tylenchidae. A: *Filenchus discrepans*. B: *Ultratenella vitrea*. C: *Tenunemellus graminis*. D: *Filenchus macramphis*. E: *Chilenchus elegans*. F: *Lelenchus leptosoma*. G: *Atetylenchus abulbosus*. H: *Tanzanius coffeae*. I: *Malenchus exiguus*. J: *Malenchus andrassyi*. K: *Filenchus thornei*. L: *Neopsilenchus magnidens*. M: *Cephalenchus hexalineatus.* N: *Tylenchus davainei*. O: *Irantylenchus clavidorus*. P: *Coslenchus costatus*. Q: *Aglenchus agricola*. R: *Tylenchus maius*. S: *Basiria duplexa*. T:

Basiria paragracilis. U: *Antarctenchus hooperi*. V: *Gracilancea graciloides*. W: *Epicharinema keralense*. X: *Tylodorus acuminatus.* Scale bar: A-W=10 μm, X=20 μm.

Female reproductive system

The female gonad cellular architecture of the female gonad in Tylenchidae is presented in Figs 9, 10. Generally, the female reproductive system is predominantly monodelphic, prodelphic, but also rarely didelphic, amphidelphic (*viz. Antarctenchus, Psilenchus, Atetylenchus*). The ovary is outstretched with oocytes arranged in a single row. In few occasions, oocytes are arranged in two rows and this can be used as a species specific character (*e.g. Boleodorus acurvus* and *B. clavicaudatus*). The oviduct in Tylenchidae comprises of two rows of three to seven cells. The oviduct of *Tylenchus, Filenchus, Coslenchus* and *Aglenchus* is composed of two rows of three or four cells. In *Basiria, Boleodorus, Neopsilenchus* and *Psilenchus,* five (exceptionally six) cells per row are present with the most proximal oviduct cells usually being slightly larger; *Cephalenchus* is characterized by a longer and slightly bent oviduct that comprises two rows of five, six or seven cells. The spermatheca is offset (*e.g.* most species in *Filenchus* and *Tylenchus, Boleodorus thylactus*) or axial (*e.g.* most of *Basiria, Boleodorus* and *Cephalenchus, Coslenchus costatus*). The spermatheca shows several variations in cellular architecture within the Tylenchidae, but usually comprises 10 to 16 cells, except the spermatheca of *Psilenchus aestuarius* is known to have 18-20 cells. Two large cells are usually present connecting the spermatheca to the uterus. The uterus cells are arranged in irregular rows, each comprising 38 to 55 cells (Bert *et al.*, 2006) as four regular rows (= quadricolumella). A constriction may be present between the uterus and the uterine sac (Fig. 9A). Uterine sac is presented anterior to the vagina/vulva. The post-vulval uterine sac (PUS) is rudimentary, usually about half to one of vulval body width, but absent in *Aglenchus, Coslenchus, Fraglenchus, Gracilancea* and some of *Filenchus* (species belong to the former *Duosulcius* and *Zanenchus*) and this has been used as generic character.

The vulva in Tylenchidae is delimited by a gradual depression of the cuticle that forms a wide sunken (*e.g. Coslenchus, Aglenchus, Malenchus*, Fig. 11 A-E), a sharp and narrow sink of one annulus (most common type, *e.g. Filenchus, Lelenchus, Basiria, Boleodorus*, Fig. 11 J) or an elevated cone (only in *Eutylenchus*, Fig 11 F). Epiptygmata are considered as cuticular protrusions of the vaginal wall (Siddiqi, 2000). When present they are usually small, sometimes only visible in SEM (*Aglenchus, Coslenchus, Fraglenchus, Gracilancea* and

Malenchus). However, *Silenchus* has a large epiptygmata which forms a distinct beak-like projection in all studied specimens (Fig. 11 G). The vulva is mostly open, but can also be covered by a longitudinal flap (*Atylenchus*) or bordered by lateral flaps which are either wide (*Aglenchus*, *Coslenchus*, *Fraglenchus*, *Gracilancea*, *Eutylenchus* and *Cephalenchus*) or small (*Malenchus*). The thickness of muscles attached to vagina wall is an important generic delimitation character in some genera (Qing *et al.* 2017). It is thin in most species, but swollen either in the more distal part (*Aglenchus* and *Coslenchus*, Fig. 11 A-D,) or in the proximal to middle part of the vagina (*Malenchus*, Fig. 11 E).

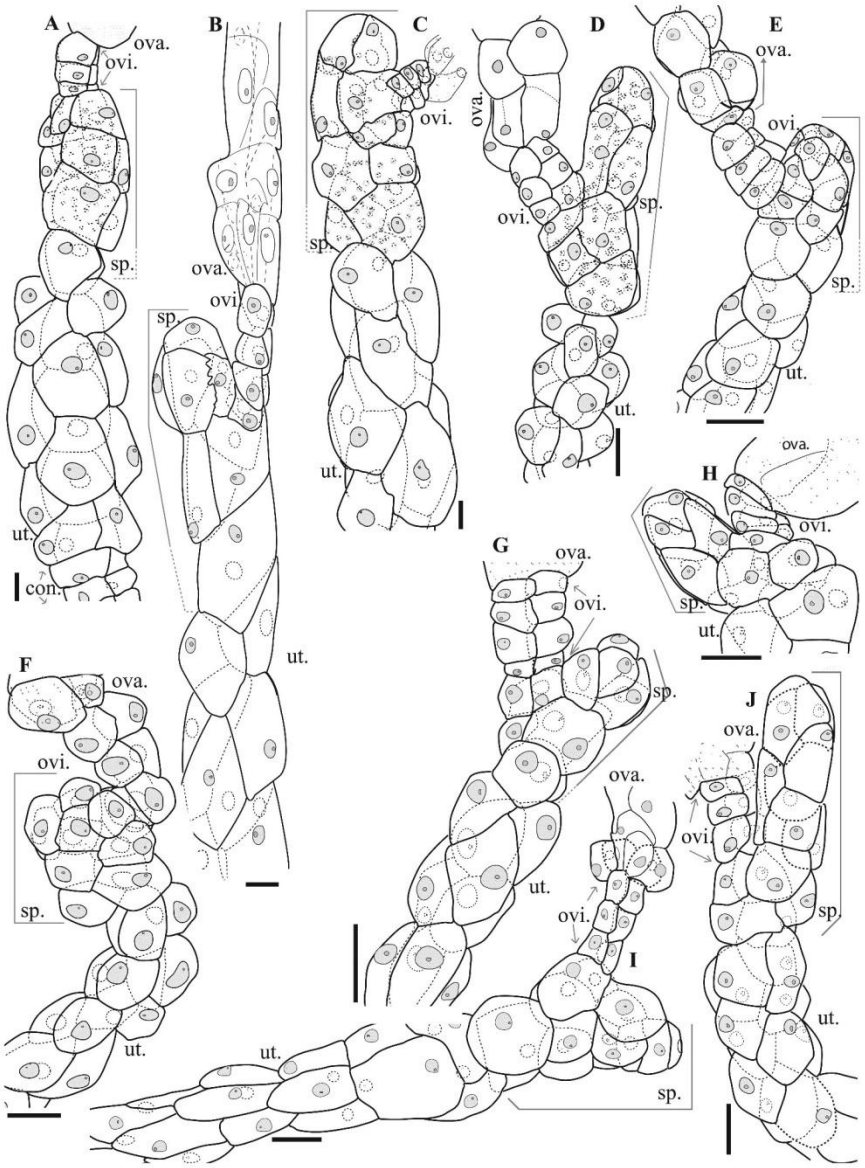

Fig. 9 The cellular architecture of oviduct, spermatheca and uterus of *Tylenchus* spp. and *Filenchus* spp. These species all have four rows of uterus, two rows of oviduct and offset

spermatheca, but different in spermatheca cell numbers. A: *T. arcuatus*. B: *T. davainei*. C: *T. elegans*. D: *F. vulgaris*. E: *F. vulgaris*. F: *F. thornei*. G: *F. orbus*. H: *F. facultativus*. I: *F.* cf. *terrestris*. J: *F*. cf. *facultativus*. ova.: proximal end of ovary; ovi.: oviduct; sp.: spermatheca; ut.: uterus; con.: constriction between uterus and uterine sac. Scale bars = 10 μm (Bert *et al.*, 2006)

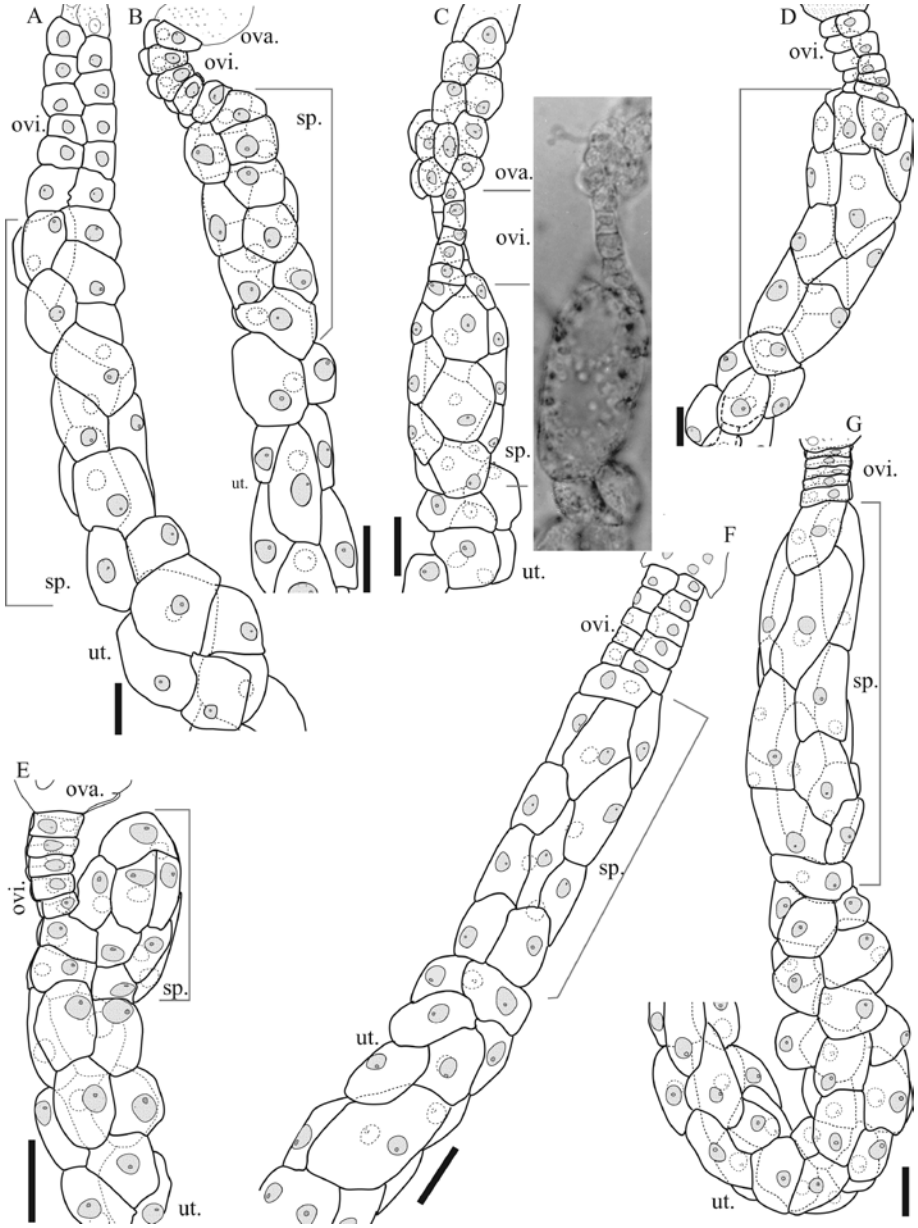

Fig. 10 The cellular architecture of oviduct, spermatheca and distal part uterus of Boleodorinae. These species all have four rows of uterus, two rows of oviduct. Most known species in Boleodorinae have an axial spermatheca (A-D, F, G), but spermatheca can also be present (E). A: *Basiria gracilis*. B: *B. graminophila*. C: *B. graminophila* D: *B. duplexa*. E: *Boleodorus thylactus*. F: *Neopsilenchus magnidens*. G: *Psilenchus aestuarius*. ova.: proximal end of ovary; ovi.: oviduct; sp.: spermatheca; ut.: uterus. Scale bars = 10 μm. (Bert *et al.*,

2006)

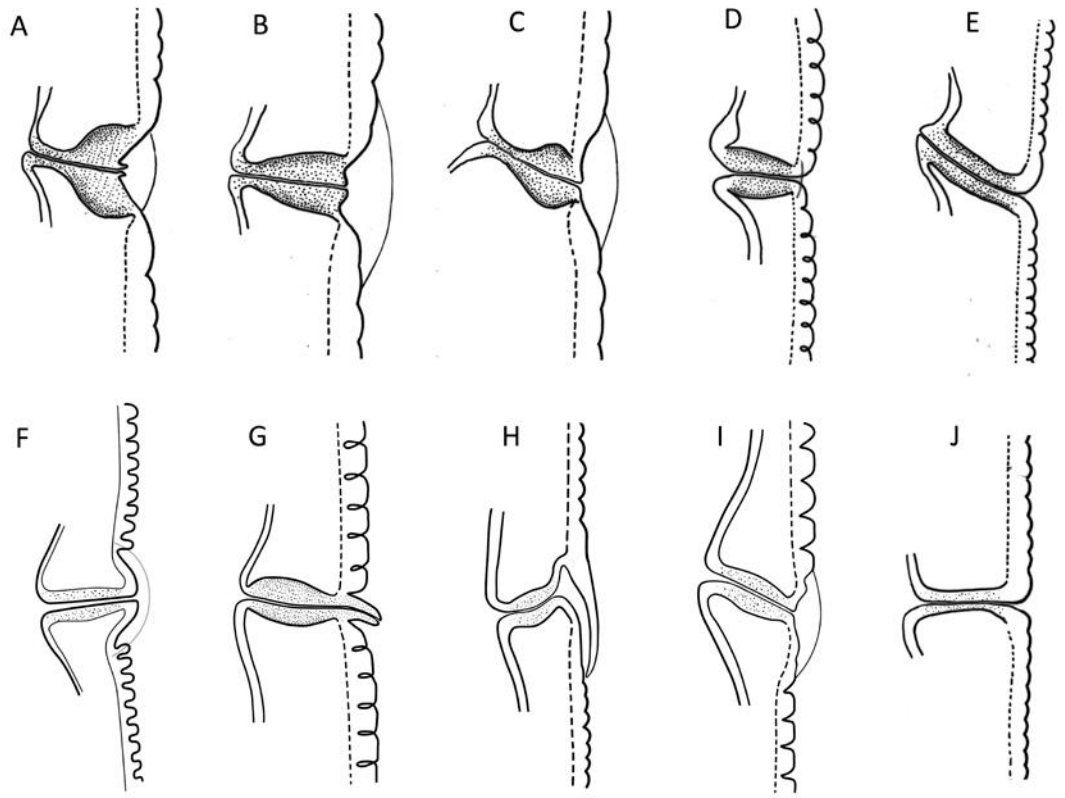

Fig. 11 Vulva regions in different genera of Tylenchidae. A-C: vulva with wide flap, vagina with swollen muscle in distal part, present genus *Aglenchus* and *Coslenchus*. D, E. vulva with small or without flap, vagina with swollen muscle in more proximal or middle part, present in *Malenchus*. F: vulva elevated, with flap, present in *Eutylenchus*. G: epiptygmata large, forming a distinct beak-like projection, vagina with swollen muscle in proximal or middle part, present in *Silenchus*. H: vulva covered by a longitudinal flap, present in *Atylenchus*. I: vulva with wide flap, vagina not or slightly swollen, present in *Cephalenchus*. J: vulva without flap, thin and straight wall without swollen muscle attached. This is the most common type in Tylenchidae, e.g. *Filenchus, Tylenchus*.

Male copulatory system

The male reproductive system in Tylenchidae is similar to the other tylenchs. The most remarkable character is the variation of male bursa (Fig. 12): most species have a short, adanal, leptodern bursa. In some genera bursa is absent (*Miculenchus*, *Atylenchus* and *Tanzanius*). In Ecphyadophorinae, the bursa is lobed, the flaps are rectangular to narrow, projecting outwards and backwards (*e.g. Tenunemellus, Tremonema*) or large, elongate-oval

shape (*Epicharinema*). In *Silenchus* the bursa is long, reaching almost to midway on tail and this has been considered as a generic character.

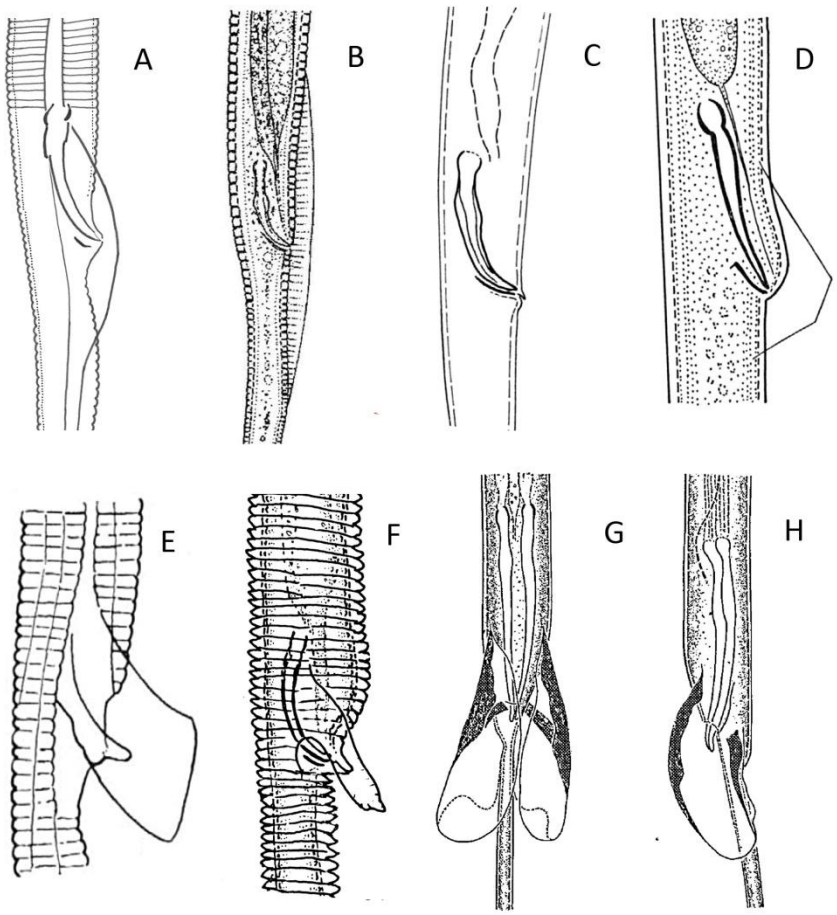

Fig. 12 Male tail region showing the variation of bursa in different genera of Tylenchidae. A. the short adanal bursa, present in most genera of Tylenchidae. B: large and long bursa, reaching almost to midway on tail, present in *Silenchus*. C: Male without bursa, present in *Miculenchus*, *Atylenchus* and *Tanzanius*. D, E: bursa flaps rectangular, lobed, projecting outward and backward, presented in *Tenunemellus*. F: Bursa narrow, lobed with narrow timp, projecting outward and backward, presented in *Tremonema*. G, H: bursa large, elongate-oval, flap-like in outline, presented in *Epicharinema*. Drawings adapted from Husain & Khan (1968); Siddiqi (1994); Raski *et al.* (1982); Raski & Geraert (1984) and Andrássy (2001).

Tail

The tail in Tylenchidae is generally filiform but rich in variety. It is one of the most important characters for the family. The *Filenchus* is the most heterogeneous genus regarding the tail morphology: a tail length from around 30 μm (in some of *F. misellus* and *F. sandneri* populations) to 300 µm; from attenuated (*e.g. F. crassacuticulus*, c=8.2-8.9, c'=5.3-7) to

extremely filiformed (*e.g. F. flagellicaudatus*, c=2.4-2.6, c'=32-37). In most of Boleodorinae (e.g. *Basiria*, *Boleodorus* and *Neothada*) the tail is shorter, mostly ranging from 50 to 80 μm with c value from 5 to 13, although *B. dolichura* has a long filiform tail of 220 to 276 μm. Also in *Malenchus* and *Tylenchus*, the tail is shorter in most species, between 12 to 60 μm with c value about 4 to 7, and the ventrally curved tail tip is typical for *Tylenchus* in comparison to *Filenchus*. Conversely, species belonging to Ecphyadophorinae are extremely slender, their tails are thin and long and can reach up to 350 μm (*e.g. Chilenchus, Epicharinema*) with c value that can be less than 2 (some of *Lelenchus leptosoma* populations), only with few shorter exceptions (*e.g.* tail in *Ecphyadophora caelata* is around 50 μm). The Tylodorinae also have long tails, in *Campbellenchus* the tail can reach up to 450 μm which is the longest tail in Tylenchidae.

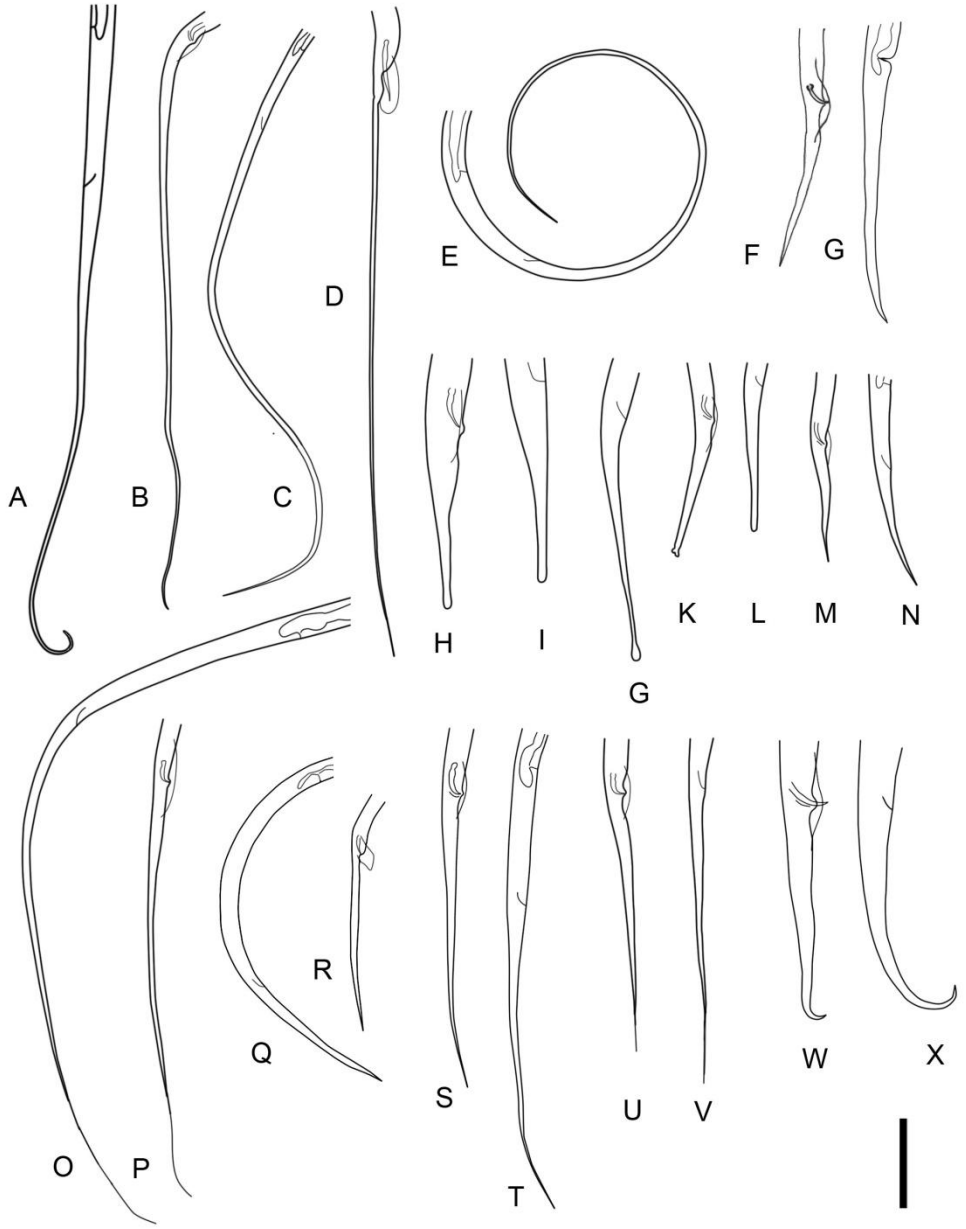

Fig.13 The tail in different genera of Tylenchidae. A, B: *Filenchus flagellicaudatus*. C: *Tenunemellus sheri*. D, E: *Epicharinema keralense*. F, G: *Malenchus exiguus*. H, I: *Boleodorus clavicaudatus*. G: *Psilenchus hilarulus*. K, L: *Basiria tumida*. M, N: *Filenchus misellus*. O, P: *Lelenchus elegans*. Q, R: *Ecphyadophoroides annulatus*. S, T: *Filenchus terrestris*. U, V: *Aglenchus agricola*. W, X: *Tylenchus davainei*. Scale bar = 50μm.

Objectives and outline of the thesis

Objectives

The general aim of the thesis is to contribute and update several aspects of the family Tylenchidae (Nematoda: Tylenchomorpha), including:

1: Obtain more data on diversity and distributions of Tylenchidae, especially the data from neglected habitats (natural ecosystem) and regions (*e.g.* China and Philippines).

2: Extract detailed morphological characters for some of important/problematic genera and examine their significance in generic delimitation.

3: Expand the molecular database by adding DNA sequences of Tylenchidae and use these sequences to study the phylogenetic relationship for each of genera.

4: Add more data for other closely related tylenchs which have supposedly ancestral characters (Sphaerularioidea) as references to study the origin and evolution of Tylenchidae.

5: Explore and apply new techniques to improve visualization and presentation of the complex morphological characters in Tylenchidae.

Outline

Chapter I comprises a general introduction of Tylenchidae, including a taxonomical background, current knowledge of the Tylenchidae phylogeny and morphological diversity in its different genera.

Chapter II focuses on the described diversity of *Malenchus*. started with study of diversity: A new species *Malenchus sexalineatus* n. sp. was discovered from Philippines, and described based on morphological and molecular data; three known species of this genus namely *M. exiguus M. nanellus* and *M. pachycephalus*, all being first records and first representative from China were characterized by their morphological data.

Chapter III presents a molecular phylogeny of Tylenchidae using 58 newly obtained 18S and 28S rRNA sequences. The light microcopy and transmission electron microscopy were used to provide details on morphological features. For the first time comprehensive morphological data are evaluated in the context of a molecular framework, thus highlighting the phylogenetic and evolutionary complexity of this structurally minimalistic group. The study also reveals the shortage of D2/D3 domain in 28S rRNA as a phylogenetic marker for early diverging

Tylenchomorpha (=tylenchs with supposedly ancestral characters).

Chapter IV redefines the generic characters of *Malenchus*, *Ottolenchus* and *Filenchus* in light of the phylogenetic study in Chapter II. A total of 22 populations including 12 type/paratype species were examined. The detailed morphology was recovered using light microscopy, scanning- and transmission- electron microscopy. All population and type slides were recorded as picture and video vouchers and provided, which are available online. Inter- or/and intraspecific variations and taxonomically informative traits are extracted.

In **Chapter V**, a new genus *Labrys chinensis* gen. n., sp. n. in Tylenchidae was described using an integrative approach: detailed morphology based on light- and electron microscopy, phylogenetic position as revealed from two *ribosomal RNA* genes, generic traits were tested for homoplasy, and the intra- and inter-population variations of four recovered populations were analyzed. For the first time, 3D printed models were incorporated in the description of a new genus as a complement to pictures and drawings to illustrate complex 3D structures and to be used in education. Hereby, we also tested the performances of different printing materials and forwarded resin as the most suitable option for the zoological field.

In **Chapter VI** The generic definitions presented in chapter II and Chapter III are applied to describe a new *Malenchus* species. Aside from *Malenchus*, three rare genera of Tylenchidae *viz*. *Miculenchus*, *Tenunemellus* and *Lelenchus*, are examined. Detailed morphology of all nematode species are provided using light microscopy (LM) and scanning electron microscopy (SEM).

Chapter VII presents a the diversity study of Tylenchidae in China. A country-wide sampling from terrestrial natural ecosystem in China revealed 25 species that belong to Tylenchidae, 17 species and 5 genera are new records for China. The detailed morphometric data are provided for these recovered populations.

In **Chapter VIII** Our research extends to the Sphaerularioidea, which also belong to the putative early diverging Tylenchomorpha (=tylenchs with supposedly ancestral characters) and are phylogenetically related to Tylenchidae. *Abursanema quadrilineatum* n. sp. was recovered from mushroom and described both morphologically from LM, SEM and TEM and molecularly based on 18S and 28S rRNA. In this chapter, secondary structures of the D2 and D3 domain of 28S rRNA were predicted for the new species and a general model for the superfamily Sphaerularioidea was built for comparative analysis.

In **Chapter IX** we summarized the 3D modeling and printing techniques that can improve the

morphology research and education. A relatively simple time-saving method using Autodesk Maya was proposed.

In the last part, **Chapter X** provides general comments on taxonomy, phylogeny and evolution of Tylenchidae. The problems of current molecular phylogeny are summarized and suggestions are provided given on marker gene selection, primer design and tree construction. In addition, the possible applications of new visualization techniques in nematology are discussed. Finally, the general conclusion, lists the major findings and future directions of taxonomy and phylogeny in Tylenchidae are provided.

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Chapter II

Description of one new, and new records of three known species of genus *Malenchus* Andrássy, 1968 (Nematoda: Tylenchidae); with notes on the development of the amphidial aperture

Chapter published as:

Qing, X.[1], Sánchez-Monge[1,2], A., Janssen, T.[1], Couvreur, M.[1], & Bert, W.[1] (2016). Description of *Malenchus sexlineatus* n. sp., new records of three known species of Malenchus Andrássy, 1968 (Nematoda: Tylenchidae) and notes on amphidial aperture development. *Nematology*, *18*(2), 155-174.

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Abstract

A new species, *Malenchus sexalineatus* n. sp. discovered from Philippines is described based on morphological and molecular data. The new species is unusual among the genus by having six lateral lines and characterized by having an exceptionally short body (270-288µm) and narrow annulations (0.7-0.8 µm). Morphological comparisons with closely related species are discussed. Furthermore, three known species of this genus namely *M. exiguus M. nanellus* and *M. pachycephalus,* all being first records and first representative from China were characterized by their morphological data. The new species was placed in a robustly supported clade containing two other *Malenchus* spp. and *M. exiguus*. Interestingly, *M. pressulus* was placed in a separate unresolved phylogenetic position. However, the phylogenetic position of these clades could not be resolved within Tylenchidae. The shapes of the amphidial aperture and fovea within *Malenchus* are also compared and its possible developmental process is illustrated and discussed.

Key Words: new species, phylogeny, SEM, taxonomy, Tylenchomorpha

Introduction

The genus *Malenchus* is one of the most specious genera within Tylenchidae and has been reported worldwide (Andrássy, 1981). This genus was established by Andrássy (1968) and is characterized by prominent annulations and dorso-ventrally flattened lip region, with *M. machadoi* as type species (formerly *Aglenchus machadoi* Andrássy, 1963). Several taxonomic changes have occurred within this genus and the first reviews by Knobloch (1976) and Siddiqi (1979) have led to the description of two species (*M. bryanti* and *M. truncatus*) and the erection of *Neomalenchus* with two species respectively.

Andrassy (1981) performed a comprehensive and detailed study of *Malenchus* and the description of seven new species and proposed *Neomalenchus* as a junior synonym of *Malenchus*, an action that was followed by Geraert and Raski (1986). Later, Siddiqi (2000) considered *Neomalenchus* as a valid subgenus and introduced another subgenus (*Telomalenchus*) to accommodate three species with straight amphidial aperture and fewer lateral lines (4 or 6 *vs* 12 or more in other *Malenchus* species), namely *M. williamsi* Geraert & Raski, 1986, *M. parthenogeneticus* Geraert & Raski, 1986 and *M. leiodermis* Geraert & Raski, 1986. Despite the flattened lip region and the long amphidial slit, Andrássy (2007) synonymized *Malenchus* with *Fraglenchus* Siddiqi, 2000 which has a rounded lip region and a short amphidial slit. Sumenkova (1988) erected the genus *Paramalenchus* for the species *P. anthrisulcus* Sumenkova, 1988. However, it was synonymized with *Malenchus* Ebsary (1991), an action that was followed by Siddiqi (2000) and Geraert (2008). *Malenchus novus* Mukhina & Kazachenko, 1981 was initially assigned to the genus *Malenchus* but later moved to the genus *Mukzia* mainly based on its unusually large body size (Siddiqi, 1986). The validity of the latter genus was not accepted by Geraert (2008) as the body size was the only used differentiating character. In this study we follow Geraert (2008) who listed 35 valid species and 3 *nomina nuda* under two subgenera (*Malenchus* and *Telomalenchus*).

Despite its importance of the genus from a phylogenetic aspect as an early diverging branch within Tylenchomorpha (=tylenchs with supposedly ancestral characters) (De Ley & Blaxter, 2002), little is known about the phylogenetic status of the genus and its inter- and intra-genus affinities. In the present study, the genus *Malenchus* is studied in China for the first time. A new species, *Malenchus sexalineatus* n. sp., is described and its phylogenetic affinities with other species and genera are depicted. Furthermore, three known species of the genus, all being first reported from China, are illustrated in detail, and the development of the

amphidial aperture of the genus is discussed.

Materials and methods

Samples collecting and processing

Samples were collected in four locations in 2012 and 2013: Mt. Hamiguitan, Philippines in August of 2012; Shimen, Hunan, China; Pingwu, Sichuan, China and Mt. Taibai, Shaanxi, China in August of 2013 (for additional details, see below). Nematodes were extracted from soil samples using a Baermann tray, collected and concentrated using a 500 mesh sieve (USA standard mesh numbers, equal to 25μm opening). After removing water, nematodes were rinsed with DESS solution and transferred to glass vials (Yoder *et al.*, 2006). DESS-preserved specimens were rinsed several times with deionized water and then transferred to anhydrous glycerin, following the protocol of Seinhorst (1962) modified by Sohlenius & Sandor (1987).

Morphological characterization

Measurements and drawings were prepared manually with a drawing tube mounted on an Olympus BX51 DIC Microscope (Olympus Optical, Tokyo, Japan), equipped with an Olympus C5060Wz camera for photography. The holotype of the new species, examined Chinese population and paratype slides of *M. williamsi* Geraert & Raski, 1986 (UGMD103427, UGMD103427, UGMD103427), *M. leiodermis* Geraert & Raski, 1986 (UGMD103431) and *M. parthenogeneticus* Geraert & Raski, 1986 (UGMD103432) were recorded as a video clips mimicking a multifocal observation through a light microscope (LM) developed by De Ley and Bert (2002). The resulting digital specimen vouchers are available at http://www.nematology.ugent.be/vce.html.

Illustrations were prepared using GNU Image Manipulation Program, GIMP 2.810 and Adobe Illustrator CS3 based on light microscope drawings. 3D models were reconstructed using Autodesk® Maya® following the procedure of Qing *et al.* (2015). For scanning electron microscopy (SEM), specimens from DESS were gradually washed with water and post fixated with 2% PFA+2.5% Glutaraldehyde in 0.1M Sorensen buffer, then washed and dehydrated in ethanol solutions and subsequently critical point dried with CO_2. After mounting on stubs, the samples were coated with gold following the procedure detailed by Steel *et al.* (2011) and observed with a JSM-840 EM (JEOL, Tokyo, Japan) at 12 kV.

Molecular characterization

Genomic DNA was extracted from DESS preserved specimens with Worm Lysis Buffer (Yoder *et al.,* 2006). PCR reaction and sequencing of the D2-D3 domains of the LSU rRNA was done following the protocol of (Múnera Uribe *et al.*,2010). *De novo* sequences were deposited in GenBank under the accession numbers KR818869 (*Malenchus sexalineatus* n. sp.), KR818870 (*Malenchus* sp. P9) and KR818871 (*Malenchus* sp. P4). These sequences were compared with other relevant available sequences in GenBank. Multiple alignments of the different genes were made using the Q-INS-i algorithm of MAFFT v. 7.205 (Katoh & Standley, 2013) which accounts for secondary RNA structure. Poorly aligned positions and divergent regions were selected and deleted by Gblocks 0.91b (Castresana, 2000) with all three less stringent options. The best-fitting substitution model was estimated using AIC in jModelTest v. 2.1.2 (Darriba *et al.*, 2012) and GTR+I+G was selected as best scored model. Maximum likelihood (ML) analysis was performed with 1000 bootstrap (BS) replicates under the GTRCAT model using RAxML 8.1.11(Stamatakis, 2006; Stamatakis *et al.*, 2008). Bayesian phylogenetic analysis (BI) was carried out with the GTR+I+G model using MrBayes 3.2.3 (Ronquist & Huelsenbeck, 2003). Analyses were run for 5×10^6 generations and Markov chains were sampled every 100 generations. Burnin was arbitrarily chosen to be 25% of the results, and evaluated using a generation/Log-likelihood scatter plot. The ML and BI analyses were performed at the CIPRES Science Gateway (Miller *et al.*, 2010). Gaps were treated as missing data for all phylogenetic analysis. A Bayesian consensus tree was created by collapsing all clades with a posterior probability (PP) below 95 or BS below 70, using TreeView v. 1.6.6 (Page, 1996). ML BS values and Bayesian posterior probabilities (PP) were summarized on the consensus tree using Adobe Illustrator CS3. To assess the significance of monophyly of the genus *Malenchus*, a constrained Bayesian analysis was ran in MrBayes 3.2.3 using the same parameters as the original analysis. Site-specific likelihoods were calculated for the unconstrained and constrained Bayesian trees using PAML v4.8 (Yang, 2007), with the same models used in the original analyses, but with the model parameters optimized by baseml. These likelihoods were compared based on Shimodaira–Hasegawa (SH) and approximately unbiased (AU) tests (Shimodaira & Hasegawa, 1999; Shimodaira, 2002) using CONSEL v. 01i (Shimodaira & Hasegawa, 2001).

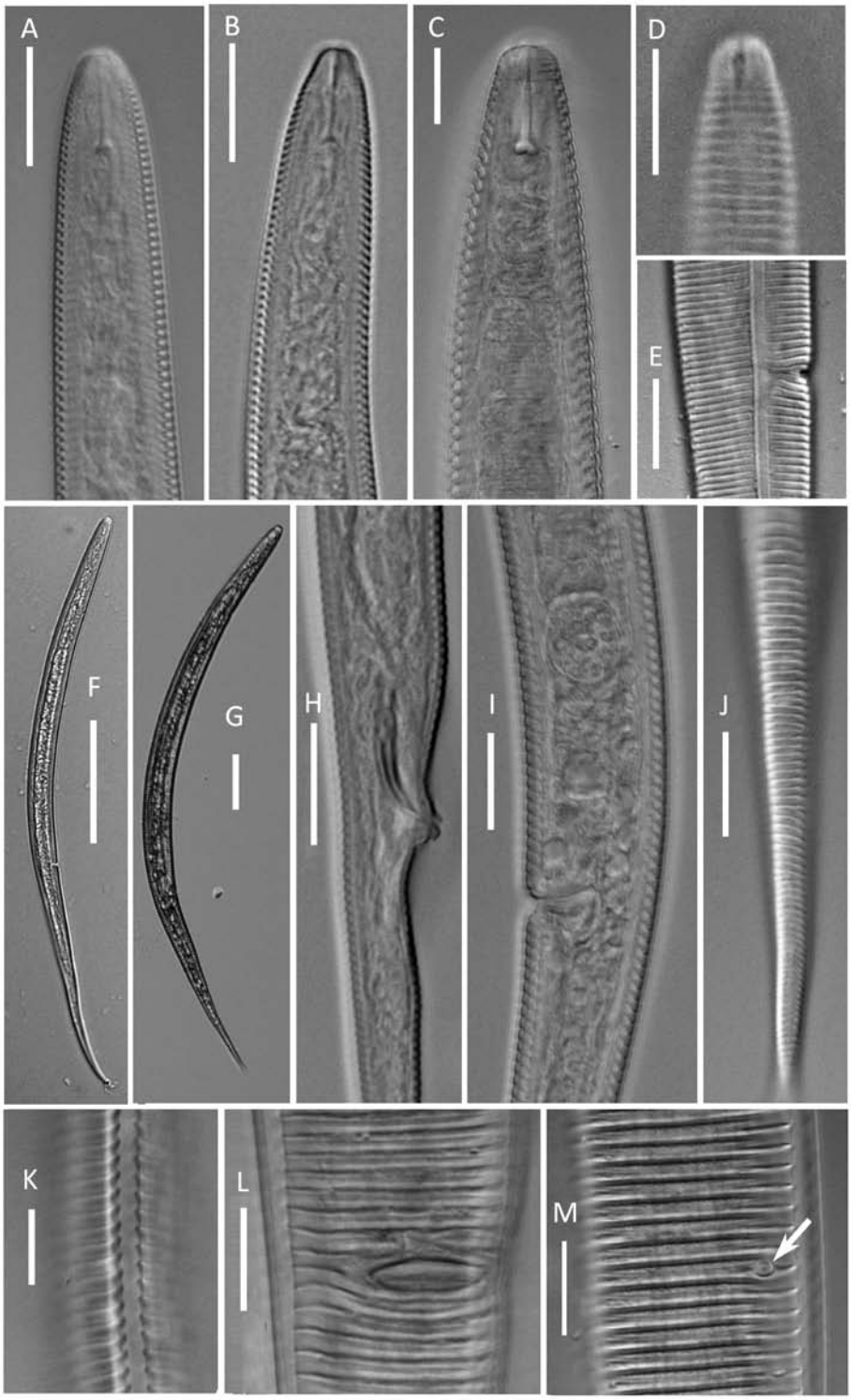

Fig. 1. LM picture of *Malenchus sexalineatus* n. sp. (A, E, F), *M. nanellus* (B, D, H) and *M. pachycephalus* (C, G, I, J, K, L, M). A-C: Female anterior end; D: Amphidial fovea; E: Lateral view of vulva region; F, G: Female habitus. H: Spicules and protruding cloacal lips; I: Vulva and spermatheca; J: annules on female tail; K: crenate female lateral lines; L: Ventral view of vulva; M: Female ventral view, arrow shows prophasmid. (Scale bar: A-E, H-M = 10 µm, F-G = 50 µm.)

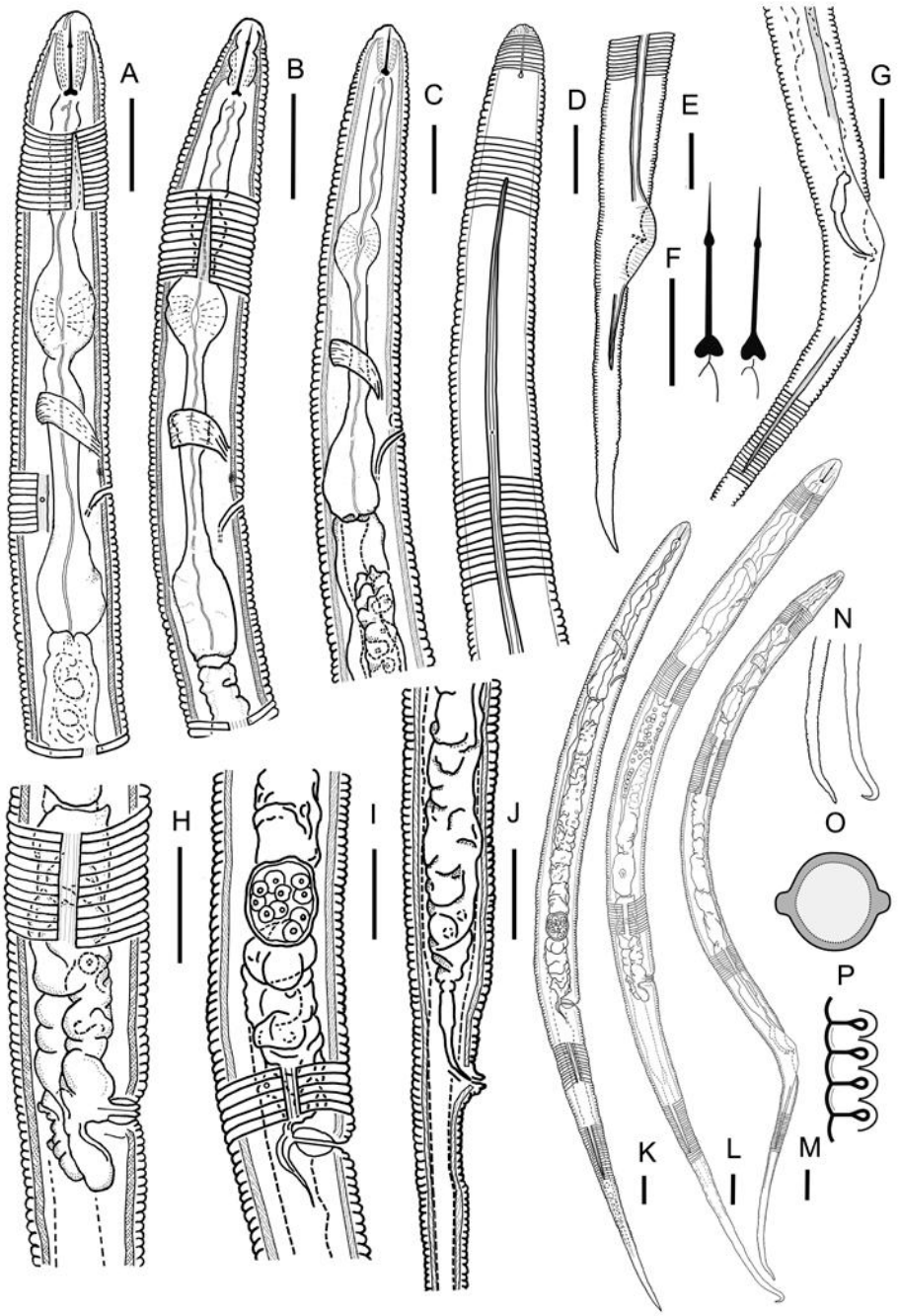

Fig. 2. Illustration of *Malenchus sexalineatus* n. sp. from the Philippines, female holotype and male paratype (A, B, F, G, H, L, M, N, O, P) and Chinese population of *M. nanellus* Siddiqi, 1979 (C, D, E, I, J, K, P). A: Female anterior body; B: Male anterior body; C, D: Female anterior body; E: Male tail; F: Female stylet; G: Male tail shows spicule, gubernaculum and bursa; H, I: Female reproductive system, showing sunken vulva, epiptygmata, thicken vaginal wall and PUS; J: Posterior male body shows spicule, gubernaculum; K, L: Female habitus; M: Male habitus shows dorsally bent tail; .N: tail tip; O: Cross-section of body shows one elevated ridge in lateral region; P: Annules. (Scale bar A-E, G-M = 10 µm, F = 20 µm.)

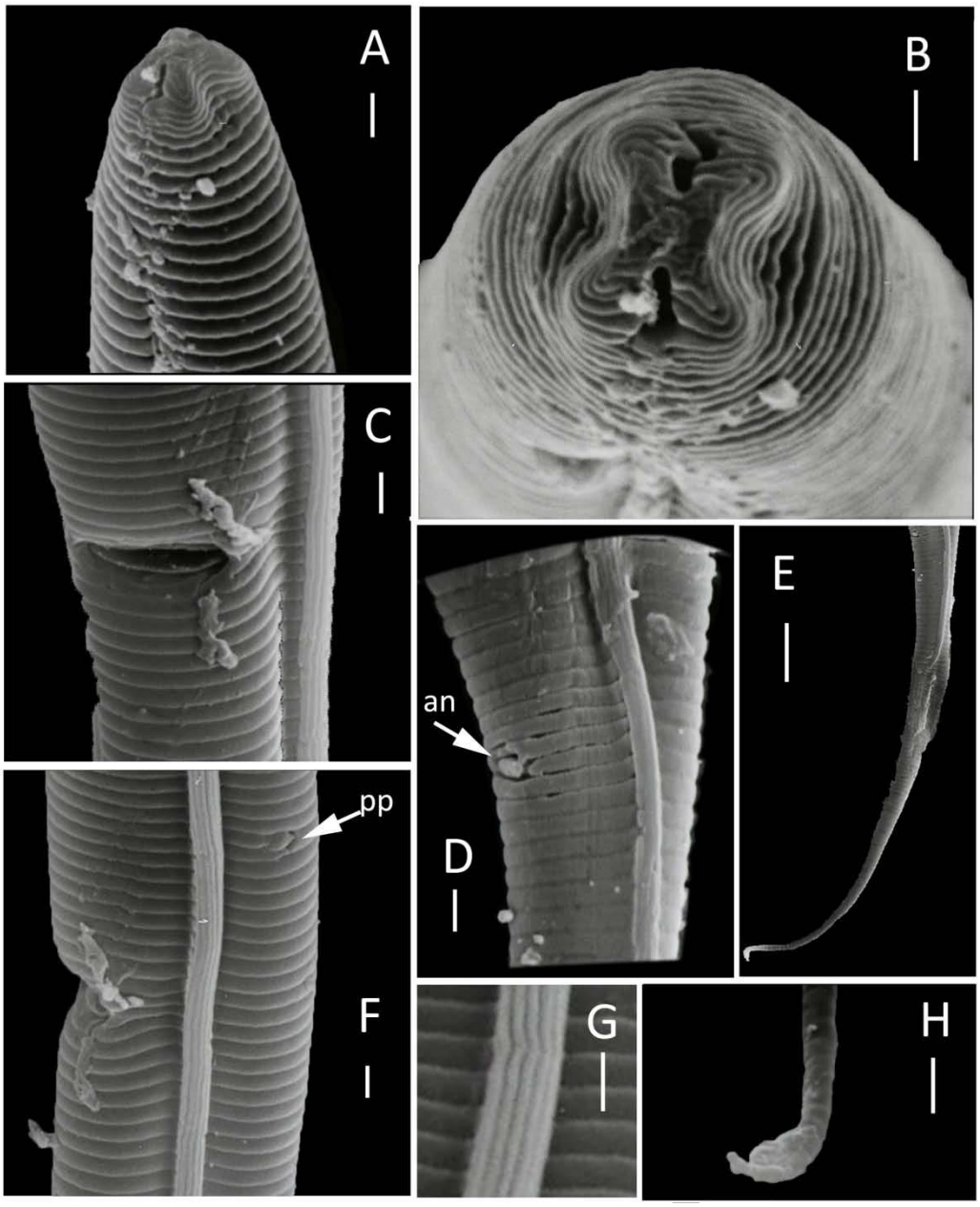

Fig. 3. SEM of female *Malenchus sexalineatus* n. sp. from Philippines. A: lip region; B: *en face* view; C: Ventral view of vulva shows epiptygmata; D: Anus (an=opening of anus); E: Tail. F: Lateral view of vulva (pp=prophasmid); G: Six incisures in lateral region; H: The hook shape tail tip. (Scale bar: A-D, F-H = 1 µm, E = 5 µm.)

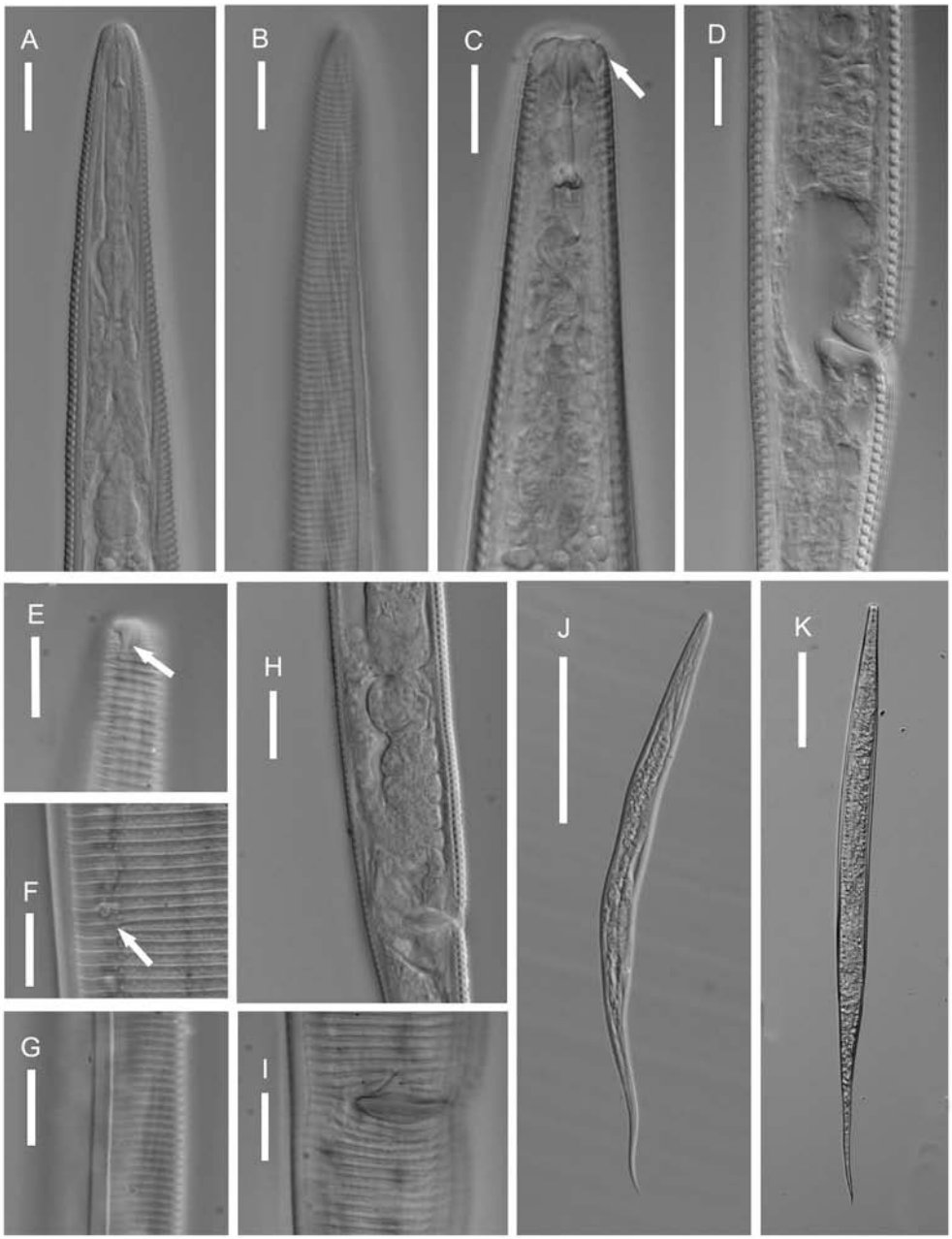

Fig. 4. LM picture of *M. exiguus* (A, B, G, H, J) and *Malenchus sp.* (C, D, E, F, I, H). A, B: Female anterior body; C: Ventral view of female anterior body (arrow shows amphidial fovea); D: Female reproductive system shows sunken vulva, thicken vaginal wall; E: Female lip region (arrow shows amphidial aperture); F: Prophasmid; G. Lateral region with offset ridge; H: Female reproductive system shows part of ovary, spermatheca, uterus, vagina and sunken vulva; I: Ventral view of vulva. J, K: Female body habitus. (Scale bar: A-I = 10 µm, J, H = 100 µm.)

Result

Malenchus exiguus (Massey, 1969) Andrássy, 1980

(Figs 4A, B, G, H, J; Figs 5A, B, D, E, F, G, I, J, M, N, O, R)

MEASUREMENTS

See Table 1

DESCRIPTION

Female

Body small to middle sized. Lip region typical of the genus, dorso-ventrally flattened. Lateral lines consisting of 2 incisures, slightly crenated, starting closed to median bulb (about 21-26 annuli from posterior to the end of lip region) and ends until half of tail. Amphidial aperture sinuous-shaped. Stylet slender, cone about one third of stylet length. Median bulb oval, valvular apparatus round, conspicuous. Prophasmid inconspicuous, 9-13 annuli anterior to vulva. Reproductive system monodelphic-prodelphic, ovary outstretched, uterus four rows with five cells in each row. Vulva sunk in body, vagina thickened, lateral flap distinct, 2-3 annuli. Spermatheca rounded, simple/unilobed, offset, filled with sperm. Tail ventrally bended, filiform with pointed terminus.

Male

Less common than females. Generally similar to female but with more elevated lip region, more delicate stylet and more elongated valvular apparatus in median bulb. Testis long, spermatids spindle shape, sperm cells round. Bursa about 30µm long, starting at the level of spicules' capitulum. Spicules and gubernaculum tylenchoid.

HABITAT AND LOCALITY

Collected from a deciduous forest around the roots of *Betula* sp. at 2772 m.a.s.l. in Mt. Taibai (34°00'46"N, 107°43'33"E), Shaanxi, China.

Remarks

Malenchus exiguus was originally described by Massey (1969) as *Aglenchus exiguus* and this species was later moved to the genus *Malenchus* by Andrássy (1980). The studied population fits the morphology, morphometry and ratios of *M. exiguus*, except for a slightly shorter stylet (7.7-8.5 *vs* 9.0-10 µm). Although, the key of Geraert (2008) brought us initially to *M. acarayensis* Andrássy, 1968, clear differences with type material of *M. acarayensis*,

include a higher tail/vulva-anus ratio (1.6-1.7 *vs* 1.3-1.4 µm), narrower annuli (1.0-1.1 vs 1.5-1.7 µm) and broader lip region (relatively round *vs* more compressed and flattened).

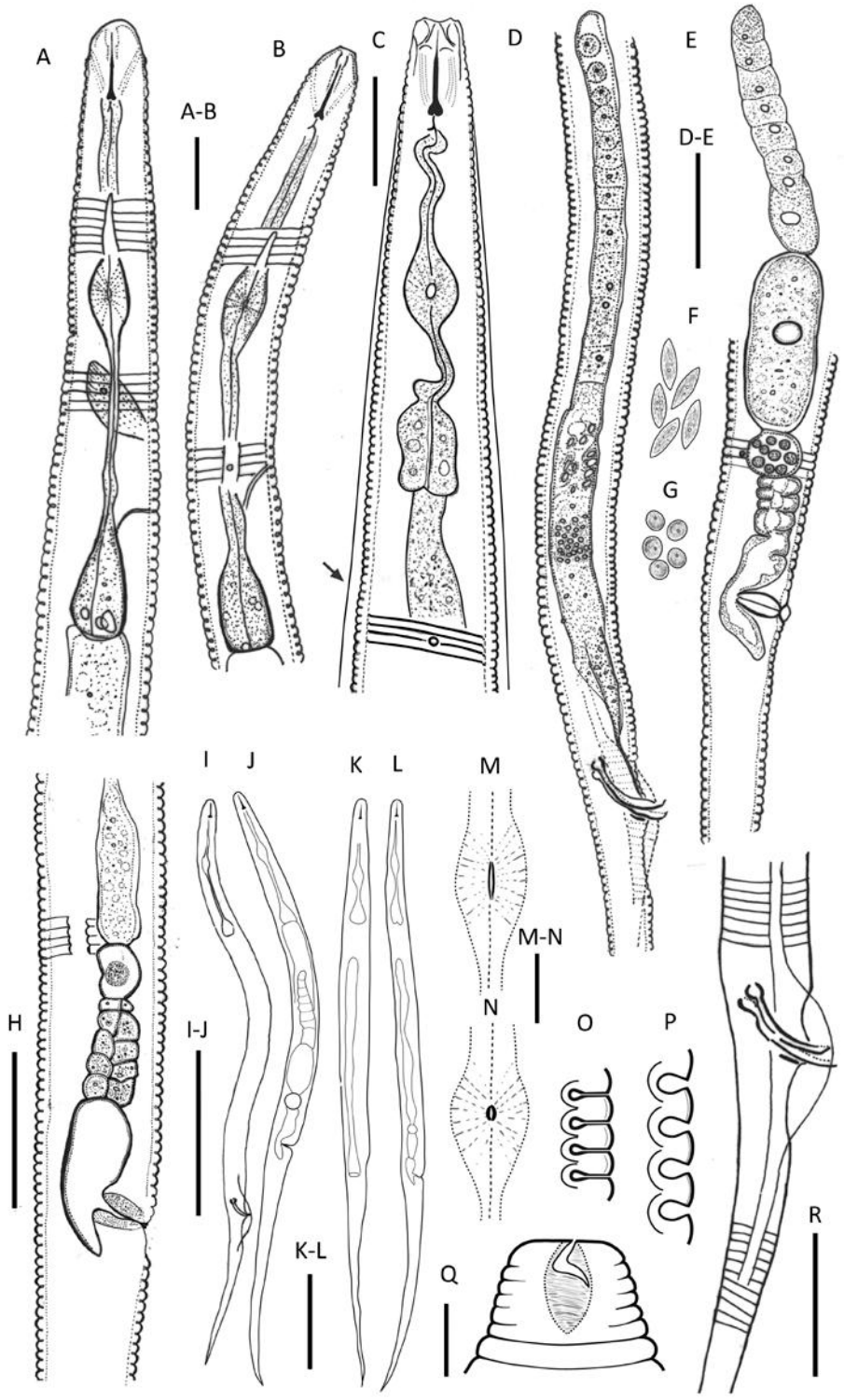

Fig. 5. Illustration of *M. exiguus* (A, B, D, E, F, G, I, J, M, N, O, R) and *Malenchus sp.* (C, H, K, L, P, Q). A: Female anterior body; B: Male anterior body; C: Ventral view of female anterior body; D: Male reproductive system; E. Female reproductive system; F: Spermatids

from *vesicula seminalis*; G: Sperm cells from *vesicula seminalis*; H: Female reproductive system; I: Male habitus; J-L: Female habitus; M: Male median bulb shows elongated valvular apparatus; N: Female median bulb shows round valvular apparatus; O, P: Folded cuticle; Q: Lateral view of lip region shows amphidial aperture and fovea; R: Male tail. (Scale A, B = 10 µm. C, D, E, H, R = 20 µm, I-L = 100 µm.)

Malenchus sexalineatus[*] n. sp.

(Figs 1A, E, F; Figs 2A, B, F-H, L-P; Fig. 3)

MEASUREMENTS

See Table 1

DESCRIPTION

Female

Body very small (one of smallest known nematode species), ventrally arcuate after fixation. Body tapers slightly toward posterior end. Cuticle thick, folded between annuli, annulations exceptionally narrow (0.7-0.8µm). Lateral field prominent, origins at half of or one stylet length behind stylet knobs ending at middle of tail, with 6 incisures in an elevated ridge with relatively smooth margin (not crenate). Number of incisures can occasionally increase to eight by irregularly short insertion of short bands. Lip region elevated, dorso-ventrally compressed, 3.52-4.15 µm wide. Oral opening surrounded by six labial papillae, which is set on a slight protuberated oral plate. Amphidial apertures S-shaped, starting at the labial plate. Labial framework weak. Stylet slender and delicate, cone about one third of total length, cone width half of anterior shaft width and one third of posterior shaft width. Median bulb oval and weakly developed, with slightly or not sclerotized valve. Isthmus long and slender. Terminal bulb short and pyriform. Excretory pore at the level of anterior part of pharyngeal bulb. Hemizonid 2-3 annuli long and 2-3 annuli before excretory pore. Deirids at the level of excretory pore. Rectum very thin, anus inconspicuous. Reproductive system monodelphic, prodelphic, ovary outstretched with oocytes arranged in a single row. Spermatheca rounded to elongated; offset, globular sperm limited in spermatheca or also in proximal part of uterus. Uterus has four rows with five cells in each row. Uterus sac spacious with thickened wall, egg not observed (not gravid). Vulva sunken in body contour,

∗ Etymology: the specific epithet “sexalineatus” refers to the number of lines in the lateral field under SEM, “six” (Latin prefix, “sex-”) and “lined” (Latin “lineatus”).

lateral flaps absent or one annuli long. Epiptygmata present. Vagina perpendicular to body with thickened vaginal wall. Prophasmid 14-16 annuli anterior to vulva. Tail tapering gradually to more or less pointed hook-shaped tip.

Male

Less frequent than females. General morphology similar to that of female except reproductive system and more slender body. Testis single, located along ventral side of body. Spermatogonia arranged in one row, spermatids few, hardly visible, spermatozoa round, filling proximal part of vesicula seminalis. Vas deferens separated from other parts of gonad. Tail strongly and dorsally bent after cloaca, giving the tail a total curvature of 130-140° to adjacent body anterior to spicule, which is unique in the genus. Cloacal opening on prominent cone with protruding lips. Bursa short but prominent, adanal, starts at the same level of spicules' capitulum. Spicules paired, slightly bent ventrally, capitulum part rounded, shaft and blade slightly tapering. Gubernaculum short and very thin.

TYPE HABITAT AND LOCALITY

Recovered from Mount Hamiguitan (6°43'51.8"N, 126°10'05.3"E), Philippines, at an altitude of 950m under the litter of *Lithocarpus llanosii* Rehder (Fagaceae).

TYPE MATERIAL

Holotype female, four female paratypes and one male paratype were deposited at the Museum Voor Dierkunde (Collection number UGMD 104304), Ghent University, Ghent, Belgium. Additional paratypes are available in the UGent Nematode Collection (slide UGnem144) of the Nematology Research Unit, Department of Biology, Ghent University, Ghent, Belgium. The new generic name has been registered in ZooBank (zoobank.org) under the identifier 6EE3BA51-E178-43C6-AD88-056083AA3D82.

DIAGNOSIS AND RELATIONSHIPS

Despite that only 12 species out of 35 listed valid species by Geraert (2008) have SEM image (7-12 lines have been detected), and that LM is unable to discern the exact number of lateral lines, still the unique combination of features in *M. sexalineatus* n. sp. differentiate it from all other *Malenchus* (*Malenchus*) species. The new species is described based on species concept that emphasis morphological difference. It is characterized by having six lines at lateral fields, exceptionally short body (270-288 µm), narrow annulations (0.7-0.8 µm) and a dorsally bent male tail after DESS relaxation.

Malenchus sexalineatus n. sp. is assigned to the genus *Malenchus* based on the

combination of the following morphological features: dorso-ventrally compressed and anteriorly flattened lip region, very prominent cuticle annulations, protruding and conspicuous lateral field and markedly narrowing body behind vulva. On subgenus level, the few lateral lines point to *Telomalenchus* Siddiqi, 2000, however, this subgenus is characterized by straight amphidial apertures while S-shaped amphidial aperture is typical for the subgenus *Malenchus* (Siddiqi, 2000). Furthermore, all *Telomalenchus* paratypes (*M. williamsi* Geraert & Raski, 1986, *M. leiodermis* Geraert & Raski, 1986, *M. parthenogeneticus* Geraert & Raski, 1986) examined by LM in the present study showed many differences with the proposed new species in morphological characters like annulations (relatively weak annulations *vs* prominent cuticle annulations), vulva flap (four or more annuli long *vs* invisible), lateral lines (four or more well separated lines in LM *vs* two lines in LM) and stylet shape (much longer *vs* short). Finally, the presence of six lateral lines differentiate the new species from all SEM available species in the subgenus *Malenchus* which have numerous lateral lines. Nevertheless, *M. sexalineatus* n. sp. comes closer to the subgenus *Malenchus* because of the 6 incisures that are tightly arranged in one protruding band (two lines in LM) and the S-shaped amphidial aperture. Therefore, phylogenetic analyses are needed to verify/test the position of this species and other species in this subgenus.

Malenchus sexalineatus n. sp. is distinguished from *M. williamsi* Geraert & Raski, 1986, the only species in the genus with six lateral lines (based on currently available SEM data), by a shorter body and weaker stylet *vs.* relatively longer body and longer stylet; narrower annulations *vs*. broader annulations; one protruding ridge *vs.* incisures in later region well separated in LM resembling the lateral lines in genus *Cephalenchus* Goodey, 1962; the presence of a S-shaped *vs*. straight amphidial aperture, and absence or one annuli of vulval flaps *vs.* distinct vulval flap. By having an exceptionally short body, *M. sexalineatus* n. sp. comes close to *M. parvus* Brzeski, 1989, *M. bryanti* Knobloch, 1976 and *M. acarayensis* Andrássy, 1968. However, there are significant differences in the lateral lines, annuli width and most morphometric ratios. The morphological and morphometric differential traits of above mentioned species are compared in Table 2.

MOLECULAR CHARACTERIZATION

Tree topologies inferred by ML and BI were largely congruent, except for several unresolved clades which were collapsed (original BI and ML tree available at http://nematodes.myspecies.info). Bootstrap values and posterior probabilities are summarized on the Bayesian consensus tree (see Fig. 7.).

In all analyses, *M. sexalineatus* n. sp. was robustly supported (PP=100, BS=98) as sister taxa to *M. exiguus* (Massey, 1969) Andrássy, 1980. However, these two species are morphologically separated. This clade together with two *Malenchus* spp. P4 and P9, *M. labiatus* Maqbool & Shahina, 1985 and *Lelenchus leptosoma* (de Man, 1884) Andrássy, 1954 form a fully supported clade (PP=100, BS=100) where P4 and P9 (unidentified due to only two juveniles in total, recovered at *ca.* 500 m distance from the new species location) showed no genetic distance and only differed in sequence length. However, our phylogenetic analysis could not reveal supported relationships of this clade with other taxa in Tylenchidae. Surprisingly our analyses did not prove the monophyly of the genus *Malenchus*, as *M. pressulus* (Kazachenko, 1975) Andrássy, 1981 is placed in a separate unresolved position. The result also supports *M. pressulus* to be *Malenchus* instead of its original description as a species of *Aglenchus.* but also that relationship is not supported. The alternative topology showing the monophyly of the genus was tested (graphical representation of the topology of constrained tree is given in Fig. 8B) and this morphologically based hypothesis was rejected based on SH and AU tests (SH test $p = 0.031$, AU test $p = 0.026$).

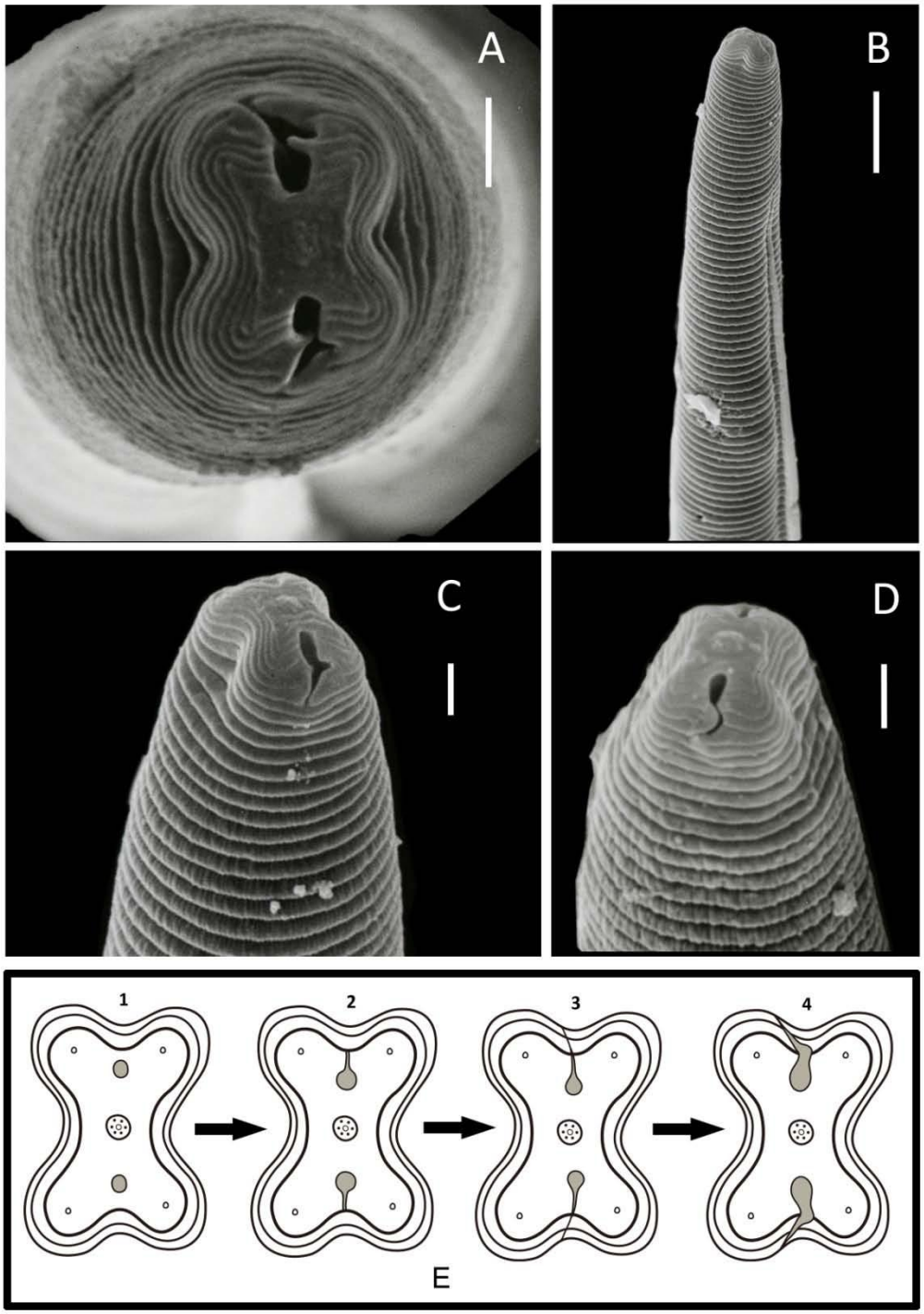

Fig. 6. SEM of female and juvenile of *M. nanellus* from China, and the possible development process of amphidial aperture. A: *en face* view of female shows oval hole in anterior part of amphidial aperture; B: Anterior part of female; C: Lateral view of female lip region; D: Lip region of juvenile; E: Possible development process of amphidial aperture. (Scale bar: A, C, D = 10 µm, B = 50 µm.)

Malenchus nanellus Siddiqi, 1979

(Figs 1B, D, H; Figs 2C, D, E, I, J, K, P; Fig. 6)

MEASUREMENTS

See Table 1

DESCRIPTION

Female

Body short. Lip region typical of the genus, dorso-ventrally flattened. Cuticle strongly annulated. SEM shows fine, longitudinal striae on annuli (Fig. 6). Lateral field smooth, about 1/6 body width, starts at mid-way of procorpus or 16 annuli from anterior end (or about one stylet length behind stylet base) and ends 3/4 of tail. SEM shows large amphidial holes at the lateral borders of labial plate, which continues as sinuous slit along lateral side of the lip region. Stylet slender, cone about 1/3 of total length, cone width 1/3 of anterior shaft and 1/4 in posterior shaft. Median bulb oval with distinct valve. Excretory pore located midway between nerve ring and basal bulb. Deirid at the level of excretory pore. Prophasmid 9-10 annuli anterior to vulva. Reproductive system monodelphic, prodelphic, ovary outstretched with oocytes arranged in a single row. Uterus has four rows with five cells in each row. Uterus sac spacious with thickened wall. Vulva sunken in body, epiptygmata indistinct, vagina slightly sloping, lateral flap small but visible, 2-3 annuli wide. Spermatheca small, offset, simple, rounded to elongated (only one elongated spermatheca observed, 10µm in length and 6.6µm in width), and with oval sperm cells. Tail 67-91µm, tail tip fine, ventrally bent.

Male

Less common than female. Resembles female in most features except for genital system and more narrower annulations. Bursa about 28µm long, starts at the level of spicules' capitulum.

HABITAT AND LOCALITY

Recovered from soil around roots of fern and moss in forest of Pingwu (N 32°25'26.2" E 104°37'02.4"), Sichuan province, China, 552 m.a.s.l..

Remarks

M. nanellus was originally described by Siddiqi (1979) from maize rhizosphere from Nigeria. It has been reported from Hungary (Andrássy, 1981), India (Siddiqui & Khan, 1983), Pakistan (Maqbool & Shahina, 1985), Colorado, USA (Geraert & Raski, 1986), Papua New

Guinea (Troccoli & Geraert, 1995) and Poland (Brzeski, 1998). This is the first report of *M. nanellus* from China. The general morphology and measurements of the Chinese population fits with the description of the type material from Nigeria, but some minor differences including slightly wider annulations (1.1-1.3 *vs* 0.8-0.9), shorter tail (67-91*vs* 80-90) and some variation of MB (46-52 *vs* 42-45).

The study of amphidial aperture shows that the lateral slit is not visible using LM in early juvenile stages, indicating the presence of only oval holes in the labial plate (Fig. 6E1). In late juvenile stages very narrow sinuous slits are visible both in SEM (Fig.6 D) and LM, indicating a gradually laterally expansion of the slit (Fig. 6 E2-3). In the adult stage, the width of this S-shape slit increases (Fig. 6 E4).

Notably, although the starting point of the lateral field was used as species specific character (Geraert & Raski, 1986), it shows remarkable variation according to several authors (Siddiqi, 1979; Andrássy, 1981; Siddiqui & Khan, 1983; Geraert & Raski, 1986; Troccoli & Geraert, 1995; Geraert, 2008) from stylet knob level, mid-region of procorpus to procorpus base. Since the level of these variations among populations is high enough to define multiple species listed in the species identification key (No. 7 and No.13) of Geraert (2008), the importance and reliability of this morphological trait for species delimitation remains under open question mark. However, in spite of some variation in the starting point of lateral lines, it is always located at more or less the mid-region of procorpus in present Chinese population, indicating that this feature is stable within the studied population herein.

Malenchus spermatheca's shape has been described with intra-specific variation, as simple offset, rounded to elongated (Siddiqi, 1979; Geraert & Raski, 1986) or bilobed (Andrássy, 1981; Troccoli & Geraert, 1995). The variability of the spermatheca shape in Chinese population is high, *i.e.* from rounded to elongated; sperm only in spermatheca or also present in proximal of uterus which appearing as a bilobed spermatheca. Therefore, in agreement with (Geraert & Raski, 1986), we believe that spermatheca morphology (simple/unilobed or bilobed), is not a useful trait for species delimitation in *Malenchus.*

Malenchus pachycephalus Andrássy, 1981

(Figs 1C, G, I, J, K, L, M)

MEASUREMENTS

See Table 1

DESCRIPTION

Female

General morphology typical of the genus. Body relatively large in genus, ventrally curved. Cuticular annulations coarse and wide. Lateral field consisting of 2 incisures as seen by light microscopy, deeply crenate, originating 3-4 annuli anterior to stylet base, end about half of tail length. Lip region less dorso-ventrally flatted than other species in genus. Stylet robust, cone about 1/3 stylet length, 1/4-1/5 width of shaft, knobs slightly asymmetrical with longer dorsal side. Median bulb weakly developed, valvular apparatus not distinct. Vulva sunk in body, epiptygmata present, vulva flap indistinct, about one annulus wide. Vagina perpendicular to body axis, about 10µm long. Spermatheca elongated, simple/unilobed or bilobed (sperm presence in proximal region of uterus), with round sperm cells, about 27-49 µm long and 12-15 µm wide. Prophasmid around 11 annuli anterior to vulva. Tail slightly ventrally curved, tip sharply pointed.

Male

Not seen.

HABITAT AND LOCALITY

Soil samples were collected in deciduous forest at 1835 m.a.s.l in Shimen (30°01'55.2"N, 110°39'54.0"E), Hunan province, China.

Remarks

M. pachycephalus was originally described by Andrássy (1981) from fern grass in South Carolina, USA. Later, it was reported from Hungary, Bulgaria, Italy (Andrássy, 1981) and Spain (Gómez-Barcina *et al.*, 1992). This is the first report of the species from China. Morphology and morphometric data of this population strongly resemble those given in original description (Andrássy, 1981), except for a slightly longer tail (74-78 *vs* 65-72 µm) and ending point of lateral field (at 1/2 *vs* 1/3-1/4 of tail). This Chinese population also resembles the Spanish population (Gómez-Barcina *et al.*, 1992), but has a longer tail (74-78 µm *vs* 56-69 µm).

Malenchus sp.

(Figs 4C-F, I, H; Figs 5C, H, K, L, P, Q)

MEASUREMENTS

See Table 1

DESCRIPTION

Female

From this species only a single specimen was collected. Body large. Cuticle coarsely annulated and folded between annuli. Lateral field not crenate, consisting of 2 incisures, starts at 5 annuli posterior to stylet knobs and ends in the middle of tail. Lip region continuous, not elevated, not or slightly flattened or not, 9.1 µm wide at base. Amphidial aperture sinuous-shaped. Stylet prominent, cone occupied 5.93 µm in a total, cone base width 1/4 of anterior and 1/5 of posterior shaft width. Median bulb relatively robust in the genus. Basal bulb more rectangular, covered with sheath like structure. Vulva sunken in body contour, epiptygmata weak, flap absent, vagina wall thickened. Prophasmid conspicuous, 21-22 annuli anterior to vulva. Spermatheca small, round, offset. Tail straight but slightly dorsally bent at the end with a pointed terminus.

Male

Not seen.

HABITAT AND LOCALITY

Recovered from soil sample collected in deciduous forest near the root of *Quercus* sp. at 1963 m.a.s.l. in Mt. Taibai (34°03'40"N, 107°41'09"E), Shaanxi, China.

Remarks

The single recovered specimen has an exceptional large body, which makes it closed to *M. novus* Mukhina & Kazachenko, 1985. This rare species was first and only described in eastern Russia in 1985. General morphology of single female fits well to original description except for a more muscular median bulb and minor difference in some measurements. However, it is not possible to assign species identity based on only one single specimen.

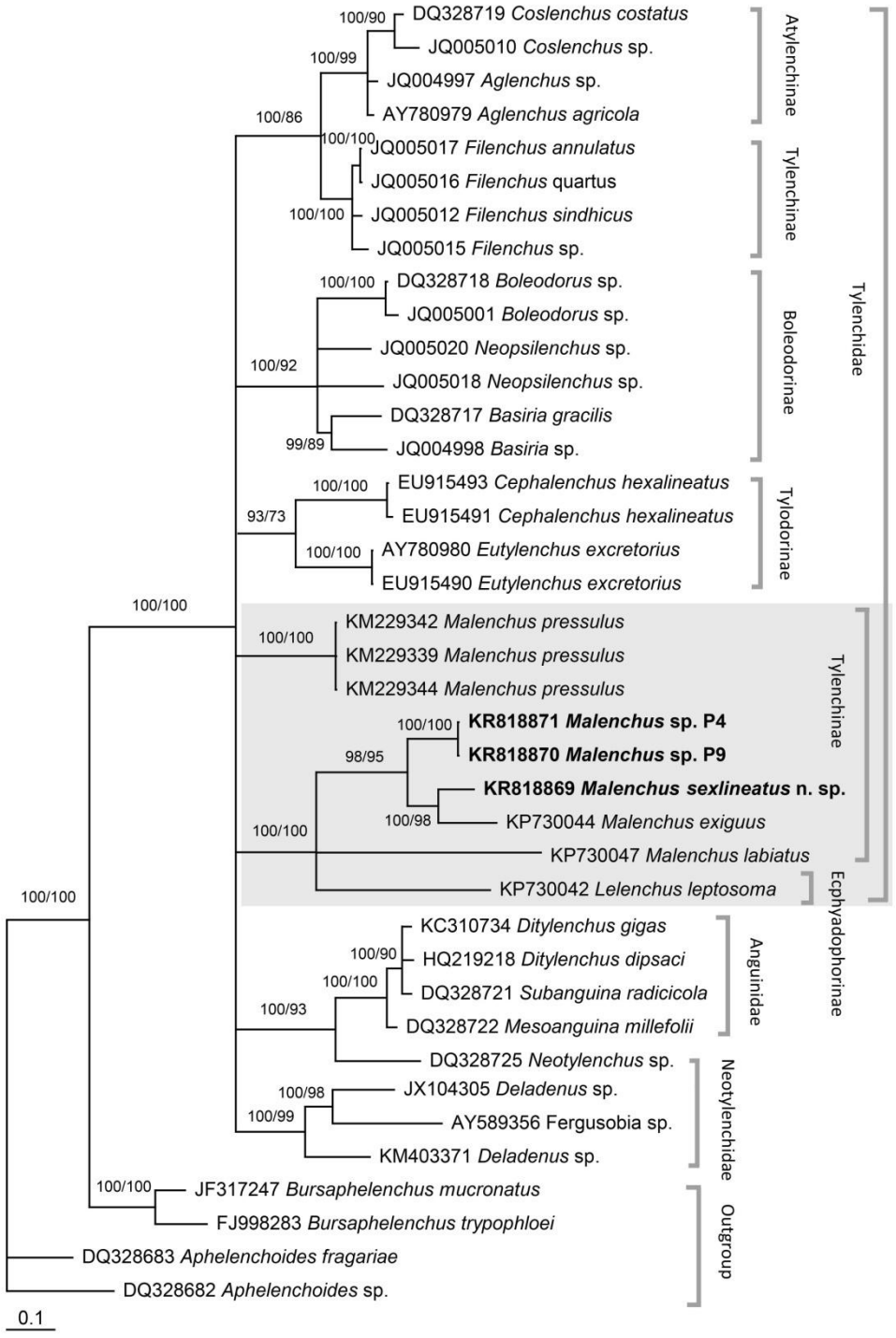

Fig. 7. Bayesian strict consensus phylogeny highlighting the phylogenetic position of *M. sexalineatus* n. sp. in relation with other relevant sequences from GenBank based on the D2-D3 domain of LSU rRNA sequences. Branch support is indicated in following order: PP value in BI analysis/ BS value from ML analysis. New sequences generated in this study are highlighted in bold.

Discussion

Molecular characterization and phylogeny

Recent studies (van Megen *et al.*, 2009; Bert *et al.*, 2010; Atighi *et al.*, 2013) based on 18S rRNA indicated that *Malenchus* is nested within *Filenchus.* However this was based on a single *M. andrassyi* Merny, 1970 sequence (AY284587), for which no morphological information nor geographic location was provided by (Holterman *et al.*, 2006). Recently, a 28S rRNA-based phylogeny indicated a moderately (PP=69) or robustly supported (BS=99) clade harboring all *Malenchus* spp. species and *Lelenchus* (Yaghoubi *et al.*, 2015). However, this result is not reproducible (especially the high BS value), even with identical data and described methods; nevertheless, AU and SH tests cannot reject this topology at the 90% significance level (SH p=0.145, AU p=0.137) (Fig. 8). Here we could only demonstrate the relationship of *M. sexalineatus* n. sp., *M. exiguus* and an unidentified *Malenchus* species but the relationship of *M. labiatus* and *Lelenchus leptosome,* as well as the position of *Malenchus* within Tylenchidae could not be clearly established.

Bert *et al.*, (2010) mentioned that the grouping of *M. andrassyi* and certain *Filenchus* spp. shared the presence of a single ridge in the lateral field. However, *M. presulus* also has a single ridge and appears within non-single ridge *Filenchus* spp. in our phylogeny, indicating the multiple origin of a single offset ridge. This is in line with the heterogeneity of cuticle morphology. Although the folded cuticle and dorso-ventrally compressed lip region were traditionally considered as synapomorphies for the genus (Andrássy, 1981), these similarities may not be homologous since multiple cuticle folded patterns and lip region shape variations were observed in different *Malenchus* species of this study. This would be in agreement with the polyphyly of *Malenchus* showed in our phylogenetic analysis. Furthermore, AU and SH tests appear to reject the monophyly of the genus *Malenchus* at the 95% significant level (Fig 8.).

Thus, the characterization and the position of *Malenchus* within Tylenchidae is still unsettled. Moreover, morphological data in combination with very limited available molecular data do not permit corroboration of any alternative for the current generic definition. Hence, we have described *M. sexalineatus* n. sp. as a new species within *Malenchus*. Nevertheless, a wider and more comprehensive analysis using additional genetic markers is needed for this genus *Malenchus*, and for Tylenchidae in general, to define molecularly based clades and associated morphological apomorphies.

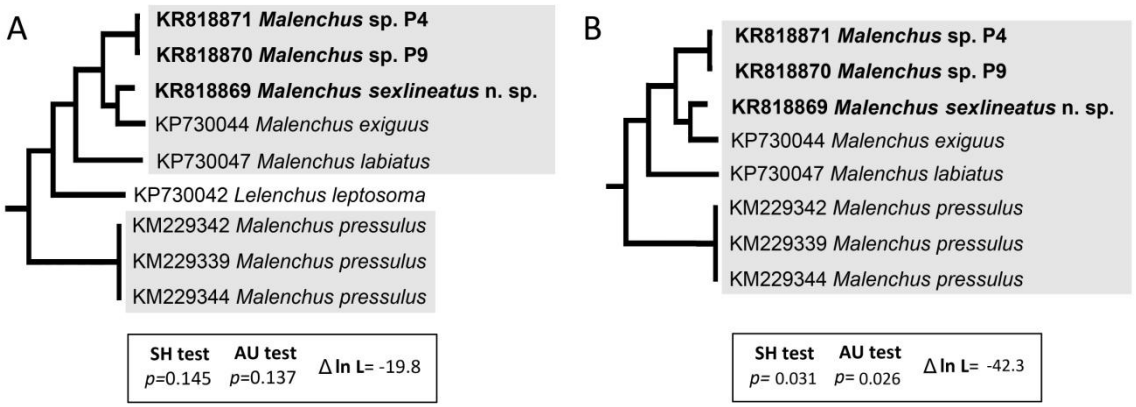

Fig. 8. Comparing alternative hypotheses using AU and SH test. The topological schemas (hypotheses) are compared with the originally obtained topology (Fig. 7). Clades containing *Malenchus* species are highlighted in gray. A: The hypothesis of paraphyly of *Malenchus* as robustly supported (BS=99) in the analysis of Yaghoubi *et al.*, (2015). B: The hypothesis of monophyly of *Malenchus*. Δln L: the Log likelihood difference of the two alternative hypotheses. The two hypotheses are less likely than the original topology, but only hypothesis B can be significantly rejected.

Remarks on amphidial aperture development

The amphidial apertures of the genus *Malenchus* were generally described as large S-shaped openings reaching the lip region base with an also large fovea (Andrássy, 1981) or the opening was interpreted as very wide and covered by cuticular outgrowths, sheltering most part of fovea, resulting in finer zigzag clefts (Gómez-Barcina *et al.*, 1992). On the other hand, Geraert and Raski (1986) introduced a second type, the straight-aperture found in three species that later on was used as basic trait to erect the subgenus *Telomalenchus*. Both amphidial aperture types were modeled following Qing *et al.* (2015) (Fig. 9.). As an internal structure, the amphidial fovea is generally invisible in family Tylenchidae, however, a conspicuous spindle shaped fovea is clearly visible in all studied *Malenchus* specimens in this work.

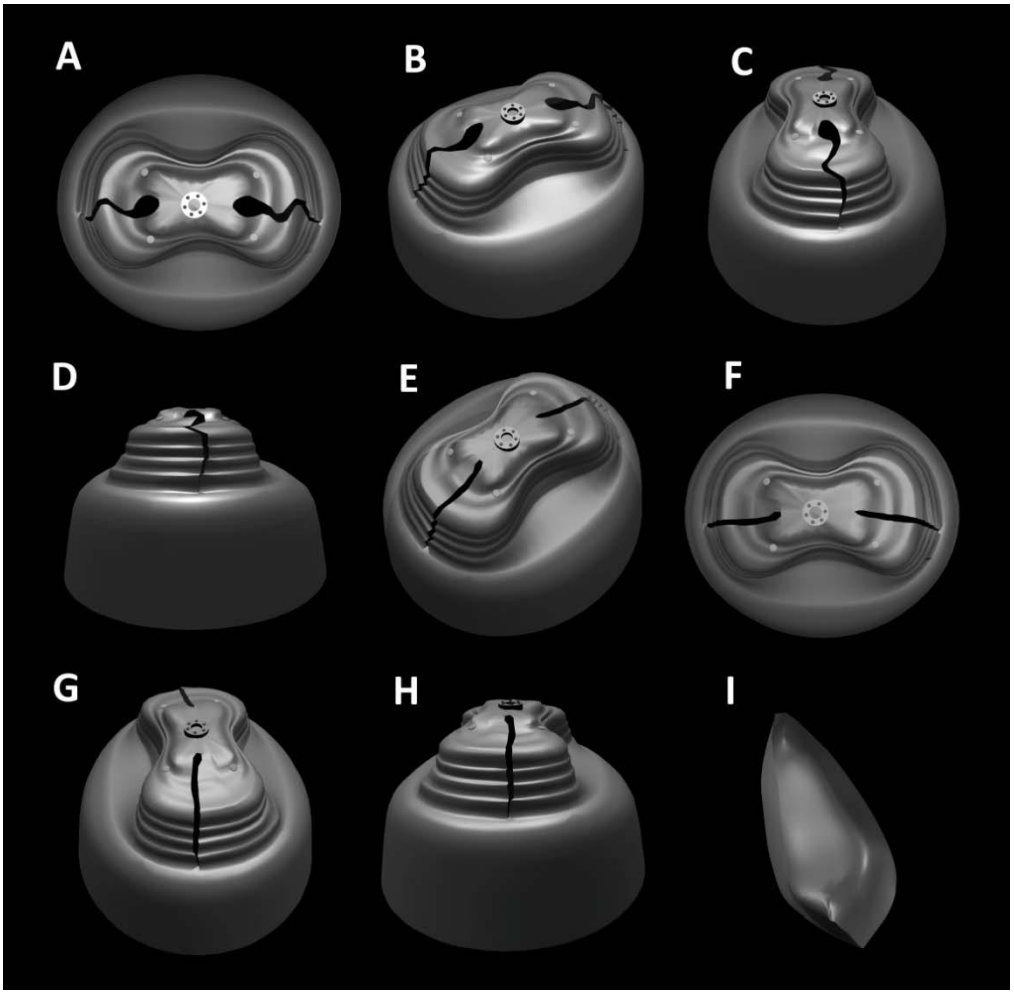

Fig. 9. 3D models of the lip region of the two subgenera in genus *Malenchus.* A-D: S-shaped amphidial aperture, subgenus *Malenchus*; E-H: straight amphidial aperture, subgenus *Telomalenchus*; I: Lateral view of amphidial fovea.

Generally, present observations agree with studies of Andrássy (1981) and Gómez-Barcina *et al.* (1992) in which the aperture is a large round to oval-shaped hole, sharply narrowing to a slit and ending at the base of the lip region. Remarkably, inspecting of Chinese population of *M. nanellus* showed that the morphology of the amphidial aperture changes according to the life stage of the species (Fig. 6A, C, D, E). However, a straight aperture, as known for the subgenus *Telomalenchus*, was never observed and the original oval hole remained constant in all stages. This is an indication that the amphidial aperture morphology is not shaped by the cuticular outgrowths as noted by Gómez-Barcina *et al.*, (1992) but the intrinsic shape of the subgenus *Malenchus* amphidial aperture, *i.e.* starting anteriorly within the labial plate as a hole and continuing at the lateral side of the lip region as longitudinal slits. The alterations during the development may be explained as adaptation to its multiple functions *e.g.* feeding habit, mating, moving, sensing chemicals or moisture

(Bumbarger *et al.*, 2009) in different life stages or simply as structural changes in different developmental stages without functional link.

Acknowledgement

We thank Dr. Irma Tandingan De Ley, Nic Smol and Joeseph Quisado for their help during sampling in Philippines; Mr Ruel Colong, Protected Area Superintendent for Mount Hamiguitan and San Isidro Mayor Edgar Saulon for granting the sampling permit; Dr Lourdes Generalao, Dr Irvin Generalao, Dr Lea Angsinco Jimenez and Dr Emily Fabregar for generously providing logistical support during sampling and nematode extraction in Philippines. We thank Wang Meng from NWSUAF for providing multiple Chinese samples. The first author thanks China Scholarship Council (CSC) for providing a PhD grant. The second author acknowledges the MICITT, CONICIT, University of Costa Rica and PEACE Project (Erasmus Mundus) for financial support. This work was also supported by a special research fund UGent 01N02312.

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Chapter III

Molecular phylogeny of *Malenchus* and *Filenchus*

Chapter published as:

Qing X., Decraemer W., Claeys M. and Bert W. (2017). Molecular phylogeny of *Malenchus* and *Filenchus* (Nematoda: Tylenchidae). *Zoologica Scripta*. DOI: 10.1111/zsc.12236.

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Abstract

The family Tylenchidae is phylogenetically important to understand early diverging Tylenchomorpha (=tylenchs with supposedly ancestral characters) and to assess soil ecosystems. In the present study we focus on *Malenchus* and *Filenchus* as representatives of the Tylenchidae. Samples collected worldwide result in 58 new sequences and light microcopy and transmission electron microscopy provide details on morphological features. For the first time comprehensive morphological data are evaluated in the context of a molecular framework, thus highlighting the phylogenetic and evolutionary complexity of this structurally minimalistic group. Results show that the genus *Filenchus* is polyphyletic in both the 18S and 28S rRNA phylogeny, while *Malenchus* is polyphyletic and monophyletic in the 28S rRNA and the 18S rRNA, respectively. Ultrastructural study demonstrate specific aspects of lateral cuticular incisures, cuticular layering and the amphideal fovea are surprisingly congruent with the obtained molecular phylogenies, while classical characteristics such as cuticle annulations are evolutionary highly plastic and mosaic in distribution. The study also reveals the shortage of D2/D3 domain in 28S rRNA as a phylogenetic marker for early diverging Tylenchomorpha.

Key words: Tylenchomorpha; nematode; ultrastructure; transmission electron microscopy

Introduction

Tylenchidae are abundant and diverse such that they may constitute up to 30% of the nematodes in any given soil sample (Yeates & Bird, 1994; Ferris & Bongers, 2006). Despite the abundance, the taxonomy of Tylenchidae is notoriously problematic: most species combine a low observational resolution with high intraspecific variability, and DNA sequence representing these taxa is usually not available. As a result, there is no consensus regarding their classification from species level up to family level (Brzeski, 1998; Siddiqi, 2000; Andrássy, 2007; Geraert, 2008).

In the present study we focus on this neglected group, and select two common genera of differing appearance: *Malenchus,* supposedly characterized by the presence of a robustly annulated cuticle, and *Filenchus,* considered to be a catch-all genus lacking morphological synapomorphies (Bert *et al.*, 2010). *Malenchus* was found to be monophyletic or polyphyletic on the basis of 28S rRNA (Qing *et al.*, Yaghoubi *et al.*, 2015) but 18S rRNA phylogeny is absent, while *Filenchus* is polyphyletic based on both 18S and 28S rRNA (Bert *et al.*, 2010; Atighi *et al.*, 2013). Representatives from these two genera and additional Tylenchidae representatives were collected from worldwide sources, resulting in 58 new DNA sequences. Light microscopy (LM) and transmission electron microscopy (TEM) provided detailed morphological observations that were evaluated in a phylogenetic context. This study aims to evaluate for the first time comprehensive morphological data within the context of a molecular phylogenetic framework of early diverging (=with supposedly ancestral characters) plant-parasitic nematodes, and to highlight the phylogenetic and evolutionary complexity of this structurally minimalist group. The study also aims to delineate the boundary between *Malenchus* and *Filenchus,* to distinguish morphological features that are potentially important for future generic delimitation in Tylenchidae and to evaluate the suitability of 28S and 18S rRNA genes as phylogenetic markers for early diverging Tylenchomorpha.

Materials and methods

Taxonomic sampling

Analyzed specimens, voucher numbers, GenBank accession codes and site details are presented in supplement Table S1. Nematodes were extracted from soil samples using a Baermann tray and concentrated using a 500 mesh sieve (25µm opening). Samples collected outside Belgium were divided into two parts: fixed with 4% formalin for the morphological

analyses, and fixed with DESS solution (Yoder *et al.*, 2006) at room temperature for molecular analyses.

Morphological analyses

Formalin fixed specimens were rinsed several times with deionised water and gradually transferred to anhydrous glycerin for permanent slides, following the protocol of Seinhorst (1962) as modified by Sohlenius and Sandor (1987). Slides were examined and photographed using an Olympus BX51 DIC Microscope (Olympus Optical, Tokyo, Japan), equipped with an Olympus C5060Wz camera. Specimens were identified to species level based on available keys (Andrássy, 1981; Geraert & Raski, 1986; Geraert, 2008) and original descriptions. *Malenchus* sp. P5, *Filenchus* sp. C103 and C102 could not be identified to species owing to the inadequate number of individuals (i.e. few juveniles and a single adult), while *Malenchus* sp. C163 is a species new to science and will be formally described elsewhere.

To determine details of the layering of body wall cuticle, specimens were prepared for transmission electron microscopy (TEM) by fixing in 2.5% glutaraldehyde in 0.05M sodium cacodylate buffer (pH 7.4) for 30 min and rinsed by 0.05M cacodylate buffer. Post-fixation was in 1% osmium tetroxide in 0.05M cacodylate buffer for 2h followed by *en bloc* staining for 1 h in 1% uranyl acetate. The specimens were then dehydrated in an ethanol series followed by a propylene oxide series and embedded in a Spurr resin (EMS). The block face istrimmed with a Leica EM Trim device and ultra-thin sections were cut with a Leica UC7 ultramicrotome (Leica, Vienna, Austria) with a diamond knife (Diatome Ltd., Biel, Switzerland). Sections are then stained in uranyl acetate and lead citrate using a Leica EM AC20. Sections were observed with a JEOL JEM 1010 (JEOL Ltd., Tokyo, Japan) and images were recorded on imagine plates from DITABIS (Pforzheim, Germany).

Molecular analysis

DNA extraction, amplification, and sequencing: Nematode morphological vouchers were prepared prior to DNA extraction. These vouchers were made with LM of temporary mount using a combination of video clips and photomicrographs (De Ley & Bert, 2002) and these are available online at http://www.nematodes.myspecies.info. Vouchered nematodes were subsequently picked from temporary mounts and each specimen was cut into pieces and transferred to a 500µl Eppendorf tube with 20µl of worm lysis buffer (50 mM KCl; 10 mM Tris pH 8.3; 2.5mM $MgCl_2$; 0.45% NP 40 (Tergitol Sigma); 0.45% Tween 20) and frozen for

10 min at -20°C. 1µl proteinase K (1.2 mg/ml) was added to the samples before incubation, 1 h at 65°C followed by 10 min at 95°C. PCR reaction and sequencing followed the protocol of Múnera Uribe *et al.* (2010) and Bert *et al.* (2008) respectively. The D2/D3 domains of 28S rRNA were amplified with primers D2A, D3B (De Ley *et al.*, 2005), MalF (Wisniewska & Kowalewska, 2015), 1006R (Holterman *et al.*, 2008) and 826R (this study, 5'-CGATTTGCACGTCAGAACCG-3'). The 18S rRNA gene was amplified using G18S4, 18P (Blaxter *et al.*, 1998), TylF1 (This study, 5'-GCCTGAGAAATGGCCACTACG-3') and TylR2 (This study, 5'-TGRTGACTCRCACTTACTTGG-3').

Phylogenetic analyses: The obtained sequences were analyzed with other relevant sequences available in GenBank. Multiple alignments of the different genes were made using the Q-INS-I algorithm of MAFFT v. 7.205 (Katoh & Standley, 2013). Post-alignment trimming was done using Gblocks 0.91b (Castresana, 2000), however, this does not affect the tree topologies outcome other than resulting in slightly lower branch support (results not shown). The substitution saturation was assessed by DAMBE5 (Xia, 2013) implementing the method described by Xia *et al.* (2003), with gaps treated as unknown states and proportion of invariant site (P_{inv}) set to 0.17. The best-fitting substitution model was estimated using AIC in jModelTest v. 2.1.2 (Darriba *et al.*, 2012). Maximum Likelihood (ML) and Bayesian (BI) analysis was performed at the CIPRES Science Gateway (Miller *et al.*, 2010), using RAxML 8.1.11 (Stamatakis *et al.*, 2008) and MrBayes 3.2.3 (Ronquist & Huelsenbeck, 2003), respectively. ML analysis included 1000 bootstrap (BS) replicates under the GTRCAT model. Bayesian phylogenetic analysis was carried out using the GTR+I+G model for both genes, analyses were run under 5×10^6 generations (two independent runs with four chains) and Markov chains are sampled every 100 generations and 25% of the converged runs were regarded as burnin. Gaps were treated as missing data for all phylogenetic analyses. ML bootstrap values and posterior probabilities (PP) were plotted on Bayesian 50% majority-rule consensus trees after editing with TreeView v. 1.6.6 (Page, 1996) and Illustrator CS3 (Adobe).

Character evolution analysis: All analyses were performed in R version 3.2.5 (R Development Core Team). We used stochastic character mapping (Huelsenbeck *et al.*, 2003) to sample possible histories of amphideal fovea. For each character in the stochastic mapping approach, 500 stochastic trees were generated and density map were plotted using phytools (Revell, 2012). Transition matrix Q is sampled by Bayesian MCMC method and α of the γ prior was set to β*empirical (Q) by using empirical parameter to avoid bias.

To estimate the ancestral characters of body cuticle width within the genus *Malenchus*,

trees were rebuilt with taxa constrained to *Malenchus*. This excluded non-homolog cuticles in other genera (different in annulation shape, layer structures, see further discussion) thus allowing us to analyze intrageneric character evolution. Continuous traits were mapped onto the phylogeny tree using the function *contMap* by estimating the ancestral states at the internal nodes using Maximum Likelihood and interpolating the states along each edge using equation (2) of Felsenstein (1985). Prior to reconstruction, the tree was ultrametricized by applying the penalized likelihood method implemented in the *chronopl* function in the package ape (Paradis *et al.*, 2004) with a lambda= 0. The character evolutions along branches of the tree, were visualized as a color gradient using the method of Revell (2013). An additional phenogram provided a projection of the phylogenetic tree in the space defined by phenotype and relative time in order to visualize the trait distributions and examine their degree of overlap between different clades.

Results

Ultrastructure of body cuticle and annulations

The ultrastructure of the body cuticle reveals four different zones (Decraemer *et al.*, 2003) from outer to inner: (1) epicuticle, (2) cortical, (3) median and (4) basal, the latter being bordered by the basal lamina. Within Tylenchomorpha the basal zone is characterized, except at the lateral fields, by radial striae formed by longitudinal and circumferentially oriented, interwoven laminae (striaes) at a constant periodicity in longitudinal and transverse sections. The detailed description of the different zones (exc. epicuticle) of the studied species combined with information from the literature of Tylenchidae species is listed in Table 1. In the studied taxa, the four zones are always present (cortical and median zone are not always clearly differentiated) except for *Filenchus* with either a missing or very narrow median zone in the dorsal and ventral body regions. This missing zone was also in many other tylenchid taxa (Decraemer *et al.*, 2003). In the two studied species of *Malenchus*, the deep annulation results in a cortical zone extending nearly to the basal lamina (Fig. 1). As a result, the median zone becomes restricted to a ‘triangular’ area beneath mid-annulus and the basal zone with radial striae is interrupted into patches and minimal at level of the grooves in *Malenchus acarayensis* (Fig. 1 C, M). Surprisingly, in *Malenchus pachycephalus* the basal zone appears without radial striae and it is thick at the level of the annuli and thin within the region of grooves.

The annulations in the genus *Malenchus* are generally prominent and have been considered as a consistent and important generic character (Siddiqi, 1979; Andrássy, 1981; Geraert, 2008). However, results show distinct variations in i) shape, from a simple usually flattened annulus (Figs 2 A, B, G, H; 1 C) to a more complex strut-like annulus, with each slightly overlapping the adjacent annuli (Figs 2 C, D; 1 A); ii, thickness; and iii) degree of groove depth with respect to the cuticular zones. Thus, "prominent cuticular annulation" alone is a too variable and ambiguous to define and delimit genera. For example, cuticular annulations of *Filenchus balcarceanus* and *Malenchus* sp. C163 are intermediate in cuticle thickness and groove depth relative to those of other *Filenchus* spp. and *Malenchus* spp. The annulations of the other *Filenchus* species examined with TEM, *Filenchus discrepans* C172 and *Filenchus vulgaris* C179 show less pronounced annulations, restricted to the upper layers of the cortical zone (Fig. 1 E-H, O-R), resembling those of other known Tylenchidae with respect to low cuticle thickness and groove depth. Conversely, the genus *Lelenchus* that was once synonymized with *Filenchus* (Andrássy, 1976), has a smooth cuticle without annulations or transverse striae in LM (Figs 2 J; 1 I, J).

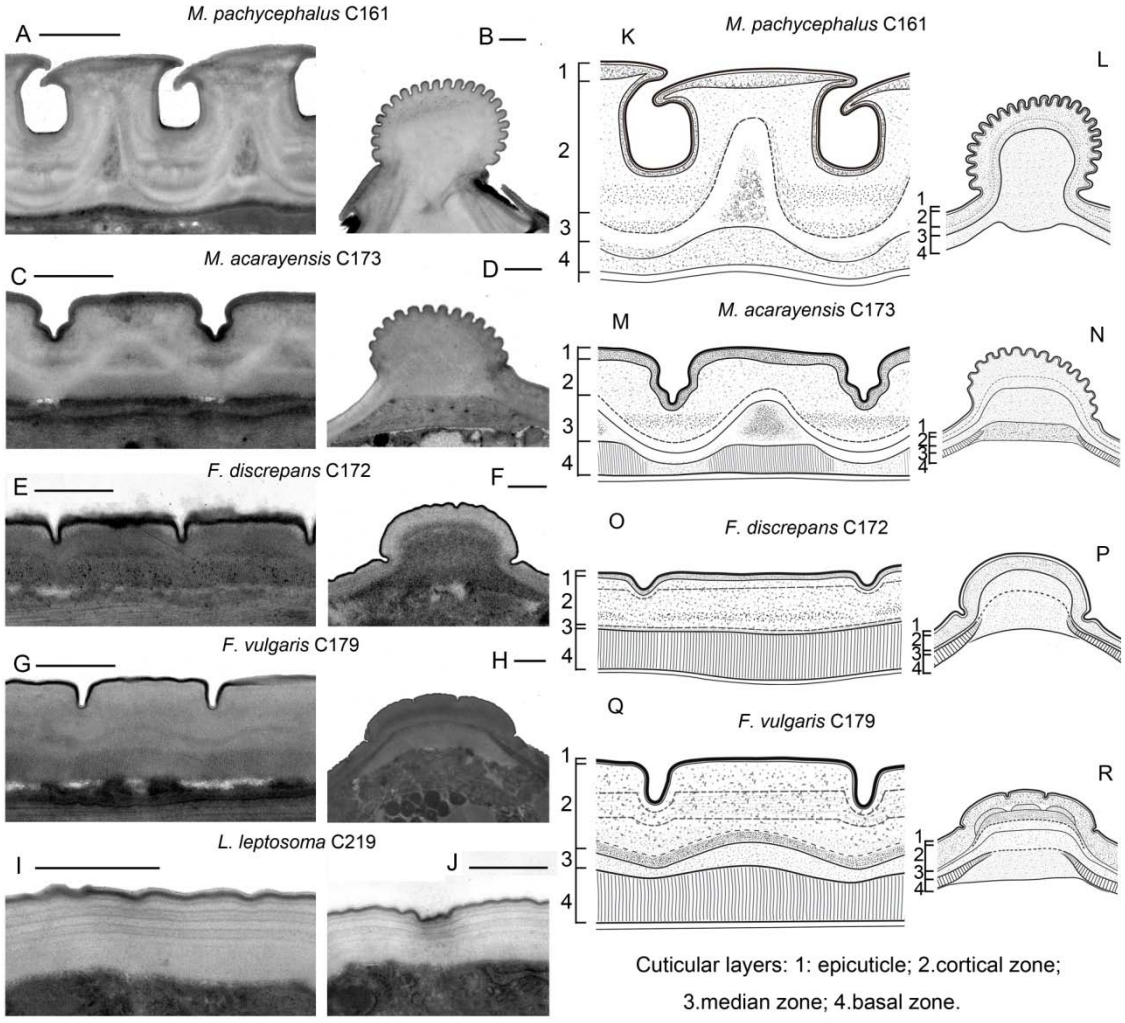

Fig. 1. The TEM of body cuticle in the genera *Malenchus* (A-D, K-N), *Filenchus* (E-H, O-R) and *Lelenchus* (I-J) and their diagrammatic representation (K-R). A, C, E, G, K, M, O, Q: longitudinal section at mid-body (left); B, D, F, H, J, L, N, P, R: transverse section of lateral field (right); I: transverse section of dorsal region. Scale bar A-G, I, J=0.5μm, H=1μm.

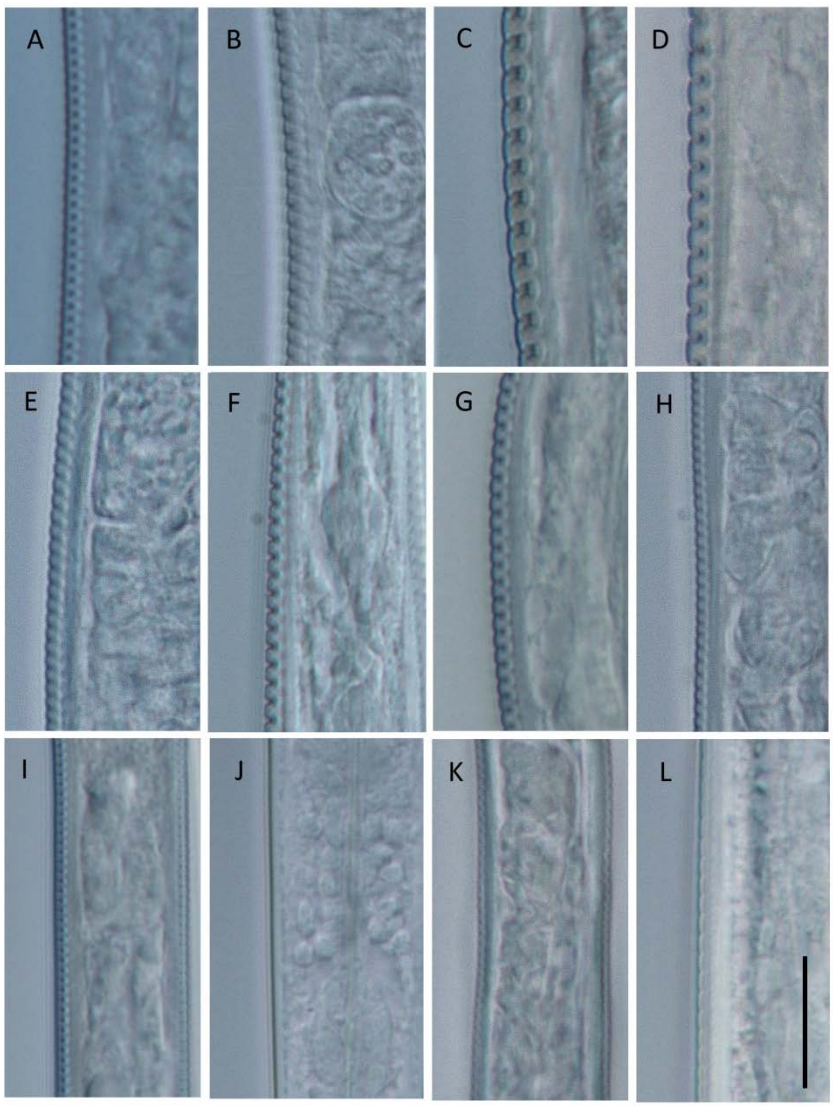

Fig. 2. LM variation of body cuticle annulation (ventrally or dorsally) in the species used in this study. A: *Malenchus* sp. P5; B: *M. pressulus*; C: *M. pachycephalus* C116; D: *M. pachycephalus* C161; E: *M. bryophilus* C171; F: *M. undulatus*; G: *M. acarayensis* C173; H: *Malenchus* sp. C163; I: *F. balcarceanus* C57; J: *L. leptosoma* C219; K: *F. discrepans* C172; L: *F. vulgaris* C179. Scale bar=10μm.

Ultrastructure of lateral region

The lateral field in *Malenchus* is prominent, visible as two incisures delimiting a single narrow elevated ridge (=protruding band) based on LM. In SEM, this ridge always shows multiple longitudinal incisures delineating small ridges, described as four, six, twelve or more, depending on the species (Geraert & Raski, 1986; Geraert, 2008; Qing *et al.*, 2015). In the present TEM study, 14 ridges were observed in *M. acarayensis* C173 and an extension of the range to 22 small ridges were observed for *M. pachycephalus* C161 (Fig. 1 B, D). Remarkably,

similar small ridges have not been found in other genera of Tylenchomorpha (Baldwin & Hirschmann, 1975; Mounport *et al.*, 1991, 1993a; Mounport *et al.*, 1993b; Mounport *et al.*, 1997; Valette *et al.*, 1997).

The lateral fields in *Filenchus* are more heterogeneous with at least two distinct patterns. For example *F. discrepans* C172 has a single elevated ridge (Fig. 1 F, P) which cannot be differentiated, based on LM, from the ridge in *Malenchus*, while *F. vulgaris* C179 has a less elevated ridge including two shallow mid-way incisures (Fig. 1 H, R) resulting in four incisures or three ridges clearly visible in LM. In contrast, *Lelenchus leptosoma* C219 has a non-protruding lateral field without longitudinal incisures.

Phylogeny of Malenchus *and* Filenchus

In this study we use 28S and 18S rRNA genes that are the two most common regions in nematode phylogenetic studies. However, both regions have some limitations in analyses of Tylenchidae. The substitution saturation test based on the 28S rRNA has revealed a high substitution rate (Table S2), suggesting that 28S rRNA is only weakly informative. On the other hand, 18S rRNA data have an appropriate substitution rate (Table S3), but PCR success using traditional 18S primers is limited giving the considerable variation of these premier-binding regions. A successful PCR from a single specimen is challenging and sometimes has to be compromised by using a primer set with a shorter targeted sequence (see new primers used in this study). Limited PCR success most likely explains considerable length variation of the reference sequences in GenBank that result in a scarcity of homologous sites in an alignment (coverage limitations). Aside from sequence limitations, 28S and 18S rRNA produced different tree topologies, and therefore alignments are presented separately and not concatenated.

The phylogeny trees were reconstructed separately (not concatenated) as the most available GenBank sequences from two genes do not representing same species. Trees inferred by ML and BI analyses are largely congruent, therefore only the Bayesian tree is shown, including the bootstrap values of the ML analysis. The tree topology based on 18S rRNA supports the monophyly of *Malenchus* (PP=1, BS=84) as a sister group to *Filenchus* species with two incisures in the lateral region (*Filenchus* clades group 2) (Fig.3 B). The *Malenchus* clade is further divided into two subclades: one well-supported (PP=1, BS=81) and a moderately supported (PP=0.65, not supported by ML). Within the well supported subclade, the *Malenchus* sequences are highly divergent (6-198 bp nucleotides difference) and

M. acarayensis and *M. pachycephalus* appear to be polyphyletic, indicating that morphology based identification is misleading and may overlook cryptic species or the less-likely alternative, *M. acarayensis* is an extremely variable species. However, due to a lack of information from type materials, no synonymization action will be taken. Based on 28S rRNA *Malenchus* is polyphyletic (Fig. 3 A, detailed tree see Fig. S1), with species in clade 1 (PP=1, BS=99) as sister of *Filenchus* + *Lelenchus* whereas species in clade 2 (PP=0.99, BS=96) are sister to *Lelenchus*. The *Malenchus* clades defined by both 18S and 28S analyses are not supported by morphological data. Indeed, combining morphology and phylogeny clearly demonstrate that the generic definitions in Tylenchidae are far from settled, displayed herein on the basis of the following four examples: (1) Two phenotypically ambiguous species are placed within the *Malenchus* clade 1: *Malenchus* sp. C163 fits the genus diagnosis based on the S-shaped amphideal aperture, distinct fovea and elevated head, but it is also similar to *Filenchus* spp. in having a relatively thin cuticle with relatively unpronounced annulations (Fig. 2 H) and an elongate-cylindrical body posterior to the vulva (instead of markedly tapered posterior to the vulva, which is a generic character for *Malenchus* as proposed by Siddiqi (1979, 2000)). Similarly, *F. balcarceanus* resembles *Filenchus* in having a lower head, elongate-cylindrical body posterior to vulva however with relatively pronounced annulations (Fig.2 I). This indicates that body markedly tapered posterior to vulva is convergent character: (2) the genus *Filenchus* consists of at least two independent well supported (PP=1, BS=100) clades: *Filenchus* clade 1, containing all species with four incisures in the lateral field, is sister (PP=1, BS=89) to the genus *Coslenchus* + *Aglenchus* whereas *Filenchus* clade 2 with a single elevated ridge is sister (PP=0.76, BS=69) to *Malenchus* clade 1. (3) *Tylenchus naranensis* is nested within *Filenchus* clade 1, the species shares similarities with the four-incisures *Filenchus* species (see also Panahandeh *et al.*, 2015b; Geraert, 2008) except for proportion of stylet cone and shaft. (4) The genus *Lelenchus*, herein represented by different populations of *L. leptosoma* is paraphyletic. Specimens C114 and C118 are sister to *Malenchus* clade 1 + *Filenchus* clade 2 (PP=1. BS=97) whereas C219, and the GenBank sequence KP7300422 are sister to *Malenchus* clade 2 (PP=0.99, BS=95).

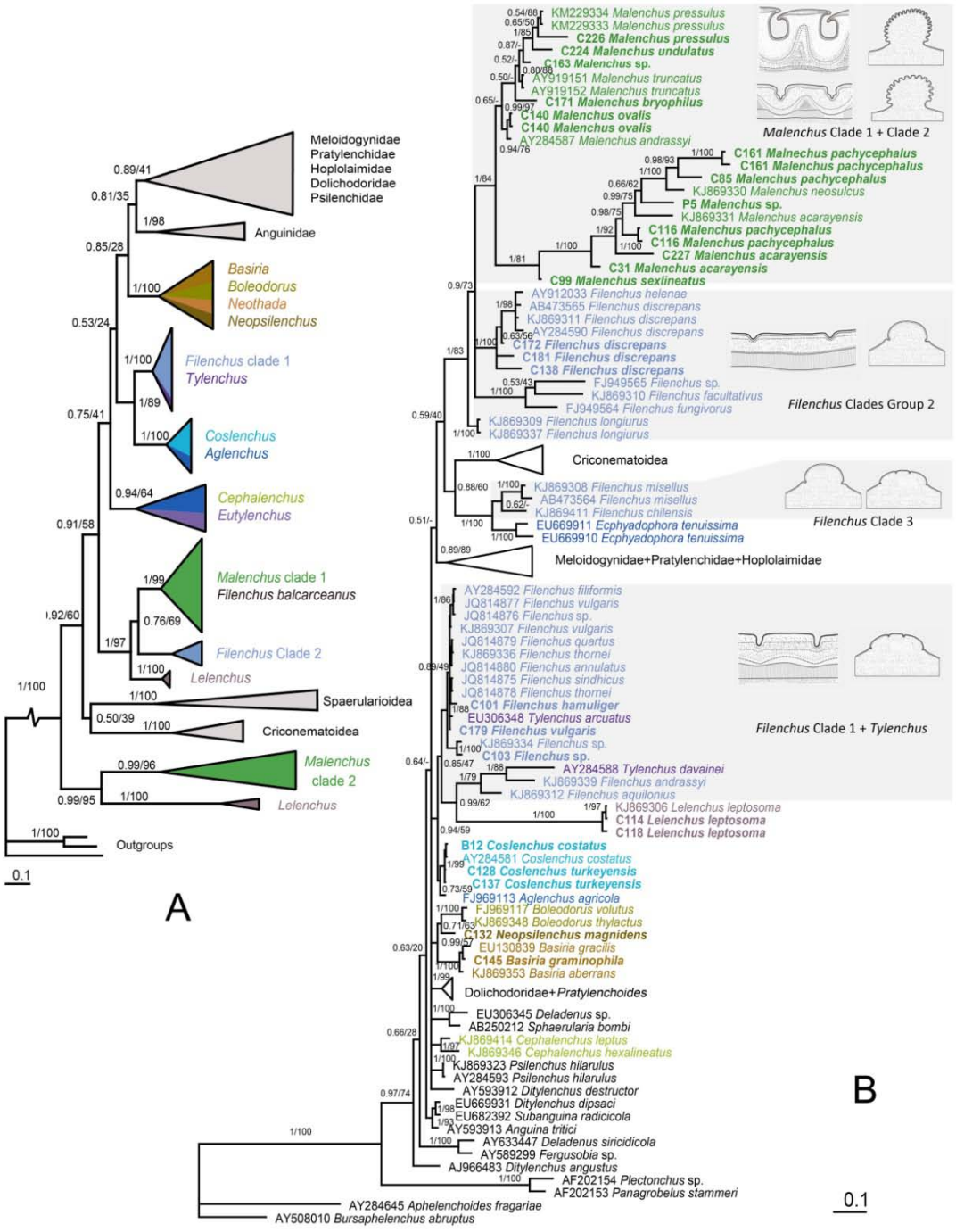

Fig. 3. Bayesian 50% majority rule consensus tree interfered on 28S (A) and 18S (B) rRNA genes. Details of 28S rRNA phylogeny see Fig. S1. New sequences original to this study are indicated in bold. Branch support is indicated as: PP value in BI analysis/BS value from ML analysis. Illustrations show cuticle structure in each clade (left: longitudinal at mid-body and right transverse at lateral field region). *Malenchus* clades 1 + 2 are characterized by small ridges; *Filenchus* clades group 2 share a two incisured lateral ridge; *Filenchus* clade 3 has a two or four incisured lateral ridge (Raski & Geraert, 1986; Okada *et al.*, 2002, Geraert, 2008); *Filenchus* clade 1 is characterized by four incisures of lateral field.

Character evolution of annuli and amphideal fovea

Annuli width and pouch-like amphideal fovea have been assumed to be taxonomic informative for *Malenchus* (Andrássy, 1981) and therefore their ancestral states and correspondence with clades as defined by molecular analyses have been analyzed (Fig. 4). For annuli width, 18S rRNA-based ancestral state reconstruction using likelihood method shows that the earliest node of *Malenchus* clade remains uncertain (Fig. 4 B). This result suggests that wide and narrow annuli have evolved several times. Consequently, no significant correspondence is found between annuli size distribution and molecular clades for 18S rRNA phylogeny (Fig 4C), thus further suggesting that annuli width does not define natural groups. Conversely, a pouch-like amphideal fovea has arisen twice, and it is very likely to be the ancestral state for *Malenchus* species although not for other closely related species (Fig. 4 A), therefore supporting its importance as a character for delimiting genera.

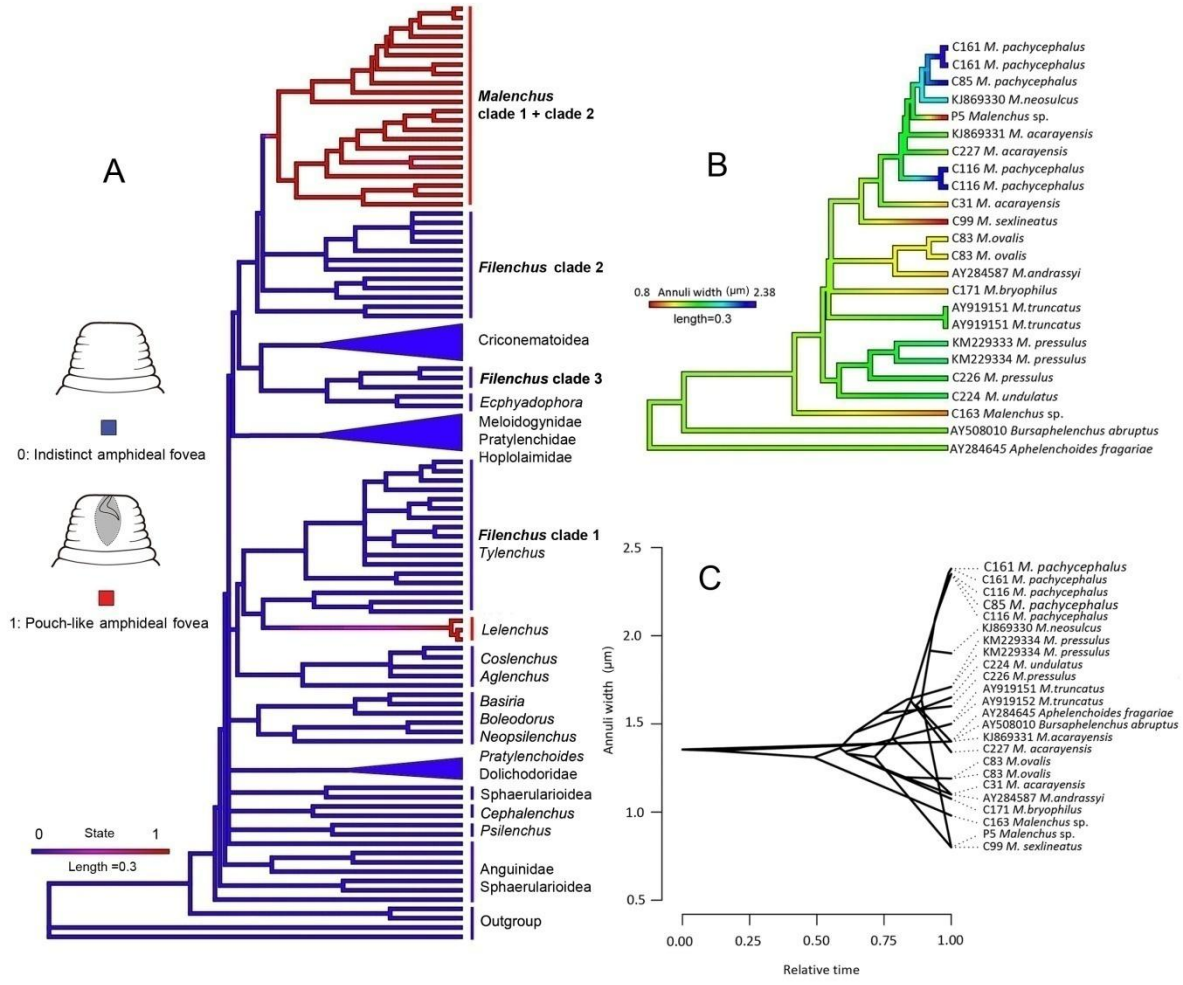

Fig. 4. Stochastic character mapping for amphideal fovea (A) and maximum likelihood

ancestral state reconstructions for annuli width (B, C) inferred from 18S rRNA sequences. The annuli width based on specimens from this study, (average measurements of 10 individuals), type material (*M. truncates, M. neosulcus* and *M. andrassyi*), Wiśniewska & Kowalewska (2015) and Panahandeh *et al*. (2014, 2015b) (*M. pressulus, M. labiatus* and *M. exiguus*). Traitgram (B, D) showing the projection of the phylogeny into a space defined by annuli width (y-axis) and relative time since divergence from the root (x-axis; not calibrated due to absent of informative fossil record).

Discussion

In the present study we focus on *Malenchus* and *Filenchus* as representatives of Tylenchidae to explore the informative value of both existing and new morphological characteristics, as well as analyze their taxonomic value in a phylogenetic framework. Our results highlight the difficulties associated with this taxonomically notorious group: morphological traits are difficult to observe consistently in very small animals; most species are not presently culturable under laboratory conditions; PCR success is variable, and the traditionally-used molecular markers often produce a conflicting signal. Discordance between different loci are also well known for other animal groups such as hominids (Ebersberger *et al.*, 2007), cichlids (Takahashi *et al.*, 2001), finches (Jennings *et al.*, 2005), grasshoppers (Carstens & Knowles, 2007) and fruit flies (Pollard *et al.*, 2006). For Nematoda, gene inconsistencies are usually found between mitochondrial and nuclear datasets (Nadler & Hudspeth, 2000; Nadler *et al.*, 2006; Derycke *et al.*, 2008; Park *et al.*, 2011). In present study, the discordance between the two nuclear genes exists for both closely-related species and more distantly-related clades. The possible reasons for these conflicting signals are numerous, including incomplete lineage sorting (Degnan & Rosenberg, 2009), hybridization (Degnan & Rosenberg, 2009), horizontal gene transfers (Tian *et al.*, 2015), recombination (Wiuf *et al.*, 2004; Than *et al.*, 2006) and saturation effects (Dolphin *et al.*, 2000). In our study, the substation saturation test confirmed that the 28S rRNA gene has multiple substitutions at the same sites, which may cause long-branch attraction (Felsenstein, 1978) and thus obscure the phylogenetic relationships among sequences (Arbogast *et al.*, 2002). Hence, the reliability of 28S rRNA phylogenies for Tylenchidae is limited, even with the use of likelihood methods, which are less sensitive to long-branch attraction (Felsenstein, 1981). However, the 28S rRNA gene has widely been used in phylogeny of Tylenchomorpha (Subbotin *et al.*, 2005; Subbotin *et al.*, 2006; Subbotin *et al.*, 2007; Subbotin *et al.*, 2008; Subbotin *et al.*, 2011), and for

Tylenchidae three of the last five studies have been based on 28S rRNA alone (Atighi *et al.*, 2013; Panahandeh *et al.*, 2015a; Panahandeh *et al.*, 2015b; Qing *et al.*, 2015; Yaghoubi *et al.*, 2015). Consequently, the obtained tree topologies should be interpreted with caution, and it is recommended that future phylogenetic studies of Tylenchidae do not solely rely on the 28S rRNA gene.

The current study also supports that the use of morphology in conjunction with molecular approaches remains essential in formulating a powerful phylogenetic hypothesis (Jenner, 2004), at least by reciprocal illumination. In this study we demonstrate that some frequently-used morphological characteristics cannot delimitate genera in Tylenchidae, while others are surprisingly congruent with molecular phylogenies.

We selected *Malenchus* as a genus with relatively well-defined morphology within Tylenchidae, particularly its prominent deep annulation in the body cuticle serving as a useful trait to delimitate the genus (Siddiqi, 1979; Andrássy, 1981; Geraert, 2008). However, we have demonstrated that this characteristic is much more variable than first assumed, and that the prominent and deep annulation is not a result of the same homologous underlying cuticular structure. Also we note that the width, groove depth and shape of the annuli are variable and that pronounced annules have independently evolved therefore being of limited use to delimitate *Malenchus*.

A pouch-like amphideal fovea (or large inner sacks) was first observed with LM by Andrássy (1981) and further illustrated by Qing *et al.* (2015), but it has never been used as diagnostic trait. As it is present in all examined *Malenchus* species, and its stability is further supported by ancestral state reconstruction, we propose the use of pouch-like amphideal fovea as a generic diagnostic characteristic for *Malenchus* (*vs* indistinct amphideal fovea in others).

Although the number of incisures in the lateral field has been considered highly variable at the genus level in Tylenchidae (*e.g.* four *vs* six in *Cephalenchus*; two *vs* four in *Basiria*; four *vs* six in *Boleodorus*; two *vs* four in *Filenchus*; absent, two and four in *Lelenchus*), we have shown that the type of lateral region remarkably corresponds to the molecular defined clades in both 28S and 18S rRNA phylogenies. Therefore, the number of lateral lines can be used to refine the “catch all” genus *Filenchus*. Although this characteristic has already been used by Siddiqi (1979, 2000) and Siddiqi and Lal (1992) to differentiate *Ottolenchus* from *Filenchus* (two *vs* four incisures), LM observations do not always provide clear information (*e.g. F. balcarceanus* was placed in *Ottolenchus* since it appears as two incisures in LM, but it

has six small ridges in SEM as shown by Torres and Geraert (1996). It is therefore necessary to identify and use detailed morphological (including SEM, TEM) and molecular traits in order to clarify generic definitions.

On the ultrastructural level, the absence of radial striae in the basal layer is remarkable, since in most plant-parasitic Tylenchomorpha, all stages have basal radial striae except when physically constrained in some way. For example, radial striaes are always interrupted at the level of the lateral field, where they are replaced by fibrillar layers, allowing small changes in body diameter, and in other cases radial striae of the basal layer are confined to small patches as in obese females (Heteroderinae). Basal radial striae showing a constant periodicity play a role in an antagonistic mechanism to high inner body pressure and contraction of longitudinal body muscles that assists in body locomotion, and are thought to be responsible for the radial strength of the cuticle. Basal radial striae are formed when the elongation of the embryo is complete, and are considered to be necessary for maintaining body shape after elongation (Priess & Hirsh, 1986). Radial striae also protect the animals in hazardous environments and are characteristic of most free-living terrestrial juvenile stages of the clades III-V *sensu* Blaxter *et al.* (1998) that includes most parasitic taxa (vertebrate as well as plant-parasitic). Males and free-living J2 juveniles of *Globodera rostochiensis* have basal radial striae, although the endoparasitic stages do not (Bird, 1968). In *M. acarayensis*, the breaking up of the basal radial striae at the region of the deep grooves in the cuticle may afford some flexibility to the body cuticle at that level. Although comparison of distantly related nematodes suggest that several structural elements of the body cuticle have independently arisen several times (Decraemer *et al.*, 2003), at the genus level such characters appear phylogenetically informative.

In conclusion, although integrated approaches have been implemented and informative taxonomic characteristics are recovered, it has been herein demonstrated that current approaches cannot completely resolve neither the phylogeny nor generic definitions. It is clear that the use of some other, either existing or new technologies (*e.g.* TEM for other structures, multiple genes phylogeny, phylogenomics) are needed to extract more informative genes and/or morphological characters. Nevertheless, even with the newest techniques, nematode taxonomists still need to test and revise as warranted, the congruence of morphology-based systematics and molecular phylogenetics. For the time being, a comprehensive understanding of a taxonomically notorious group, such as Tylenchidae, including the embellishment of the major patterns and clades surely must be the key priority, rather than a compilation of a

never-ending catalogue of single taxonomic units (De Ley, 2000).

Acknowledgements

We thank Dr. Lifei Lin from Yunnan Honghe University, China and Dr. Ursula Eisendle-Flöckner for kindly providing samples. We are grateful to Dieter Slos for the help during sampling and Toon Janssen for his valuable suggestions in primer design. We thank the anonymous reviewers for their careful reading of our manuscript and their many insightful suggestions and corrections. The first author thanks the China Scholarship Council (CSC) for providing a Ph.D. grant.

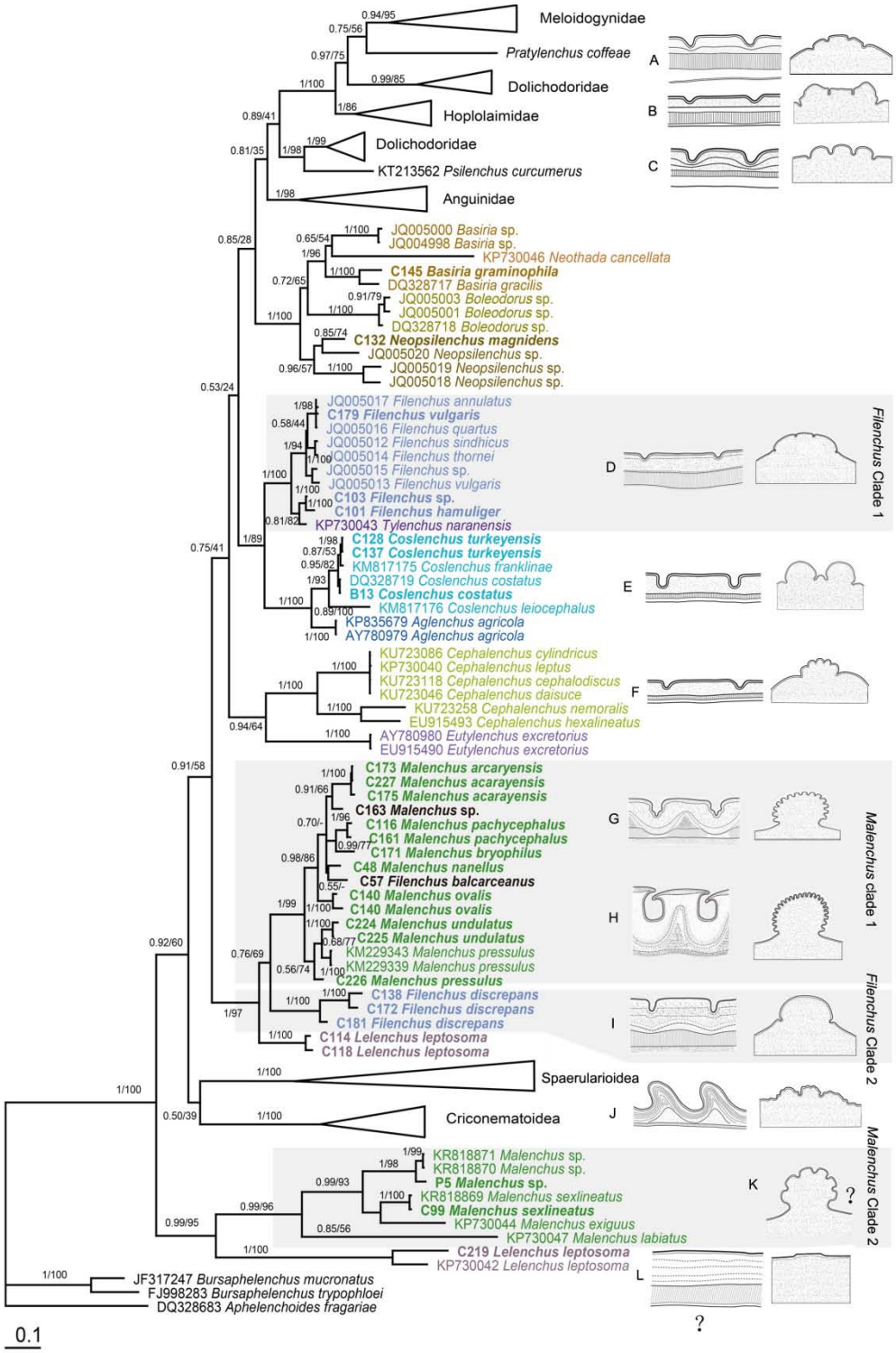

Fig. S1. Bayesian 50% majority rule consensus tree interfered on 28S rRNA gene. New sequences original to this study are indicated in bold. Phylogenetic position of the genera *Malenchus* and *Filenchus* are highlighted. Branch support is indicated in following order: PP value in BI analysis/BS value from ML analysis. Illustrations based on TEM, unless mentioned otherwise, and representative of the cuticle structure in each clade. Left, longitudinal section of mid-body and right, transverse section of lateral field. A: Male of

Meloidogyne incognita (Meloidogynidae) based on Baldwin & Hirschmann (1975); B: *Hirschmanniella oryzae* (Pratylenchidae) based on Mounport *et al.*, (1997); C: *Helicotylenchus dihystera* (Hoplolaimidae) based on Mounport *et al.*, (1993a); D: *F. vulgaris* C179; E: *Coslenchus franklinae* based on Mounport *et al.*, (1993b); F: *Cephalenchus emaginatus* based on Mounport *et al.*, 1993b; G: *M. acarayensis* C173; H: *M. pachycephalus* C161; I: *F. discrepans*; J: *Mesocriconema xenoplax* based on De Grisse, A.T. (1972); K: *M. sexlineatus* lateral region unknown, illustration proposed based on SEM picture. L: *L. leptosoma* C219, longitudinal section unknown, but scheme based on LM.

Table S1. List of sequences newly produced in the present study.

Genus	Species	Voucher	Locality	Coordinates	28S Accession no.	18S Accession no.
Malenchus	*acarayensis*	C31	Qingling, China	N 34°03'40.3" E 107°41'9.59"	-	KX156288
	acarayensis	C173, C175, C227	Groenendaal, Belgium	N 50°45'52.3" E 4°25'48.0"	KX156313 KX156316 KX156325	KX156282
	bryophilus	C171	Mt.Grossglockner, Austria	N 47°04'08.9" E 12°45'10.6"	KX156320	KX156299
	nanellus	C48	Pingwu, China	N 32°25'26.3" E 104°37'02"	KX156310	-
	ovalis	C140 C83	Poeke, Belgium	N 51°02'34.5" E 03°27'18.3"	KX156308 KX156309	KX156297 KX156298
	pachycephalus	C116	Jinping, China	N 22°58'48.9" E 103°23'34.1"	KX156314	KX156286 KX156287
	pachycephalus	C161	Poeke, Belgium	N 51°02'34.5" E 03°27'18.3"	KX156318	KX156291 KX156292
	pachycephalus	C85	Poeke, Belgium	N 51°02'35.4" E 3°26'56.3"	-	KX156290
	pressulus	C226	Göttingen, Germany	N 51°31'41.6" E 9°58'07.6"	KX156336	KX156280
	sexlineatus	C99	Mt. Hamiguitan, Philippines	N 6°43'51.8" E 126°10'05.3"	KX156319	KX156300
	undulatus	C224 C225	Göttingen, Germany	N 51°31'41.6" E 9°58'07.6"	KX156333 KX156334	KX156281
	sp.	P5	Mt. Hamiguitan, Philippines	N 6°43'50.1" E 126°10'15.4"	KX156332	KX156289
	sp.	C163	Poeke, Belgium	N 51°02'35.4" E 3°26'56.3"	KX156312	KX156302
Filenchus	*balcarceanus*	C57	Baishui, China	N 35°14'39.6" E 109°28'31.6"	KX156311	-
	discrepans	C172	Groenendaal, Belgium	N 50°45'52.3" E 4°25'48.0"	KX156321	KX156295
	discrepans	C181	Qingling, China	N 34°03'40.3" E 107°41'9.59"	KX156317	KX156305
	discrepans	C138	Göttingen, Germany	N 51°33'15.8" E 9°57'10.0"	KX156315	KX156306
	hamuliger	C101	Qingling, China	N 34°03'40.3" E 107°41'9.59"	KX156331	KX156304
	vulgaris	C179	Groenendaal, Belgium	N 50°45'52.3" E 4°25'48.0"	KX156337	KX156307

Lelenchus	sp.	C103 C102	Shimen, China	N 29°56'08.3" E 110°47'13.1"	KX156330	KX156303
	leptosoma	C114	Ghent, Belgium	N 51°02'31.9" E 3°41'11.8"	KX156322	KX156294
	leptosoma	C118	Qinling, China	N 33° 57' 31.2" E 107°45'8.56"	KX156324	KX156293
	leptosoma	C219	Groenendaal, Belgium	N 50°45'57.8" E 4°25'18.4"	KX156335	-
Basiria	*graminophila*	C145	Ghent, Belgium	N 51°02'09.3" E 3°43'18.9"	KX156326	KX156301
Neopsilenchus	*magnidens*	C132	Göttingen, Germany	N 51°33'16.0" E 9°57'19.7"	KX156323	KX156296
Coslenchus	*costatus*	B12	Ghent, Belgium	N 51°02'09.3" E 3°43'18.9"	KX156329	KX156285
	turkeyensis	C128 C137	Ghent, Belgium	N 51°02'31.9" E 3°41'11.8"	KX156327 KX156328	KX156283 KX156284

Table S2. Substitution saturation test for 28S rRNA with all taxa included.

No. subset samples	I_{ss}	I_{ss} Sym	T	P	I_{ss} Asym	T	P	DF
4	1.044	0.821	10.740	0.000	0.789	12.283	0.000	846
8	0.906	0.789	6.643	0.000	0.684	12.623	0.000	846
16	0.843	0.772	4.360	0.000	0.575	16.512	0.000	846
32	0.824	0.749	4.757	0.000	0.445	24.144	0.000	846

Table S3. Substitution saturation test for 18S rRNA with all taxa included.

No. subset samples	I_{ss}	I_{ss} Sym	T	P	I_{ss} Asym	T	P	DF
4	0.799	0.841	2.613	0.009	0.815	1.021	0.307	1681
8	0.663	0.823	10.620	0.000	0.728	4.337	0.000	1681
16	0.654	0.806	11.200	0.000	0.633	1.575	0.115	1681
32	0.634	0.790	11.928	0.000	0.520	8.699	0.000	1681

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Chapter IV

Redefinition of genus *Malenchus* Andrássy, 1968 (Tylenchomorpha: Tylenchidae) with additional data on ecology

Chapter modified from:

Qing X. and Bert W. (2017) Redefinition of genus *Malenchus* Andrássy, 1968 (Tylenchomorpha: Tylenchidae) with additional data on ecology. *Journal of Nematology* 49: 189-206.

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Abstract:

Malenchus is the second specious genus in Tylenchidae. In the presented study we examined 22 populations including 12 type/paratype species. Detailed morphology was recovered using light microscopy, scanning- and transmission- electron microscopy. All population and type slides were recorded as picture and video vouchers, which are available online. We have compared inter- or intraspecific variations and extracted taxonomically informative traits. Amended definitions of the *Malenchus* as well as the closely related *Ottolenchus* were given based on a combination of morphology and recent molecular data, and their phylogeny were analysed in a context of Tylenchidae. Furthermore, we test different fungi and moss as a food resource of *Malenchus*.

Keywords: *Duosulcius*, *Filenchus*, morphology, *Ottolenchus*, taxonomy, Tylenchomorpha, *Zanenchus*

Introduction

Tylenchidae is one of the most important soil inhabiting nematode groups (Andrássy, 1981), and species belonging to Tylenchidae may constitute up to 30% of the nematode individuals in a soil sample (Yeates and Bird, 1994; Ferris and Bongers, 2006). As early diverging Tylenchomorpha (=tylenchs with supposedly ancestral characters), they do not comprise economically important plant parasites and are characterized by ancestral characters, such as weak feeding apparatus, undifferentiated non-muscular corpus, filiform tails, and four cell rows in uterus. (Luc et al., 1987; Siddiqi, 2000; Bert et al., 2008). Knowledge of their food resources remains limited, albeit, given their numeric importance, this subject is important for trophic guild analysis or soil quality evaluation. Furthermore, their small body size and a lack of clearly homologous characters prevented us from deriving a consistent systematic framework. As a result, the delimitation of taxa in this group remains poorly documented and highly uncertain (Bongers and Bongers, 1998; Yeates, 2003; Ferris and Bongers, 2006).

In this study we focus on the cosmopolitan genus *Malenchus*, which is the second most specious (after *Filenchus*) in Tylenchidae. Although several species have been proposed, morphology details have been often only poorly described. The only genus review was made more than thirty years ago based on a limited number of morphological details (Andrássy, 1981). Recently, molecular methods have revealed a phylogenetic position for the genus *Malenchus* (Yaghoubi et al., 2015; Qing et al., 2016; Qing et al., 2017), but the need for a review is growing. In this present study we examined type or paratype of 12 species together with 10 populations worldwide. We do not intend to establish new nor to synonymize current taxon but rather to summarize morphological variations and analyse the results in a phylogenical context, as most of the taxonomically important characters are generally absent or incomplete in the original description (Qing et al., 2017).

Materials and methods

All specimen examined in this study are listed in Table1. Classification of *Malenchus* and Tylenchidae follows Geraert (2008). Geographic distributions were plotted using QGIS 2.82 based on original descriptions and other reports (Andrássy, 1981; Geraert and Raski, 1986; Gómez-Barcina et al., 1992; Geraert, 2008; Holovachov, 2014; Mundo-Ocampo et al., 2015; Panahandeh et al., 2015a; Panahandeh et al., 2015b; Yaghoubi et al., 2015; Qing et al., 2016). Measurements and drawings from slides were prepared manually with a drawing tube

mounted on an Olympus BX51 DIC Microscope (Olympus Optical, Tokyo, Japan), equipped with an Nikon DS-FI2 camera (Nikon Corporation, Tokyo, Japan) for photography. All examined populations as well as type slides were recorded as a video clips mimicking a multifocal observation through a light microscope (LM) following the video capture and editing procedures (De Ley and Bert, 2002). The resulting virtual specimens are available at http://nematodes.myspecies.info. Extraction and examination of female reproductive system was based on the method of Geraert (1973) and Bert et al. (2008). Illustrations were prepared based on light microscope drawings and modified by Adobe Illustrator CS3 and Adobe Photoshop CS6.

For scanning electron microscopy (SEM), specimens from DESS were gradually transfered to water, then dehydrated in a battery of ethanol solutions and dried by critical point dried with CO_2. After mounting on stubs samples were coated with gold and observed with a JSM-840 EM (JEOL, Tokyo, Japan) at 12 kV. For transmission electron microscope (TEM), specimens were fixed, ultra-thin sections were cut and sections were stained as detailed by Qing et al. (2017). Sections were observed with a JEOL JEM 1010.

To test the feeding type, four fungal species (*Flammulina velutipes*, *Lepista nuda*, *Botrytis cinerea* and *Pleurotus* sp.) were used as they represent different fungal groups, easily be cultured in lab condition and previous studies (Okada et al., 2002; Okada and Kadota, 2003; Okada et al., 2005) has suggested some of them can be feed by *Filenchus* spp. These fungal were inoculated on potato dextrose agar (PDA) medium with three repeats for each species and incubated at 25℃ for 10 days until the mycelium covered the culture plates. 40 individuals of *M. pachycephalus* and *M. acarayensis* were transferred to each plate and nematodes were extracted by Baermann tray after two months. Since *Malenchus* species are frequently associated with moss, it is consider as a potential host. *Eurhynchium* sp. was isolated from soil habited by *M. pachycephalus*, rinsed 5 times with distill water to remove attached detritus and then carefully transplant to culture plates with 1% agar in tap water. Controls plates were made using 1% agar in tap water to compare with the two treatments. Forty individuals were transferred to each plate and directly checked in binocular every three days for two months.

Result and discussion

Taxonomic overview

The genus *Malenchus* was established by Andrássy (1968) with *M. machadoi* (formerly

Aglenchus machadoi Andrássy, 1963) as the type species. Later several new genera have been erected and later synonymized with this genus, for details see Geraert (2008). Within the genus, three subgenera are valid: *Malenchus*, *Neomalenchus* and *Telomalenchus*. *Neomalenchus* was initially established as a genus for species with indistinct median bulb in *Malenchus* (Siddiqi, 1979), but this genus was synonymized (Andrássy, 1981) in his comprehensive review of *Malenchus* and later considered as a subgenus (Siddiqi, 2000). *Malenchus* subgenus *Telomalenchus* was introduced to accommodate three species (*M. williamsi* Geraert and Raski, 1986; *M. parthenogeneticus* Geraert and Raski, 1986 and *M. leiodermi* Geraert and Raski, 1986) with straight amphideal aperture and less lateral incisures (four or six) (Siddiqi, 2000). Although Andrássy (2007) synonymized subgenus *Telomalenchus* with genus *Fraglenchus*, such an action was rejected by Geraert (2008). Currently, *Malenchus* contains 36 valid species and 3 *nomina nuda* (Geraert, 2008; Mundo-Ocampo et al., 2015; Qing et al., 2016).

Geographic distribution

Malenchus is a cosmopolitan genus and is reported from all continents except for Antarctica (Fig. 1). Among them, *M. bryophilus* (Steiner, 1914) Andrássy 1981, and *M. acarayensis* Andrássy, 1968 are the most frequently encountered species, while 18 species are only reported once from their type location (*M. angustus* Talavera and Siddiqi, 1996; *M. anthrisulcus* (Sumenkova, 1988) Ebsary, 1991; *M. fusiformis* (Thorne and Malek, 1968) Siddiqi, 1979; *M. gratiosus* Andrássy 1981; *M. holochmatus* (Singh, 1971) Siddiqi, 1986; *M. herrerai* Mundo-Ocampo, Holovachov and Pereira, 2015; M. *kausari* Khan and Ahmad, 1991; *M. macrodorus* Geraert and Raski, 1986; *M. nobilis* Andrássy, 1981; *M. pampinatus* Andrássy, 1981; *M. paramonovi* Katalan-Gateva and Alexiev, 1985; *M. parvus* Brzeski, 1988; *M. sexlineatus* Qing, Sánchez-monge, Janssen, Couvreur and Bert, 2016; *M. shaheenae* Khan and Ahmad, 1991; *M. solovjovae* Brzeski, 1988; *M. subtilis* Lai and Khan, 1988; *M. truncates* Knobloch, 1976; *M. parthenogeneticus* Geraert and Raski, 1986; *M. williamsi* Geraert and Raski, 1986).

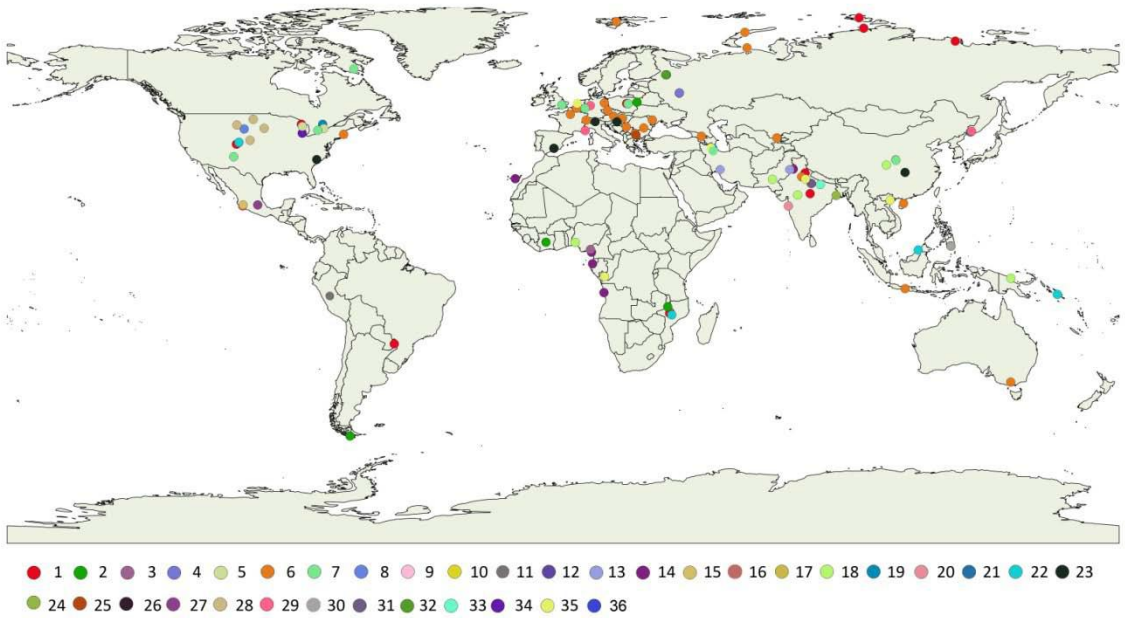

Figure 1. World distribution of *Malenchus* species. Species are labeled with different colors. 1. *M. acarayensis*. 2. *M. Andrassyi* Merny, 1970. 3. *M. angustus*. 4. *M. anthrisulcus*. 5. *M. bryanti* Knobloch, 1976. 6. *M. bryophilus* (Steiner, 1914) Andrássy, 1981. 7. *M. exiguus* (Massey, 1969) Andrássy, 1981. 8. *M. fusiformis*. 9. *M. gratiosus*. 10. *M. holochmatus*. 11. *M. herrerai*. 12. *M. kausari*. 13. *M. labiatus* Maqbool and Shahina, 1985. 14. *M. laccocephalus* Andrássy, 1981. 15. *M. leiodermis* Geraert and Raski, 1986. 16. *M. machadoi* (Andrássy, 1963) Andrássy, 1968. 17. *M. macrodorus*. 18. *M. nanellus* Siddiqi, 1979. 19. *M. neosulcus* Geraert and Raski, 1986. 20. *M. nobilis*. 21. *M. novus* Mukhina and Kazachenko, 1981. 22. *M. ovalis* (Siddiqi, 1979) Andrássy, 1981. 23. *M. pachycephalus* Andrássy, 1981. 24. *M. pampinatus*. 25. *M. paramonovi*. 26. *M. parthenogeneticus.* 27. *M. parvus*. 28. *M. platycephalus* (Thorne and Malek, 1968) Andrássy, 1981. 29. *M. pressulus* (Kazachenko, 1975) Andrássy, 1981. 30. *M. sexlineatus*. 31. *M. shaheenae*. 32. *M. solovjovae*. 33. *M. subtilis*. 34. *M. truncates*. 35. *M. undulates* Andrássy, 1981 and 36. *M. williamsi*.

General morphology

The body size of *Malenchus* ranges from 250µm to 900µm, the largest species is *M. novus*, while *M. sexlineatus*, *M. bryanti* and *M. parvus* are the three smallest species (Fig. 2). A ventrally arcuate habitus is the most common appearance, but a straight or “S” shape can also occasionally be found. Body behind vulva usually tapers markedly so that width at anus is about half of that at vulva, but an elongated-cylindrical shape similar to that of other genera

in Tylenchidae is also possible (*e.g. Malenchus* sp. C163 nested within *Malenchus* clade [Qing et al., 2017] but with elongated-cylindrical shape behind vulva).

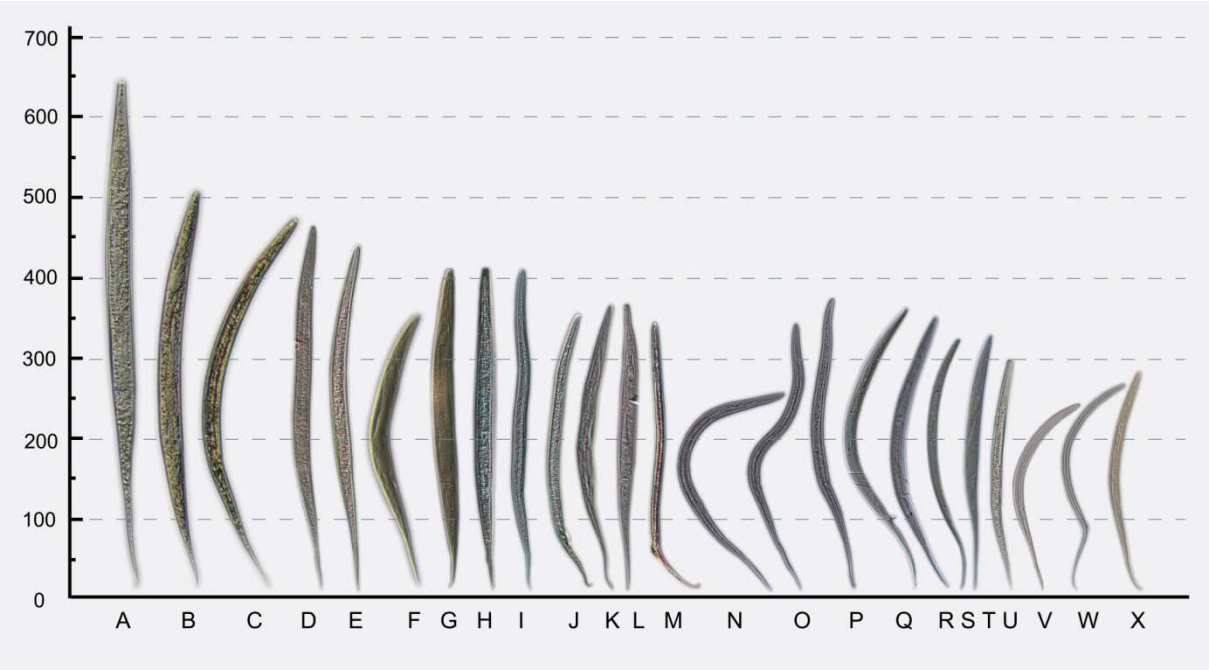

Figure 2. Body habitus and size in genus *Malenchus.* Size measured in µm and shown in longitudinal axis. A. *M. novus* from China. B-C. M. pachycephalus C116 from China. D. *M. williamsi* from Chile. E. *M. solovjovae,* from Poland. F-G. *M. pachycephalus* from Spain. H-J. *M. pachycephalus* C161 from Belgium. K-L. *M. exiguus* from China. M. *M. undulates*, from Philippines. N-P. *M. acarayensis* from Spain. Q-R. *M. tantulus*, from Malawi. S-T. *M. nanellus*, from Nigeria. U. *M. parvus*, from Mexico. V-X. *M. sexlineatus*, from Philippines. Female ventral views. A, B, D, G, H, L, T. Female lateral views. C, E, F, I, K, N, O, P, R, S, U, V, X. Male later view. J, M, Q, W.

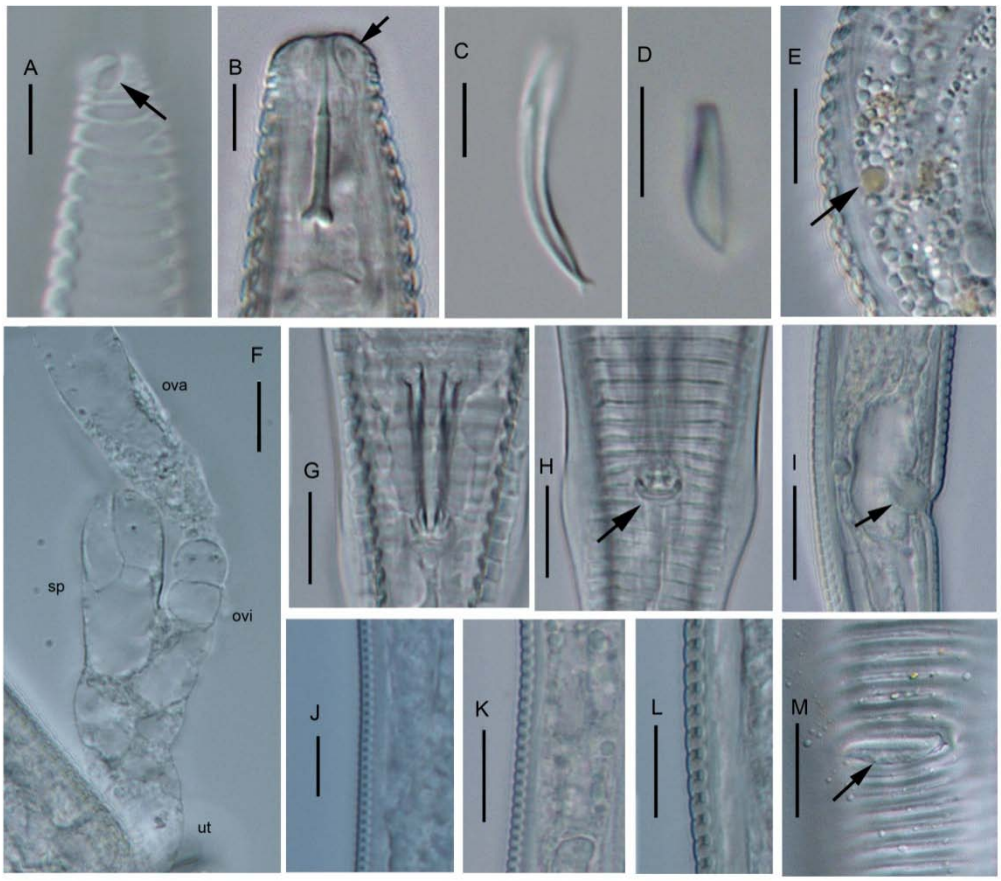

Figure 3 Selected anatomic structures in *Malenchus pachycephalus* (A-I, L, M), *M. sexlineatus* (J) and *M. exiguus* (K). A. lateral view of amphideal fovea. B. head region, arrow indicates ventral view of amphideal fovea. C. spicule, after dissection. D. gubernaculum, after dissection. E. anterior part of intestine, arrow indicates brown granule. F. female gonad after dissection. G. ventral view of spicule. H. ventral view of cloacal, arrow indicates distal end of spicule and gubernaculum. I. lateral view of vulva region, arrow indicates swollen vagina. J. folded cuticle of type 1. K. folded cuticle of type 2. L. folded cuticle of type 3. M. ventral view of vulva, arrow indicates epiptygmata. ova = ovarium. ovi = oviduct. sp = spermatheca. Scale bar: A-D, J = 5µm. E-I, K-M = 10 µm.

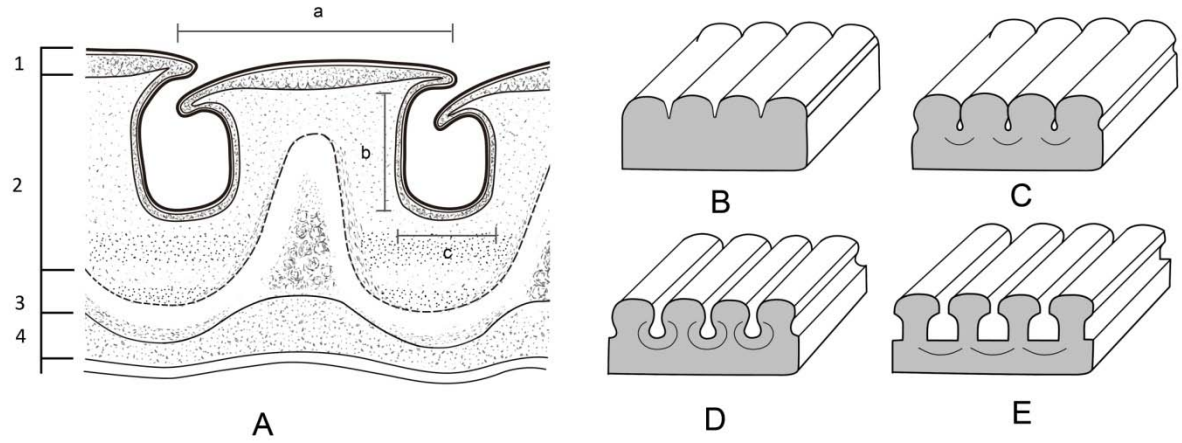

Figure 4 Diagrammatic example of cuticle layers in *Malenchus* (A) and the variation of the cuticle as observed based on LM observation (B-E). A. Illustration of ultrastructure in *M. pachycephalus* based on TEM, adapted from Qing et al. (2016). (1) epicuticle. (2) cortical zone. (3) median zone. (4) basal zone. (a) annuli width. (b) groove depth. (c) groove width. B-E. Schematically representation of the most common cuticle appearances in Tylenchidae. B. *Filenchus* type with indistinct annuli. C-E. cuticle types in *Malenchus*.

Cuticle annulation

The cuticle in genus *Malenchus* is generally thick and folded between annuli (Figs.3J-L; 4C-E; 5A) (Andrássy, 1981), in contrast to the typical finely-striated *Filenchus* (Figs 4B; 6C, E, F). The cuticle surface is smooth in most species but longitudinal striae can be observed occasionally under SEM (Fig. 7I, J). Annulations are prominent with a width of 0.76 to 2.38 µm, conspicuous even under low magnification. Although with some variations, the annulation number (especially from anterior to vulva/cloacal) and width shows different ranges interspecifically and is a taxonomically useful reference (see details in Table1).

The cuticle has been considered as an important generic character ever since this genus was proposed (Siddiqi, 1979; Andrássy, 1981; Geraert, 2008). However, a recent study shows that annulations can vary from prominent and folded to rather faint (Qing et al., 2017). These variations can be explained by different combinations of annuli width (a in Fig. 4A), groove height (b in Fig. 4A) and groove width (c in Fig. 4A) and therefore can be roughly clustered into three groups: (1) with indistinct folded part (Figs. 3J; 4C), annuli narrow and groove hardly visible in LM (a>4c, usually annuli width less than 1.2 µm), represented by species *M. sexlineatus*, *M. parthenogeneticus*, *M. leiodermis* and *Malenchus* sp. C163; (2) with

moderated folded cuticle annuli width (Figs.3K; 4D), groove narrow but visible (4c<a<2c, usually annuli width 1.2-1.6 μm), with species *M. parvus, M. acarayensis, M. exiguus, M. nanellus, M. ovalis*; (3) cuticle prominently folded (Figs. 3L; 4E), with spacious grooves and wide annuli (a<2c, usually annuli wider than 1.6μm); typical species include *M. pachycephalus, M. solovjovae, M. pressulus, M. novus.*

Within each type, the groove appears with a narrow opening, forming a nearly-enclosed space. In TEM this groove lumen was embedded by unknown organisms which resemble conidia, zoospore or hypha of fungus (Figs. 5B, C, E, F). Remarkably, we recovered 18S rRNA of the fungus *Malassezia* sp. from *M. pachycephalus*, the sequence similar to a fungus associated with *Malenchus* sp. in forest soil (Renker et al., 2003). Such fungal sequences have been obtained five times during our studies on *Malenchus* using “nematode specific” primers (Qing et al., 2017). Fungi from the genus *Malassezia* are opportunists, causing infection in humans and animals; they are commonly isolated from the skin and scalp of humans (Cunningham et al., 1990; Marcon and Powell, 1992; Hay and Midgley, 2010) and also from insects (Zhang et al., 2003). Although it has been reported from several species (*Malenchus* spp., *Meloidogyne* sp., *Acrobeloides* sp. and *Cephalobus* sp.) and assumed to be selectively associated with nematodes (Renker et al., 2003) as a vector (Karabörklü et al., 2015) or in random adherence (Adam et al., 2014), the relationship of *Malassezia* and nematodes remains unknown. In this study, the recovered unknown cuticula-associated organisms confirm the association of nematodes with another organism, and such an organism is likely to be *Malassezia* sp.

Cuticle ultrastructure

The ultrastructure in the cuticle was conventionally divided into four layers (Decraemer et al. 2003): (1) epicuticle, (2) cortical zone, (3) median zone, (4) basal zone (including basal lamina) and all of these layers are present in *Malenchus* (Fig. 4A). The epicuticle and cortical and median zones generally resemble those of other Tylenchomorpha, whereas the radial striae in the basal zone are reduced in *M. pachycephalus and M. acarayensis* (Qing et al., 2017). Although the cuticle ultrastructure shows intergeneric variation within Tylenchomorpha (Johnson et al., 1970; Mounport et al., 1991; Mounport et al., 1993b; Mounport et al., 1997; Valette et al., 1997), a radially striated layer in the basal zone was considered to be always present (Decraemer et al., 2003; Geraert, 2006). Although several structural cuticular elements are homoplasious within Nematoda, at less inclusive taxonomic

levels (e.g. on a family or genus level) the cuticle appears to be a more reliable phylogenetic marker (Decraemer et al., 2003). Thus, the divergent cuticle structure supports *Malenchus* as an evolutionarily divergent lineage within Tylenchomorpha and this character therefore important to define the genus.

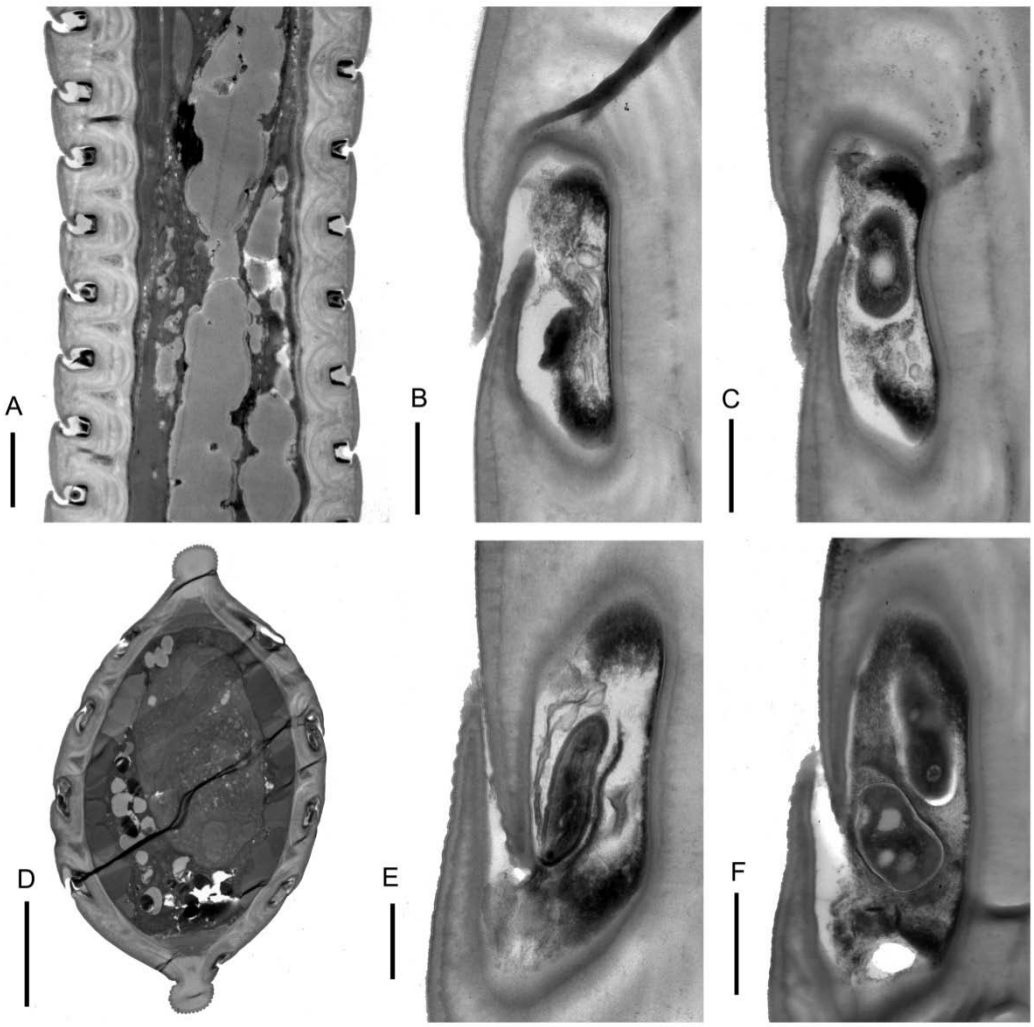

Figure 5 Cuticle ultrastructure of *M. pachycephalus* C161. A. longitudinal section in female middle body. B, C, E, F. unknown organisms present in annulation grooves. D. Cross section in female middle body. Scale bar: A = 2 μm. B, C, E, F = 0.5 μm. D = 5 μm.

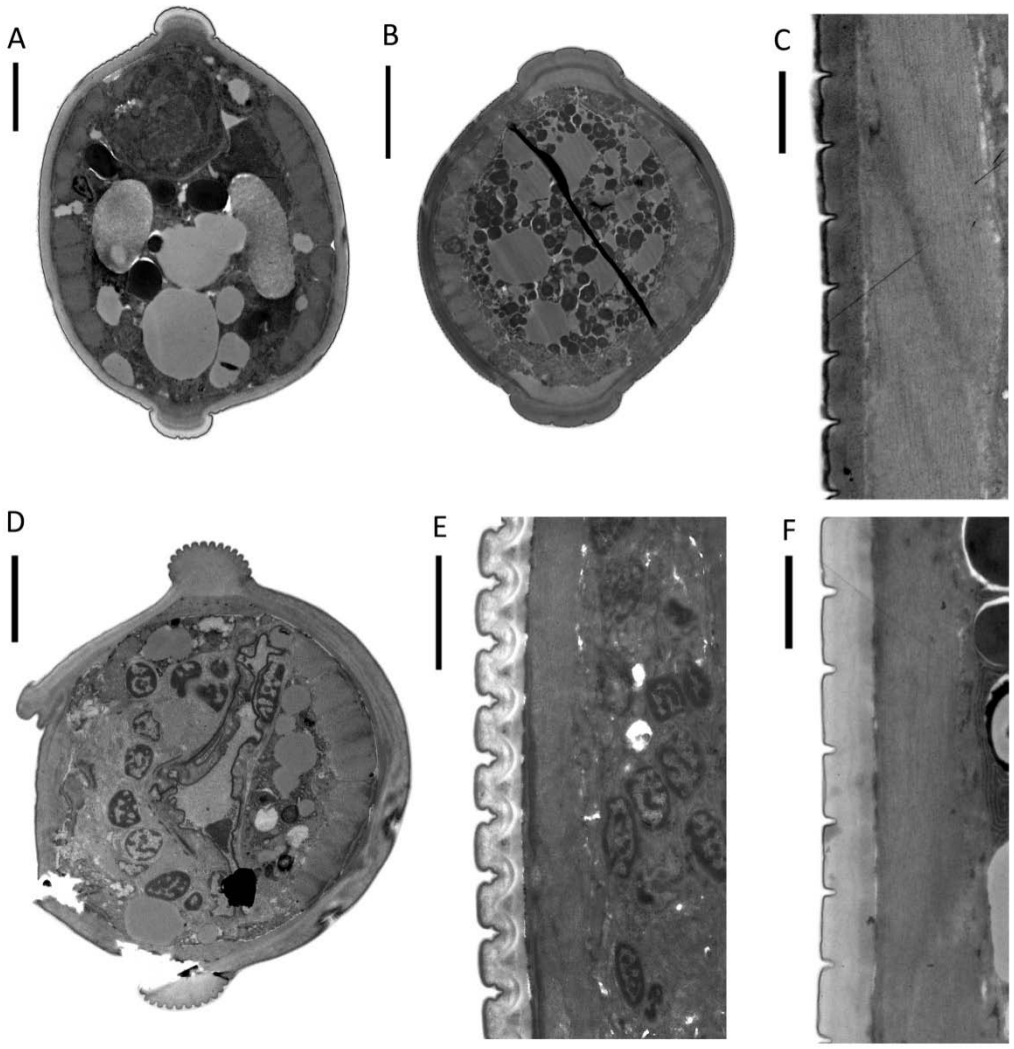

Figure 6 Ultrastructure of cuticle and lateral region in *Malenchus* and *Filenchus*. A, C. *F. discrepans*. B, F. *F. vulgaris.* D, E. *M. acarayensis*. A, B, D. cross section of female middle body. C, E, F. longtitudinal section in female middle body. Scale bar: A, D, E = 2μm, B = 4 μm, C, F=1.

Head region

The head of genus *Malenchus* is generally elevated, dorso-ventrally compressed (Andrássy, 1981) but more continuous in some species such as *M. exiguus, M. parthenogeneticus* and *M. williamsi*) (Figs.8; 9). Stylet usually delicate, comparable to *Filenchus,* but can be robust in some species (e.g. *M. macrodorus, M. novus, M. pachycephalus, M. solovjovae*). Cone part of stylet always heavier sclerotized but distinctly shorter (1/3-1/2 *v*s shaft) and thinner than shaft (Fig. 3B). Basal knobs flattened, directed backwards, forming a triangle-like base in stylet.

Amphideal fovea is usually invisible in Tylenchidae but is conspicuous spindle shaped (=large inner sacks) in *Malenchus* (Fig. 3A, B), a trait that corresponds to molecularly defined lineages and thus potentially useful in *Malenchus* delimitation (Qing et al., 2017). The amphideal fovea is wrapped in cuticular outgrowths, which form the finer clefts (Gómez-Barcina et al., 1992) resulting in either an S-shaped (Andrássy, 1981) or straight (Geraert and Raski, 1986) amphideal aperture. Although the aperture shape can change during development by the modification of the two outgrowths, it never switches from S-shaped to straight (Qing et al., 2016). The most common S-shaped aperture varies among species and can be roughly divided into two groups: (1) aperture starts with large round to oval shaped hole, sharply narrowing to a slit and ending at head base, represented by *M. macrodorus, M. nanellus, M. pachycephalus, M. solovjovae,* and *M. sexalineatus*; (2) the aperture slit is equally wide throughout its length, represented by *M. acarayensis*. Interestingly, the S-shape aperture is also present in some *Filenchus* species, which have only two lateral field incisures such as *F. normanjonesi*, *F. facultativus*, and *F. helenae* (Raski and Geraert, 1986b), but not in *F. fungivorous* (Bert et al., 2010), and never in *Filenchus* with four incisures. This is in line with the molecularly-based observation that *Filenchus* species with two incisures are more closed related to *Malenchus* than *Filenchus* species with four incisures (Atighi et al., 2013; Qing et al., 2017).

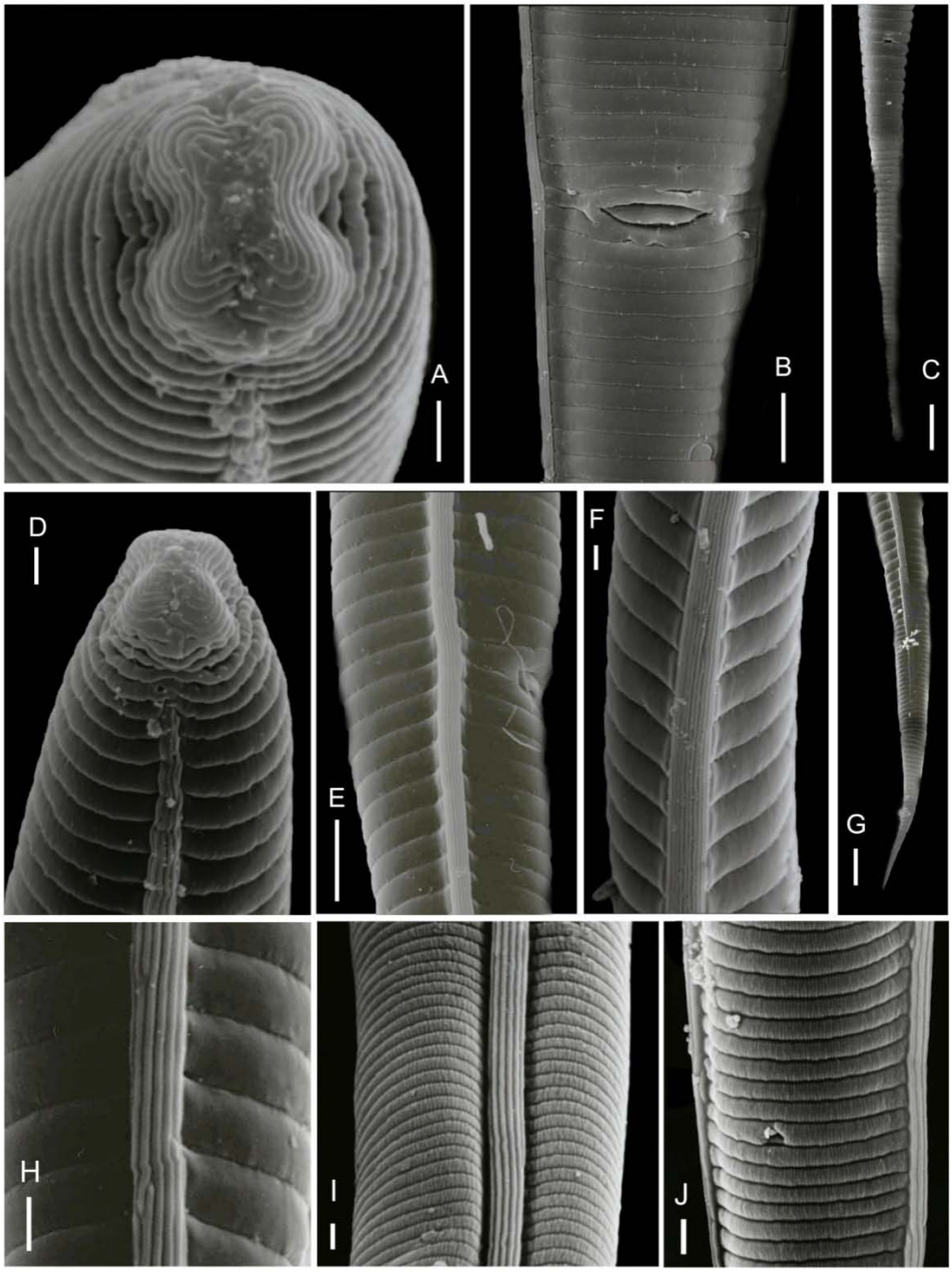

Figure 7 SEM of female *M. pachycephalus* C116 (A-H) and *M. nanellus* (I, J). A. *en face* view. B. vulva. C. ventral view of tail. D. lateral view of female head. E. lateral view of vulva region. F. lateral view of middle body showing smooth cuticle surface. G. lateral view of tail. H. lateral region of tail showing small ridges are stopped or interrupted. I. lateral region appears slightly crenated due to the extension of the cuticle annulations until ridge beneath. J. ventral view of anus showing cuticle surface with longitudinal striae. Scale bar: A, D, F, H, I, J = 1 µm. B, C, E, G = 5 µm.

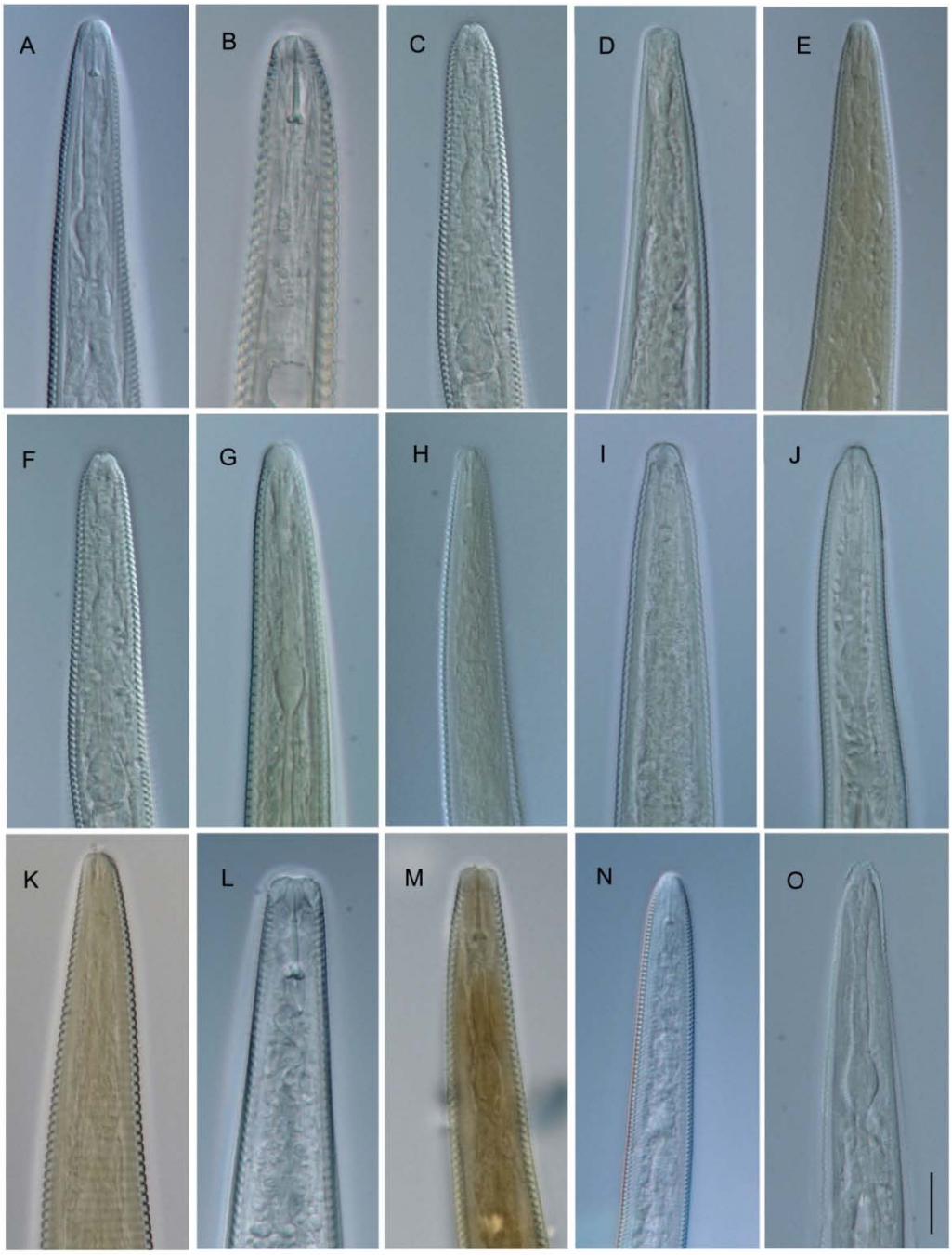

Figure 8 Anterior part of different species in genus *Malenchus*. All specimens are from examined type/paratypes, except for *M. exiguus* from Chinese population. More picture and video vouchers see http://nematodes.myspecies.info. A. *M. exiguus*. B. *M. pachycephalus*. C. *M. parvus*. D. *M. leiodermis*. E. *M. nanellus*. F. *M. ovalis*. G. *M. solovjovae*. H. *M. tantulus*. I. *M. williamsi*. J. *M. acarayensis*. K. *M. macrodorus*. L. *M. novus*. M. *M. malawiensis*. N. *M. sexlineatus* O. *M. parthenogeneticus*. Scale bar = 10 µm.

Lateral region

The lateral region is prominent, two incisures delimit a single narrow but elevated ridge (= protruding band, by Geraert and Raski [1986]; Geraert [2008]). In LM it appears as a plain ridge but in SEM or TEM several small ridges can be discerned (Figs. 5D; 6D; 7F, H, I). This feature is different from genus *Filenchus* (Fig. 6A, B) as well as other known species in Tylenchomorpha (Baldwin and Hirschmann, 1975; Mounport et al., 1991, 1993a; Mounport et al., 1993b; Mounport et al., 1997; Valette et al., 1997). The number of these small ridges is an interspecific variable, ranging from 3 to 14 based on SEM (Geraert and Raski, 1986; Brzeski, 1988; Gómez-Barcina et al., 1992; Mundo-Ocampo et al., 2015; Qing et al., 2016). However, even based on SEM the actual number can be underestimated, as small ridges can be present below the elevated ridge of the lateral region and these are hard to observe based on a single SEM image plane (Figs. 7I; 10B). Therefore, a cross section is crucial to determine the correct number of small ridges, which can be up to 22 based on TEM (Figs. 5D, 6D) (Qing et al., 2017).

The boundary of lateral lines sometimes appears to be a crenated margin, based on LM (Knobloch, 1976; Siddiqi, 1979; Andrássy, 1981; Geraert and Raski, 1986; Siddiqi, 2000). However, unlike other species, this crenated lateral field appears to correspond with the width of the ridge base, and if narrow then the crenation can extend below the lateral ridge that appears as a crenated margin from a lateral view (the longitudinal lateral ridge overlap with transversal crenation in two focus planes, see Fig. 10A-C), while if the base is wide there is no overlap and the margin of the lateral field is smooth (transversal crenation cannot reach to bottom of longitudinal ridge, see Fig. 10D-F).

The beginning of the lateral field range from few annuli after the head to the median bulb level (Figs. 9; 11) and ends at 1/4 to 1/3 of the tail. Interestingly, at least two start patterns have been observed (Fig. 10G, H), and the number of small ridges can be reduced at the anterior- or posterior-most part (Fig. 10I-M); they are clearly dissimilar to *Cephalenchus* (Mizukubo and Minagawa, 1985; Raski and Geraert, 1986a), which start from single ridge then hierarchically split three times to form five small ridges (six incisures). The start position of the lateral field has been used in species diagnosis and is indeed, consistent intraspecifically and varies interspecifically, based on our observations of 22 examined populations over 18 species. However, interpopulation differences have also been observed, for example the lateral field of *M. nanellus* starts at knob level (Troccoli and Geraert, 1995),

the mid-region of procorpus (Siddiqi, 1979; Andrássy, 1981; Siddiqui and Khan, 1983; Geraert, 2008) or even at the base of the procorpus (Geraert and Raski, 1986). If this is a matter of real variation or the presence of cryptic species (the examined paratype start at mid-region of the procorpus, other different reports may be cryptic species) remains to be investigated, but based on our data the starting position of the lateral ridge is a consistent character and taxonomically informative. This also concurs with the key to species provided by Andrássy (1981) and Geraert (2008).

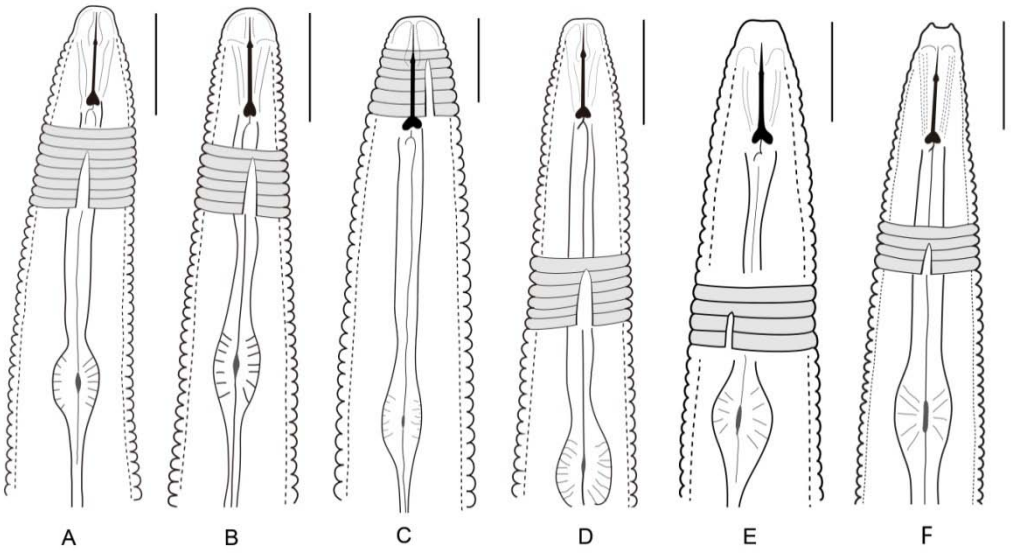

Figure 9 Illustration of anterior part of five *Malenchus* species showing general head shape, stylet and start position of lateral lines. A. *M. acarayensis*. B. *M. exiguus*, C. *M. pachycephalus*. D. *M. nanellus*. E. *leiodermis*. F. *M. labiatus*. Adapt from Andrássy (1981), Geraert and Raski (1986) and Maqbool and Shahina (1985). Scale bar = 10 µm

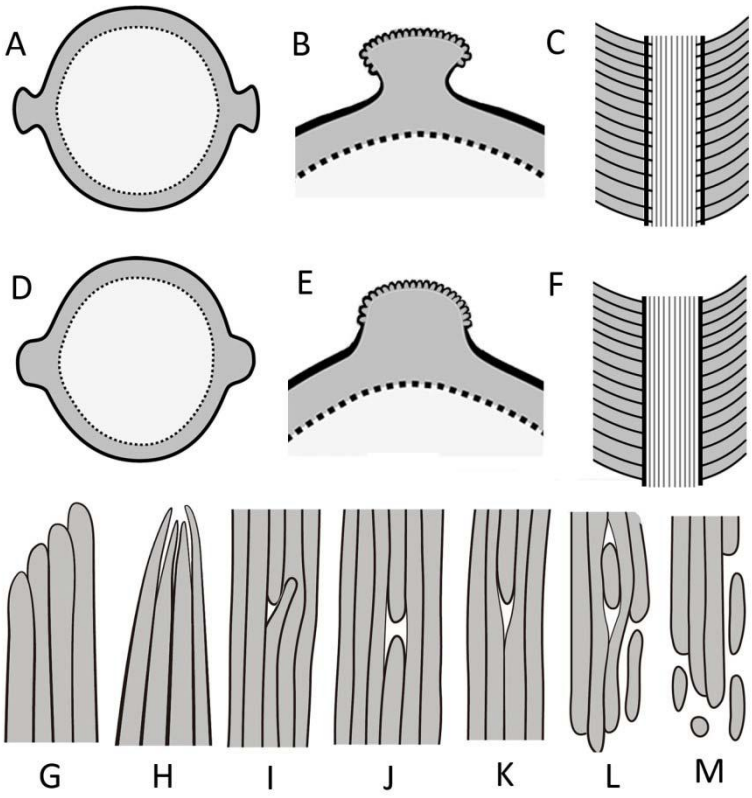

Figure 10 Illustration of lateral region in genus *Malenchus.* A-C. longitudinal lateral ridge narrow at the base, forming overlap with transversal crenation at two image plane and appears as crenated margin. A, B. cross section of lateral ridge. C. lateral view of lateral ridge. D-F. transversal crenation cannot reach bottom of lateral ridge, no overlap from lateral view and appears as smooth margin. D, E. cross section of lateral ridge. F. lateral view of lateral ridge. G, H. anterior start of lateral ridge. I-K. lateral ridge with small ridges stopped or interrupted. L, M. posterior end of lateral ridge.

Prophasmid

The phasmid usually occurs in the lateral region of the tail, but In Tylenchidae it is situated postmedian, just outside the lateralfields and termed prophasmid (Siddiqi, 1978). In females, the position ranges from 2-8 annuli anterior to 4-5 posterior vulva. Andrássy (1981) considered the prophasmid position as taxonomically informative at species level, ignoring the considerable variation presented in the same paper. Similar variations are also observed in this present study (Fig. 12); the intraspecific variation is often as large as the interspecific variation. Even in the same specimen both prophasmids can differ in up to 5 annuli from one another. Hence, this character is not reliable to distinguish species, except for *M. williamsi* with an unusual but conserved prophasmid position (post- vulval *vs* anterior to vulva in other

species, see Fig. 12).

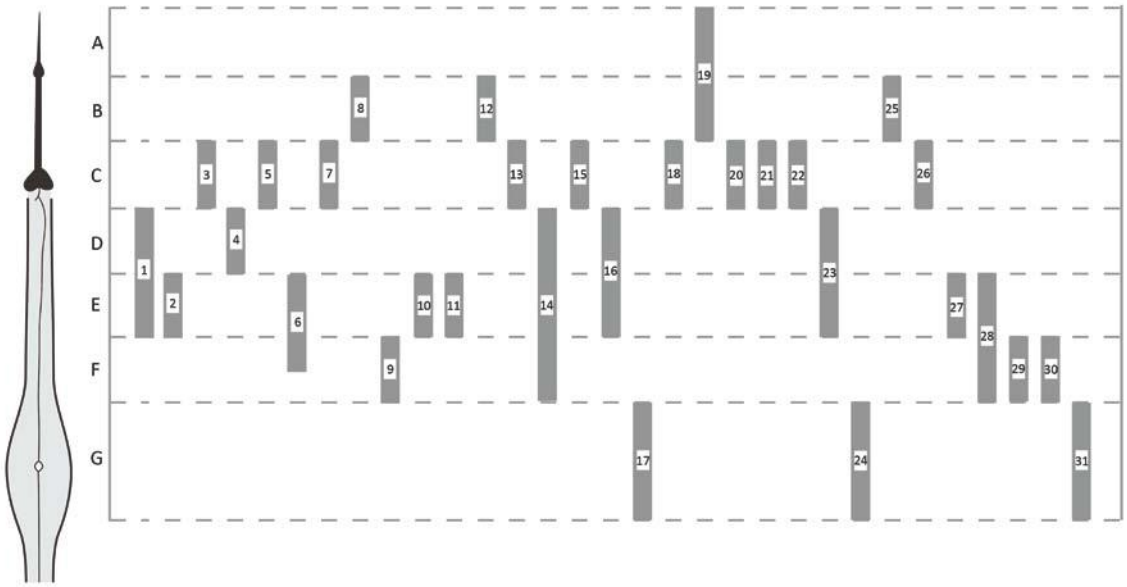

Figure 11 The relative origin positions of lateral lines in genus *Malenchus.* A. anterior of stylet. B. mid-region of stylet. C. level of knob. D. anterior of procorpus. E：mid-region of procorpus. F. base of procorpus. G. median bulb region. 1. *M. acarayensis*. 2. *M. andrassyi*. 3. *M. angustus*. 4. *M. bryanti*. 5. *M. bryophilus*. 6. *M. exiguus*. 7. *M. gratiosus*. 8. *M. herrerai*. 9. *M. kausari*. 10. *M. labiatus.* 11. *M. laccocephalus*. 12. *M. machadoi*. 13. *M. macrodorus*. 14. *M. nanellus*. 15. *M. neosulcus*. 16. *M. nobilis*. 17. *M. novus*. 18. *M. ovalis*. 19. *M. pachycephalus*. 20. *M. pampinatus*. 21. *M. parvus*. 22. *M. pressulus*. 23. *M. sexlineatus*. 24. *M. shaheenae*. 25. *M. solovjovae*. 26. *M. subtilis*. 27. *M. truncates*. 28. *M. undulates*. 29. *M. leiodermis*. 30. *M. parthenogeneticus*. 31. *M. williamsi.*

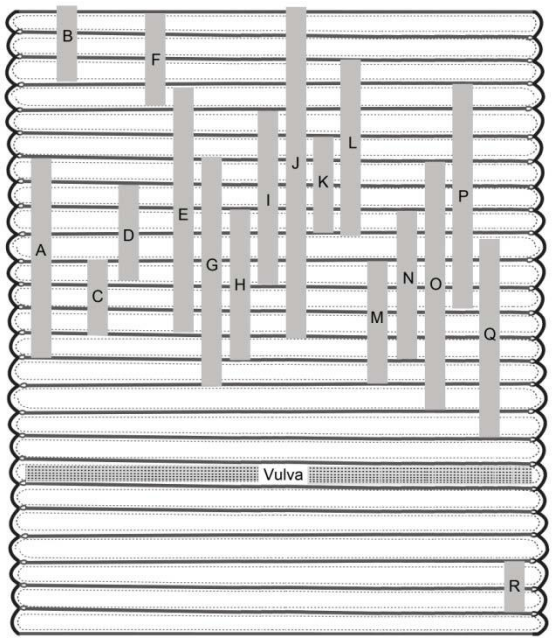

Figure 12 The relative position of prophasmid in *Malenchus.* All prophasmids located dorsal side near lateral lines, bars here only shows range of phasmid locations measured by number of annulations anterior/posterior to vulva. A. *M. acarayensis*. B. *M. andrassyi*. C. *M. bryanti*. D. *M. bryophilus*. E. *M. exiguus*. F. *M. sexlineatus*. G. *M. macrodorus*. H. *M. malawiensis*. I. *M. nanellus*. J. *M. nobilis*. K. *M. ovalis*. L. *M. pachycephalus* (Andrassy's population). M. *M. pachycephalus* C161. N. *M. parthenogeneticus*. O. *M. parvus*. P. *M. pressulus*. Q. *M. solovjovae*. R. *M. williamsi*. Based on data from Andrássy (1981), Geraert and Raski (1986) and Qing et al. (2016) and this study.

TABLE 2. Detail counts of female gonad cellular architecture.[a]

Species	Oviduct		Spermatheca	Uterus	
	Rows	Cells per row	Cells	Cell rows	Cells per row
M. pachycephalus	2	3	16 (+2)	4	5
M. acarayensis	2	4	17 (+2)	4	5
M. ovalis	2	3	14 (+2)	4	5
Malenchus sp. C163	2	4	10 (+2)	4	5

[a] Numbers in brackets indicate connecting cells between spermatheca and uterus.

Reproductive system

Female reproductive system monodelphic, ovary outstretched with oocytes arranged in a single row. Uterine sac spacious with thickened wall, eggs only present exceptionally (non-gravid) (Brzeski, 1988), post-vulval uterine sac (PUS) about half of body width. Vagina

has well developed muscles, perpendicular to body or slightly anteriorly direct. Vulva sunken, cavity shape with epiptygmata and lateral flaps (=dikes in Andrássy [1981]).

Based on dissected gonoducts, the oviduct comprises two rows of three (*M. pachycephalus*, *M. acarayensis* and *M. ovalis*) or four cells (*Malenchus* sp. C163), the spermatheca is offset, comprises 10 to 17 cells (Table 2) and is connected to the uterus by two cells (uterus except for *M. ovalis*), and the uterus cells are arranged in four regular rows (=quadricolumella) of five cells (Figs. 3F; 13; Table 2). Our observations concur with other gonoduct studies of Tylenchidae (Bert et al., 2006); the oviduct and uterus rows have been considered as an evolutionary stable structure: two oviduct cell rows were considered as an apomorphy of the order Rhabditida and four rows in uterus were typical for Tylenchidae and Anguinidae (Geraert, 1983; Bert et al., 2006; Geraert, 2006; Bert et al., 2008). The cell number of the spermatheca is intraspecifically consistent in all examined specimens, supporting spermatheca number as a species-specific indicator (Bert et al., 2006; Bert et al., 2008). However, additional observations based on more species are necessary to validate this character for *Malenchus* species identification. According to *in vivo* observations, the spermatheca of *Malenchus* appear as rounded to elongated and offset or bilobed-offset. However, examination of the expelled *M. ovalis* gonoduct shows that the bilobed appearance is the result of the non-offset part of spermatheca being filled with sperm. This confirms the observations of Qing et al. (2016) who presumed that the observed bilobed structure is the effect of sperm cells in the proximal part of the uterus and further limits the use of this trait in species diagnosis (Geraert and Raski, 1986).

The vulva is delimited by a depression of the cuticle, usually a gradual sinking that extends over two or three adjacent annuli, this in contrast to a sharp sink of one annulus in *Filenchus*. Lateral flaps (*i.e.* lateral dikes by Andrássy [1981] or vulval membranes by Carta et al. [2009]) are the cuticular outgrowths lateral and perpendicular to vulval slit. Two-annuli-long lateral flaps (Fig. 14B) is most common but they can be also indistinct (*e.g. M. pachycephalus, M. solovjovae, M. macrodorus*) or extend to 7-8 annuli (*M. williamsi*) (Fig. 14C). Interestingly, lateral flaps usually reduced in species with wider annuli. Epiptygmata (Fig. 14A-C) are found in all studied specimens and are considered as cuticular protrusions of the vaginal wall (Siddiqi, 2000). Although indistinct in LM, they can be clearly distinguished in SEM (Fig. 7B). A vagina with swollen muscle (Figs. 3I, 15D, E) is most promising character we recovered. Although the level of swollenness can vary among species (can be less swollen, *e.g.* Fig. 15E), the muscles in *Malenchus* are always thicker and darker in LM compare with *Filenchus.* This character has been noticed by several authors (Siddiqi, 1979;

Andrássy, 1981; Geraert, 2008), none of them used it as generic delimitation character. A swelling of the proximal or middle part of the vagina is presents in all examined *Malenchus* and we consider this character as an important generic character. In *Aglenchus* and *Coslenchus* the vulva is also swollen but more in the distal part (Fig. 15) and this trait may have evolved independently.

The male is generally less frequent than the female. Testis single, spermatogonia normally arranged in one row, spermatids few, indistinct. Spermatozoa always round but size can differ among species, filling proximal part of *vesicula seminalis*. Cloacal opening bears prominent cone with protruding lips. Spicules are variable in size and shape and thus taxonomically important in some species (Nickle, 1970; Hechler, 1971; Geraert and De Grisse, 1982; Adams and Nguyen, 2002). Within Tylenchomorpha spicule is less informative in species diagnosis, however four characters are potentially useful on genus level: (1) curvature; (2) the length/diameter ratio; (3) the presence/absence of a velum and (4) the shape of spicule tip (Geraert and De Grisse, 1982; Geraert, 2006). The typical "tylenchid-like" shape of capitulum, shaft and blade varies among the four examined species (Fig. 16). Remarkably, the spicule tip is twisted in *M. pachycephalus* and *M. acarayensis*, the edges curve in at level of blade but abruptly twist 180° and curve outwards at the end of the blade, which appears as a C-shape in the cross view of distal end (Figs. 3C, G; 16). Such a structure is unique to Tylenchidae. The gubernaculum is similar to other Tylenchomorpha (Clark et al., 1973; Wen and Chen, 1976; Wang and Chen, 1985), being centrally concave with ridge and two curved sides expanding laterally (Figs. 3D, H; 16E, F).

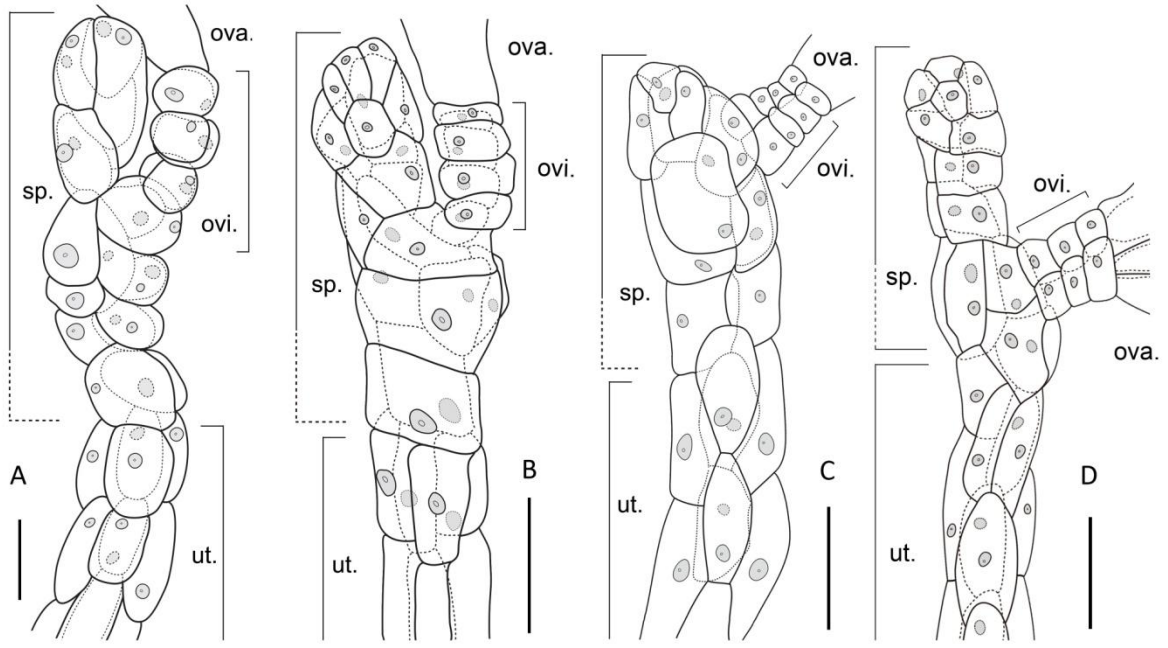

Figure 13 Line drawings of the cellular composition of oviduct, spermatheca and distal part of uterus of representative of the genus *Malenchus*. A. *M. pachycephalus*. B. *M. acarayensis.* C. *Malenchus* sp. C163. D. *M. ovalis*. Scale bar: A-C = 10 µm, D = 5 µm.

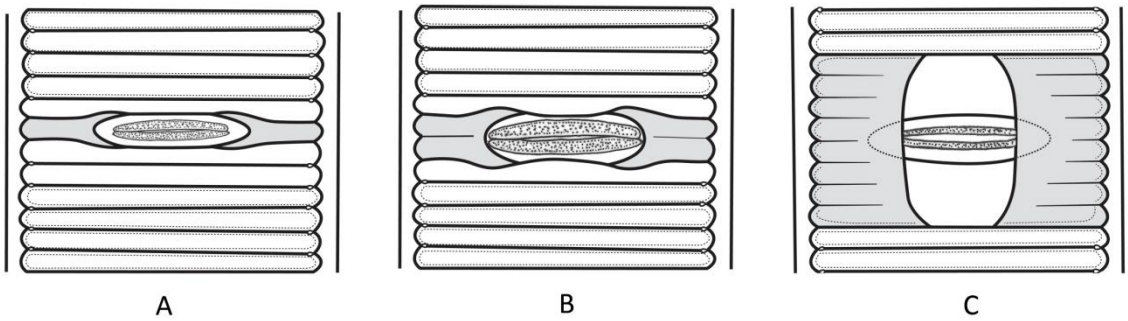

Figure 14 Ventral view of typical vulval flap and epiptygmata in the genus *Malenchus*. A. flap occupies one annulus without overlapping on vulva. B. flap occupies about two annuli slightly overlapping vulva. C. flap occupies more than four annuli covering half of vulva.

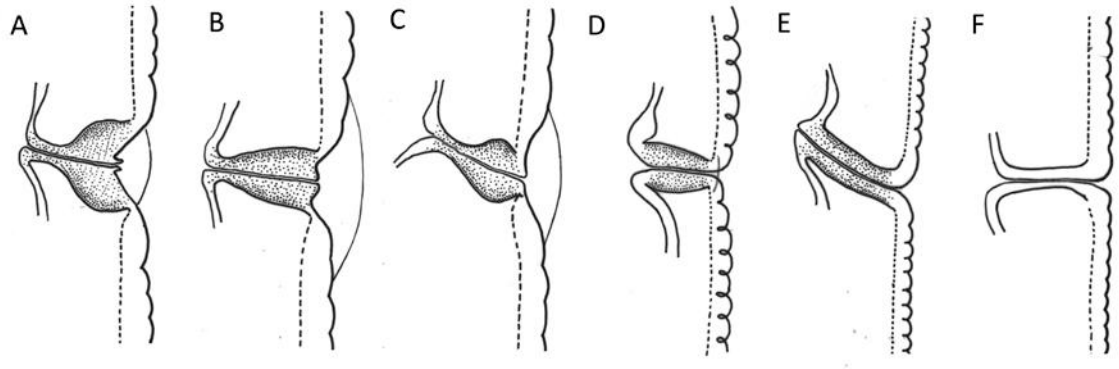

Figure 15 Vagina with different types of swollen walls. A-C. vagina with swollen wall in distal part, present in *Coslenchus* and *Aglenchus.* D, E. vagina with swollen wall in more proximal or middle part, present in *Malenchus.* F. thin and straight wall, most common type in Tylenchidae.

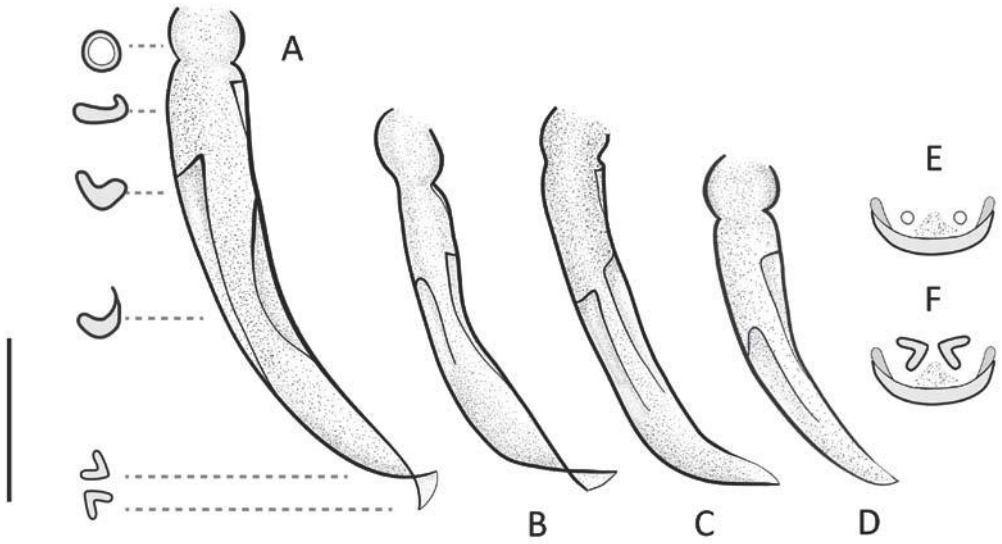

Figure 16 Spicules and gubernaculum in four *Malenchus* species. A, E, F. *M. pachycephalus*. B, C. *M. acarayensis.* D. *Malenchus* sp. C163. A, B, D. Lateral view of spicule. C. Lateral-ventral view of spicule. E, F. Distal end of spicule and gubernaculum. Scale bar = 5 µm

Revised generic definitions

Genus *Malenchus* Andrássy, 1968

Syn. *Neomalenchus* Siddiqi, 1979

Mukazia Siddiqi, 1986

Paramalenchus Sumenkova, 1988

Body straight or ventrally arcuate, dorso-ventrally flatted in cross view. Cuticle thick, most species have prominent folded annuli, occasionally with faint annuli, 0.76 to 2.38 µm, conspicuous even under low magnification. Head can be dorso-ventrally compressed or more rounded, with **pouch-like amphideal fovea**. Amphideal aperture usually S-shaped, but can also straight. Basal plate of cephalic framework is not flat (appears as M-shaped). Stylet weakly sclerotized, cone part of stylet always heavier sclerotized but distinctly shorter (1/3-1/2 *v*s shaft) and thinner than shaft. Basal knobs flattened, directed backwards, forming a triangle-like base in stylet. **Lateral field with offset ridge, comprising 6-22 small sub-ridges,** starting from stylet to level of median bulb and ending at middle of tail. Pharynx slender, median bulb from very weak to moderately developed, valvular apparatus present. Basal bulb short, pyriform. Female reproductive system monodelphic, prodelphic, straight, post-vulval uterine sac about half of body width. Prophasmid dorso-lateral, usually anterior

but rarely posterior to vulva. Vulva sunken, usually in a definite vulval cavity. Lateral flaps often present in species with narrow annuli (less than 1.8μm), but reduced or absent in species with wider annuli (more than 1.8μm). Epiptygmata present but may obscure in LM. **Vagina with swollen wall** in proximal or middle part. Body behind vulva markedly tapering so that width at anus is about half of that at vulva in most species, but can also be elongated behind vulva. Tail similar in both sexes. Male less frequent than females. Cloacal lips protruding. Bursa adanal, short, heavily curved. Spicule ventrally curved, tip is twisted in some species. Gubernaculum small.

TABLE 2.3. Comparison of generic definitions of *Malenchus*. [a]

This study	Andrássy, 1968	Siddiqi, 1979
1: Most species have prominent folded annuli, occasionally with faint annuli. 2: head can be dorso-ventrally compressed or more rounded, with **pouch-like amphideal fovea**. 3: basal plate of cephalic framework is not flat (appears as M-shaped) 4: **lateral field with offset ridge, comprising many small sub-ridges** 5: **vagina with swollen wall** in proximal or middle part. 6: body behind vulva markedly tapering in most species, but can also elongated.	1: prominent annulations of cuticle 2: elevated head, dorso-ventrally compressed 3: no description about basal plate of cephalic framework. 4: plain and conspicuous lateral fields 5: no description about vagina wall 6: markedly narrowing body behind vulva	1: thicker and folded annuli 2: cephalic region is elevated (about four or more adjacent annuli high, is striated and prominently compressed dorso-ventrally) 3: basal plate of cephalic framework is not flat (appears as M-shaped) 4: lateral fields with two closely spaced incisures, in cross-section each field appearing as a narrow, rounded ridge. 5: no description about vagina wall 6: body behind vulva markedly tapering so that width at anus is about half of that at vulva, overall shape is elongate-fusiform.

[a] Most important generic characters proposed in this study are marked in bold.

TABLE 2.4. Comparison of generic definitions of *Ottolenchus*. [a]

This study	Husain and Khan, 1967 (as subgenus)	Wu, 1970	Siddiqi, 1979
1: annulations usually less prominent, but can be relatively smooth. 2: lateral region with **one offset ridge** which forms two incisures. 3: head with low cephalic region, smooth and not prominently compressed 4: **amphideal fovea indistinct in LM**. 5: **vagina wall not well swollen,** vulva not sunken**.** 6: body behind vulva not markedly tapers, elongate-cylindrical overall body shape.	1: Body cuticle strongly annulated 2: Lateral field with only two crenate incisures 3: Head rounded with a slight depression at the base of lip region, without clear annulations 4: no description on amphideal fovea 5: no description on vulva 6: no description on body behind vulva	1: body annulation generally coarse. 2: lateral field with two incisures 3: no description on cephalic framework, *en face* rectangular with four lips, two subdorsal and two subventral, lateral lip regions in the form of two depressed areas. 4: no description on amphideal fovea 5: no description on vagina wall, rudimentary membrane of vulva present or not distinct 6: no description on body behind vulva	1: cuticle less thick and annulation less prominent. 2: lateral field with two incisures 3: head with low cephalic region, smooth and not prominently compressed 3: basal plat is somewhat flat and demarcates the cephalic region. 4: no description on amphideal fovea 5: no description on vagina wall, vulva closed 6: body behind vulva not markedly tapers, elongate-cylindrical overall body shape.

[a] Most important generic characters proposed in this study are marked in bold.

Comments on amended generic definitions

Based on characters recovered in the present study as well as available molecular evidence (Qing et al., 2016; Qing et al., 2017), we propose an amended definition of the genus *Malenchus* emphasizing on amphideal fovea, lateral region and vaginal structure. The most important traits of *Malenchus*, in comparison with earlier definitions, are presented in Table 3.

Ottolenchus are intimately related to *Malenchus* and *Filenchus* clades group 2 by sharing two incisures. Indeed, such a similarity has been noticed and repeatedly discussed (Siddiqi, 1979; Brzeski and Sauer, 1982; Raski and Geraert, 1986b; Brzeski, 1998; Siddiqi, 2000; Geraert, 2008). The two prevailing opinions are either *Ottolenchus* as a valid genus distinguished from *Filenchus* spp. by two incisures and ventral curved amphideal aperture (Siddiqi, 2000) or a synonym of *Filenchus* due to the high variability of lateral incisures (some species show faint interrupted inner lines in SEM) and an amphideal aperture similar with other known *Tylenchus* spp. and *Filenchus* spp. (Raski and Geraert, 1987; Andrássy 2007; Geraert, 2008). Molecular analysis indicates the two-incisures *Filenchus* (Fig. 6A) is separated from four-incisures *Filenchus* (Figs. 6B; 17) and suggests the lateral region is an important character to define genus (Qing et al., 2017). In such a scenario, we consider *Ottolenchus* as a valid genus and revised definitions are listed in Table 4. Given that SEM and other informative character are largely unknown in *Filenchus* or *Ottolenchus*, any action allocating species to one of the genera is difficult. Here we forward three taxonomic proposals for current *Filenchus*/*Ottolenchus* species: (1) species that fit definitions listed in Table 3 should move to *Malenchus*, (2) species with two clear incisures, no pouch-like amphideal fovea, and non-swollen vaginas should move to *Ottolenchus* (further splits into more genera are still possible, as several molecular lineage present, but so far without morphological support), (3) type species of *Filenchus* (*F. vulgaris*) bear four incisures, thus all four-incisures species should stay in *Filenchus.* Probably some *Tylenchus* species also need to be included in the latter group.

Observations on ecology

The species in genus *Malenchus* generally appears in an undisturbed environment, preferably forest soil, often associated with moss or litter or aquatic sediments (all known species have at least once reported from these habitats). Occasionally, *Malenchus* is also found in agricultural fields (Few populations from *M. acarayensis*, *M. andrassyi*, *M. labiatus*, *M. laccocephalus* and *M. ovalis*, details see Table 5). Allocation of the feeding behavior in

Tylenchidae is a recurrent discussion point among nematologists (Bongers and Bongers, 1998). Normally, *Malenchus* species are considered as epidermal and root hair feeders (Bongers and Bongers, 1998) or algal, lichen and moss feeders and parasites of lower and higher plants (Siddiqi, 1986, 2000; Andrássy, 2007). The feeding studies in Tylenchidae (Okada et al., 2002; Okada and Kadota, 2003; Okada et al., 2005) suggested a fungal-feeding habit for three *Filenchus* species. Our feeding test on four different fungal species and one moss species failed to culture either *M. pachycephalus* or *M. acarayensis*. However, we observed numerous brown to green granules consistently presented in the anterior intestine of two analyzed *Malenchus* species, but not for other fungal feeding nematodes from the same sample (Fig. 3E). Interestingly, such pigments resemble to moss and/or soil algae and this is consistent with the most reported habitats of *Malenchus*, indicating that moss and/or algae are likely to be a natural food resource. However, the direct feeding on moss or algae was not observed, thus further study is necessary to understand the exact feeding behavior of *Malenchus* as well as other Tylenchidae.

Acknowledgement

The authors thank Marjolein Couvreur for SEM and Myriam Claeys for TEM analyses. The first author thanks the China Scholarship Council (CSC) for providing a grant. This work was also supported by a special research fund UGent 01N02312.

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Table1. Number of cuticle annules and width in different species or populations of the genus *Malenchus*. Annuli number is arranged as ♀/♂ when both gender are present. Annuli width is given in Average ± SD. All number counts start from anterior end. *M. pachycephalus* from Gomez-Barcina et.al., 1992 probably contain a mixed population, the counts marked with (?) are probably not belongs to *M. pachycephalus.*

Species and voucher No.	Specimen No.	Pharynx	Vulva/Cloacal	Total	Annules width	Material	Slide No.
M. acarayensis	2♀♀	66,68	188, 207	320,356	1.26±0.08	Qinling, China	XQ048
M. acarayensis C173	5♀♀1♂	59-64/83	163-166/231	280-291/303	1.23±0.10/1.03	Qing et.al. 2017	XQ148
M. bryophilus C171	3♀♀	55-70	203-219	351-382	1.38±0.13	Qing et.al. 2017	XQ149
M. exiguus	3♀♀1♂	72-74/81	194-198/310	330-333/426	1.14±0.09/1.01	Qing et.al. 2016	XQ090, XQ091
M. leiodermis	1♀	79	208	340	1.05	Paratypes, Geraert and Raski, 1986	UGMD103431
M. macrodorus	3♀♀1♂	72-75/79	180-185/229	304-320/311	1.43±0.24/1.68	Paratypes, Geraert and Raski, 1986	UGMD103434, UGMD103435
M. malawiensis *	1♀1♂	55/63	185/284	295/360	1.43/1.03	Paratypes, Siddiqi,1979	UGMD100230, UGMD100231
M. nanellus	1♀1♂	63/92	202/234	320/325	0.93/0.84.	Paratypes, Siddiqi,1979	UGMD100223, UGMD100224
M. novus	1♀	60	236	378	1.71	Qing et.al., 2016	XQ088
M. ovalis C140	3♀♀1♂	62-64/68	162-164/314	263-277/380	1.19±0.11/1.06	Qing et.al., 2017	XQ155
M. ovalis **	1♀	71	219	343	1.2	Paratype, Siddiqi,1979	UGMD 100229
M. pachycephalus C116	3♀♀ 1♂	46/50	115-118/177	194-196/260	2.34-2.38/1.68,1.96	Qing et.al., 2017	XQ156
M. pachycephalus C161	3♀♀1♂	48-51	129-132/152	209-220/210	2.38±0.15/2.03	Qing et.al., 2017	XQ157
M. pachycephalus	3♀♀	58-60	185(?),138-142	320(?),229-225	2.15±0.07	Gomez-Barcina et.al., 1992	UGMD103002 UGMD103003 UGMD103004
M. parthenogeneticus	1♀	66	199	293	1.02	Paratypes, Geraert and Raski, 1986	UGMD103432
M. parvus	2♀	51, 54	143-147	281-305	1.35±0.14	Paratypes, Brzeski,1988	UGMD 100851
M. sexlineatus	4♀	70-75	182-185	289-296	0.76±0.03	Holotype, Qing et.al., 2016	UGMD104304
M. tantulus ***	1♀1♂	64/70	173/245	308/334	1.4±0.31	Paratypes, Siddiqi,1979	UGMD100225
M. solovjovae	4♀♀	49-50, 59?	133-135,196?	230-241, 320?	2.3±0.3	Paratypes, Brzeski,1988	UGMD 100852
M. undulatus	2♀♀	50,51	139,141	251,273	1.81±0.05	Qing et.al. 2017	XQ158
M. williamsi	1♀1♂	67	194/294	320/395	1.6/1.2	Paratypes, Geraert and Raski, 1986	UGMD103428
Malenchus sp. C163	3♀♀	80-85	271-280	436-460	0.98±0.1	Qing et.al. 2017	XQ159
Duosulcius acutus	1♀	131	364	>550	0.97	Paratypes, Siddiqi,1979	UGMD 100227

*Paratype of *Neomalenchus malawiensis* Siddiqi, 1979, synonym of *M. malawiensis* (Siddiqi, 1979) Andrássy, 1981

**Paratype of *Neomalenchus ovalis* Siddiqi, 1979, synonym of *M. ovalis* (Siddiqi, 1979) Andrássy, 1981

***Paratype of *M. tantulus* Siddiqi, 1979, synonym of *M. acarayensis* by Geraert and Raski (1986)

Table 2. Recovered habitats of different species in genus *Malenchus*

Species	Habitats	Reference/Comments
M. acarayensis	Tropical rain forest litter	Andrássy, 1968
	Sand dune forest	Wasilewska, 1970
	Soil around white birch (*Betula papyrifera*) near bog and lake area.	Knobloch, 1976
	Soil around roots of tomato	Siddiqi, 1979, Syn. *M. tantulus*
	Grass root, park near lake	Andrássy, 1981, Syn. *M. cognatus*
	Forest soil around root of *Albizia prosera*, *Quercus incana* and *Terminallia belerica*	Lal and Khan, 1988
	Soil around root of *Quercus rotundifolia*	Gomez-Barcina et al., 1992
M. andrassyi	Flooded rice field	Merny, 1970
	Soil around *pennisetum purpureum*	Siddiqi, 1979
	Soil around root of pear (*Pyrus communis*); mango (*Mangifera indica*); Wheat (*Triticum aestivum*).	Maqbool and Shahina, 1985
	Forest soil	Coosemans, 2002
M. angustus	Soil around moss	Talavera and Siddiqi, 1996
M. anthrisculus	Rhizosphere of *Anthriscus sylvestris* in flood land meadow	Sumenkova, 1988 Syn. *Paramalenchus anthrisculus.*
M. bryanti	Soil around white birch (*Betula papyrifera*) near bog and lake area.	Knobloch, 1976
	Moss soil	Andrássy, 1981
M. bryophilus	Arctic island	Loof, 1971
	Moss from rock; near root of reed grass; root of willow; sandy soil in the vicinity lake; moss from soil; forest litter; forest soil.	Andrássy, 1981
	Meadow, Moss	Coomans, 1989
	Grassland	Bert et al., 2003
M. exiguus	Root of grass in *Picea engelmanni* infected by Engelmann spruce beetle.	Massey, 1969, Syn. *Aglenchus exiguus*
	Soil around maple tree (*Acer saccharum*); birch tree (*Betula sp.*); *Dryas* sp. near lake area; red cedar (Thuya plicata); spruce (*Picea glauca*); Douglas fir (*Pseudotsuga menziesii*); spruce (*Picea engelmanni*); trembling aspen (*Populus tremuloides*); alpine fir (*Abies lasiocarpa*); Pine (*Pinus contorta*); wet moss; grass	Wu,1970, Syn. *Ottolenchus sulcus*
	Soil around root of horse chestnut (*Aesculus hippocastanum*).	Siddiqi, 1979
	Root of strawberries near lake	Szczygiel, 1974
	Soil from deciduous forest near the root of birch tree (*Betula* sp.)	Qing et. al., 2016
	rhizosphere of *Bromus sp*.	Panahandeh et al., 2014
M. fusiformis	Prairie soil	Thorne and Malek, 1968 Syn. *Tylenchus fusiformis*
M. gratiosus	Moss *Sphagnum* sp. from virgin forest	Andrássy, 1981
M. herrerai	Epiphyte moss associated with coffee plants	Mundo-Ocampo et al. 2015
M. hexalineatus	Tropical rainforest, litter under of *Lithocarpus llanosii*	Qing et al., 2016
M. holochmatus	Rhizoids of moss	Singh, 1971, Syn. *Tylenchus holochmatus*
M. kausari	Soil around roots of grass *Cyanodon dactylon*	Khan and Ahmad, 1989
M. labiatus	Soil near root of sugarcane (*Saccharum officinarum*)	Maqbool and Shahina, 1985

	rhizosphere of sugarcane	Yaghoubi et al., 2015
M. laccocephalus	Moss from Muhapa tree; moss from trunks in rain-forest	Andrássy, 1981
	Soil around root of pear (*Pyrus malus*)	Maqbool and Shahina, 1985 Syn. *M. pyri*
M. leiodermis	Freshwater soil beneath thick tundra	Geraert and Raski,1986
	Volcanic soil of a pine-oak forest,	Brzeski, 1988
M. machadoi	Moss from Moua tree	Andrássy, 1963
M. malawiensis	Soil around roots of *Eucalyptus saligna*; around root of *Pennisetum purpureum*	Siddiqi, 1979, Syn. *Neomalenchus malawiensis*
M. nanellus	Soil around root of maize (*Zea mays*) in experimental plot.	Siddiqi, 1979
	Moss from trunk of a willow; Sand soil in the vicinity of a small lake	Andrássy, 1981
	Soil near root of sugarcane (*Saccharum officinarum*)	Maqbool and Shahina, 1985
	Benthos from stagnant brooklet, mud; border of mangroves under pandanus tree; sagu tree; coconut plantation, among grass; secondary rainforest, clay under leaves; cowpat puddle with duck-weed; Bank of swamp	Troccoli and Geraert, 1995
	Soil around root of fern and moss in forest	Qing et. al., 2016
	Rhizosphere of grasses	Panahandeh et al., 2015b
M. neosulcus	*Sphagnum sp*. moss from virgin forest	Geraert and Raski, 1986
M. nobilis	Soil around grass root from a garden	Andrássy, 1981
M. novus	Soil near the root of *Echinopanax elatum*, *Abies nephrolepis* and *Pinus koraiensis*	Mukhina and Kazachenko, 1981
	Deciduous forest soil around root of *Quercus* sp	Qing et al., 2016
M. ovalis	Soil around roots of Chili (*Capsicum annuum*)	Siddiqi, 1979, Syn. *Neomalenchus ovalis*
	Soil around root of *Quercus rotundifolia*	Gomez-Barcina et al., 1992
	Wet humus from the base of a palm	Andrássy, 1981
M. pachycephalus	Fern grass; soil around root of *Alnus glutinosa*; soil around grass root, dry moss	Andrássy, 1981
	Soil around root of *Quercus rotundifolia*	Gomez-Barcina et al., 1992
	Soil from deciduous forest	Qing et al., 2016
	Moss mixed with soil from base of birch tree (*Betula* sp.) in forest	Qing et al., 2017
M. pampinatus	Soil around grass root.	Andrássy, 1981
M. paramonovi	Rhizosphere soil from mixed forest of scots pine (*Pinus sylvestris*) and spruce (*Picea sp.*)	Katalan-Gateva and Alexiev, 1989
M. parthenogeneticus	Freshwater soil beneath thick tundra	Geraert and Raski,1986
M. parvus	Sandy soil near *Vaccinium sp.* root	Brzeski, 1988
M. platycephalus	Soil near root of grass and aquatic plants near river; brush thicket	Thorne and Malek, 1968, Syn. *Tylenchus platycephalus*
M. pressulus	Soil of coniferous forest	Kazachenko, 1975 Syn. *Aglenchus pressulus*
	Soil of grass root	Andrássy, 1981
	Beech forest soil	Zell, 1988

	Rhizosphere of *Vaccinium sp*. in forest	Wisniewska and Kowalewska, 2015
M. shaheenae	Soil around root of unidentified wild trees in forest.	Khan and Ahmad, 1991
M. solovjovae	Sandy soil near root of various shrubs, close to a lake; sandy soil near birch tree (*Betula sp.*)	Brzeski, 1988
M. subtilis	Forest soil around root of Bakan (*Melia azedirach*)	Lal and Khan, 1988
M. truncatus	Soil under moss and leaf litter in low, bog-like area near woods.	Knobloch, 1976
M. undulatus	Rainforest litter; tropical, soil under leaf	Andrássy, 1981
	Rhizosphere of grasses	Panahandeh et al., 2015b
	Soil form moss	Qing et al. 2017
M. williamsi	Freshwater soil beneath thick tundra	Geraert and Raski,1986

Chapter V

3D printing in zoological systematics: an integrative taxonomy of *Labrys chinensis* gen. nov., sp. nov. (Nematoda: Tylenchomorpha)

Chapter published as:

Qing X., Bert W. (2017). 3D printing in zoological systematics: an integrative taxonomy of *Labrys chinensis* gen. nov., sp. nov. (Nematoda: Tylenchomorpha). *Journal of Zoological Systematics and Evolutionary Research*. In press

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Abstract

3D printing technology has showed its importance in many fields. In present study, the potential of such technique in zoological systematics was assessed. For the first time, 3D printed models were incorporated in the description of a new genus as a complement to pictures and drawings to illustrate complex 3D structures and to be used in education. Hereby, we also tested the performances of different printing materials and forwarded resin as the most suitable option for the zoological field. As a case study, *Labrys chinensis* gen. nov., sp. nov. was described using an integrative approach: detailed morphology based on light- and electron microscopy, phylogenetic position as revealed from two *ribosomal RNA* genes, generic traits were tested for homoplasy, and the intra- and inter-population variations of four recovered populations were analyzed. The new genus belongs to subfamily Tylenchinae, family Tylenchidae and infraorder Tylenchomorpha. It is characterized by unique labial plate that has four narrow lobes with tips detached from adjacent cuticle, laterally broad elongated amphidial apertures, a strong sclerotized excretory duct, round spacious postvulval uterine sac, and spicule with a sharp protrusion at blade.

Keywords: 3D modeling – Nematode - new genus - new species - Tylenchidae.

Introduction

Integrative taxonomy was introduced as a comprehensive framework to delimit and describe taxa by gathering together information from different types of data and methodologies (Dayrat 2005; Will et al. 2005) and has been considered as the most efficient and theoretically grounded approach to define robust species hypotheses (De Queiroz 2007; Samadi and Barberousse 2006). The commonly-used complementary perspectives include phylogeography, comparative morphology, population genetics, ecology, development and behavior. In present study we introduce 3D printing in generic description. Models are incorporated as a complement to pictures and drawings to illustrate complex 3D structures. Aside from taxonomy, we show its potential applications in linking research frontiers to education. We also compared the performance of printing materials and proposed the most suitable option.

Nematodes belong to Tylenchidae are abundant and diverse. Ecologically, they are important soil fauna which may constitute up to 30% of the nematodes in any given soil sample (Ferris and Bongers 2006; Yeates and Bird 1994). However, it is taxonomically notorious as most species combine a low observational resolution with high intraspecific variability. As a result, many descriptions are ambiguous (mainly base on light microscopy) and several genera were polyphyletic (Qing et al. 2017). Here we add a new genus, *Labrys chinensis* gen. nov., sp. nov. Integrative approaches were applied to increase descriptive resolution: detailed morphology based on light microscopy (LM) and scanning electron microscopy (SEM); 3D models were built and printed; selected generic traits were tested for phylogenetic homoplasy; four populations were recovered and their intraspecific variation was analyzed. The results expanded our knowledge on Tylenchidae and provided an example for future species description in taxonomically difficult group.

Materials and methods

Sample collecting and processing

A total of 38 individuals were collected in four locations in China (Table 1). Soil samples were incubated for 48 h on plastic trys lined with paper towels. Nematodes were concentrated using a sieve (25 μm opening). After removing water, nematodes were rinsed with DESS solution and transferred to glass vials for preservation and transportation. DESS-preserved specimens were rinsed several times with deionised water and then transferred to anhydrous glycerin for morphological analyses (Yoder et al. 2006).

Table 1. Sampling locations and GenBank accession numbers of four *Labrys chinensis* gen. nov., sp. nov. populations used in this study.

Populations	Individuals.	GenBank accession number		Locations
		28S	18S	
P1	13	KY776621, KY776622, KY776623, KY776624	KY776632	Taibai, China (34° 03'40"N, 107° 41' 9.6"E)
P2	8	KY776616, KY776617, KY776618, KY776619, KY776620	KY776633	Meixian, China (34°05'18.5" N 107°47'26.6" E)
P3	8	KY776611, KY776612, KY776613, KY776614, KY776615	KY776630	Shimen, China (29°56'08.3" N 110°47'13.1" E, 30°01'55.2" N, 110°39'54.0" E)
P4	9	KY776625, KY776626, KY776627, KY776628, KY776629	KY776631	Zhouzhi, China (107°47'9.4" E, 33°54'6.5" N)

Morphological analyses

Measurements and drawings were prepared manually with a drawing tube mounted on an Olympus BX51 DIC Microscope (Olympus Optical, Tokyo, Japan). The holotype of the new species was recorded as video clips mimicking LM multifocal observations (De Ley and Bert 2002) and these are available online at http://www.nematodes.myspecies.info. Illustrations were prepared manually based on light microscope drawings and edited by Adobe Illustrator CS3 and Adobe Photoshop CS3. For SEM, specimens from DESS were gradually washed

with water and post-fixed with 2% PFA + 2.5% glutaraldehyde in 0.1M Sorensen buffer, then washed and dehydrated in ethanol solutions and subsequently critical point-dried with CO_2. After mounting on stubs, the samples were coated with gold and observed with a JSM-840 EM (JEOL Ltd., Tokyo, Japan) at 12 kV.

Molecular phylogenetic analyses

Genomic DNA was extracted from DESS-preserved specimens with worm lysis buffer (Yoder et al. 2006). The extracted samples were frozen for 10 min at 20 °C. 1 μL proteinase K (1.2 mg/mL) was added to the samples before incubation, 1 h at 65 °C followed by 10 min at 95 °C. The D2D3 domains of 28S *ribosomal RNAs* (*rRNA*) were amplified with primers D2A (5'-ACAAGTACCGTGAGGGAAAGT-3') and D3B (5'-TGCGAAGGAACCAGCTACTA-3'). The 18S *rRNAs* were amplified with primers TylF1 (5'-GCCTGAGAAATGGCCACTACG-3') and TylR2 (5'-TGRTGACTCRCACTTACTTGG-3'). The PCR conditions were 30 s at 94 °C, 30 s at 54 °C and 2 min at 72 °C for 40 cycles. Newly obtained sequences were deposited in GenBank (Table 1). Multiple alignments of the different genes were made using the Q-INS-i algorithm implemented in MAFFT v. 7.205 (Katoh and Standley 2013) and alignments are available at TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S21161). The best-fitting substitution model was estimated using AIC in jModelTest v. 2.1.2 (Darriba et al. 2012) and GTR+I+G was selected as best scored model for both markers. Maximum likelihood (ML) analysis was performed with 1000 bootstrap (BS) replicates under the GTRCAT model using RAxML 8.1.11 (Stamatakis et al. 2008) and Bayesian inference (BI) was carried out with the GTR+I+G model using MrBayes 3.2.3 (Ronquist et al. 2012). Analyses were run for 5×10^6 generations and Markov chains were sampled every 100 generations. Burnin was arbitrarily chosen to be 25% of the results, and evaluated using a generation/Loglikelihood scatter plot. The ML and BI analyses were performed at the CIPRES Science Gateway (Miller et al. 2010). Gaps were treated as missing data for all phylogenetic analysis. All trees were visualized with TreeView v. 1.6.6 (Page 1996). ML BS values and Bayesian posterior probabilities (PP) were summarized on the consensus tree using

Adobe Illustrator CS3.

Homoplasy test

To provide an objective estimation on evolutionary conservation of lip pattern and its robustness as generic delimitation marker, we calculated the homoplasy indices, the retention index (RI), the consistency index (CI), the observed number of character transitions (obs.) and the permutation of character values (perm.) (Maddison and Slatkin 1991) across the BI consensus tree. We consider high RI and CI values (≥0.80) or low obs./perm. ratio (≤0.45) to be indicative that the analyzed traits evolved slowly enough to retain phylogenetic information and low homoplasy. All analysis was performed in Mesquite 3.10 (Maddison and Maddison 2016).

Table 2. Homoplasy test for lip region arrangement. RI: retention index, CI: consistency index, obs: observed number of character transitions; permu, permutation number of character transitions.

	18S	28S
RI	0.91	0.96
CI	0.80	0.87
obs.	10	8
perm.	28	29
obs/perm.	0.36	0.27

Table 3. Nucleotide diversity of 28S rRNA among four recovered populations (P1-P4) of *Labrys chinensis* gen. nov., sp. nov. In bold is nucleotide diversity between populations measured by $F_{st.}$ In the diagonal: nucleotide diversity within each population measured by θ_π and θ_S , indicated in order of θ_π / θ_S. All F_{ST} estimates were highly significant at $p<0.05$.

	P1	P2	P3	P4
P1	0.50/0.54			
P2	**0.89**	1.4/0.96		
P3	**0.92**	**0.93**	9.8/2.9	
P4	**0.94**	**0.94**	**0.93**	2.8/1.9

Analyses of population genetic structure

To visualize population structure and display conflicts in the data by taking into account incompatible phylogenetic signals, we generated phylogenetic networks by employing the NeighborNet algorithm (Bryant and Moulton 2004) with uncorrected pairwise p-distances in the program SplitsTree v4.10 (Huson and Bryant 2006). 1000 pseudo-replicates (result only showed among populations) bootstrap analysis was conducted to assess the support for splits in the network. We also estimated nucleotide diversity (θ_π and θ_S) within population and genetic variation among the four populations by fixation index (F_{st}). All diversity and demographic analyses were performed using Arlequin 3.1 (Excoffier et al. 2005).

3D modeling and printing

To visualize important morphological characters and facilitate zoological education, 3D models were reconstructed by Autodesk Maya following the procedure of Qing et al. (2015). Next to the new genus, three other Tylenchidae genera (*Tylodorus* sp. *Cephalenchus* sp. and *Cucullitylenchus* sp.) were modeled in order to visualize intra-family lip region variations and test printing performance through a variety of nematode taxa. The constructed models were converted to .stl format and MiniMagics 3.0 was used to inspect bad edges and multiple shells. Each model was printed by three commercial materials: PLA (polylactic acid) and ABS (Acrylonitrile Butadiene Styrene) were printed by MakerBot Replicator 2 using FDM (fused deposition modeling) method, while resin was printed by RSPro 450 Industrial 3D printer using stereolithography method. All the model files and the printing video/pictures are freely available at worldwide 3D designer community Thingiverse (www.thingiverse.com), allowing our designs to be discussed and improved by the science community.

Results

Phylogenetics analysis and homoplasy test

In both analyses the tree topologies regarding the major clades of Tylenchidae are

congruent with recently published studies (Atighi et al. 2013; Qing et al. 2017; Qing et al. 2015). The monophyly of all *Labrys chinensis* gen. nov., sp. nov. populations is fully supported (BI=1, BS=100) across two genes. The new genus is sister to a clade containing *Filenchus* + *Malenchus* based on 28S (BI=1, BS=98) or to *Filenchus misellus* (Andrássy 1958) Raski & Geraert, 1987 based on 18S (BI=1, BS=91) (Figs 4, 5). As already noted by Qing et al. (2017), the polyphyly genus *Filenchus* contains at least 3 clades. Among these clades, *F. misellus* and *F. chilensis* Raski & Geraert, 1987 formed a separate clade, separated from the type species of the genus (*F. vulgaris* (Brzeski 1963) Lownsbery & Lownsbery 1985). Such separation is also morphologically supported by their unique amphidial aperture pattern (Brzeski and Sauer 1982; Karegar and Geraert 1998; Torres and Geraert 1996). Therefore, *F. misellus* and *F. chilensis* can be designated as one or even two separate genus/genera. However, this phylogenetic grouping is based on only few GenBank sequences without morphological vouchers, limiting the validity of further taxonomic actions.

Tylenchidae taxonomy is controversial and problematic with several invalid or homoplastic generic characters (Qing et al. 2017) meaning that an objective selection for morphological characters that define phylogenetic clades is necessary. In this study homoplasy tests of lip region pattern in 18S and 28S *rRNA* phylogeny trees indicated strong phylogenetic signals (RI>0.85, CI≥0.80, obs/permu<0.45) (Table 2), indicating it is a relatively conserved character that can be used as generic character.

Population structure

The Neighbor-net analysis based on 28S *rRNA* revealed four major clades (Fig. 6A), supported by high inter-population genetic divergence (F_{st}>0.8) (Table 3) and with the geographic distribution: Population No. 1 (P1), Population No. 2 (P2) and Population No. 4 (P4) are *ca.* 10-20 km far from each other and Population No. 3 (P3) is distantly separated from the other populations at around 600 km (Fig. 6B). The most interesting general result is the presence of multiple lineages (P1, P2, P4) occurring in a relatively small geographical region. Although the divergence is relatively low, and network analyses shows that historical admixture across the range may exist, all lineages are well separated. This suggests that either the contemporary gene flow was interrupted for an unknown reason or that ancestral

polymorphisms have been retained. The intra-population nucleotide also shows diversity, the lowest divergence in P1 (θ_π=0.50, θ_S=0.54) and the highest in P3 (θ_π=9.8, θ_S=2.9). However, all populations do not show morphological or morphometrical inter- or intra-population differences.

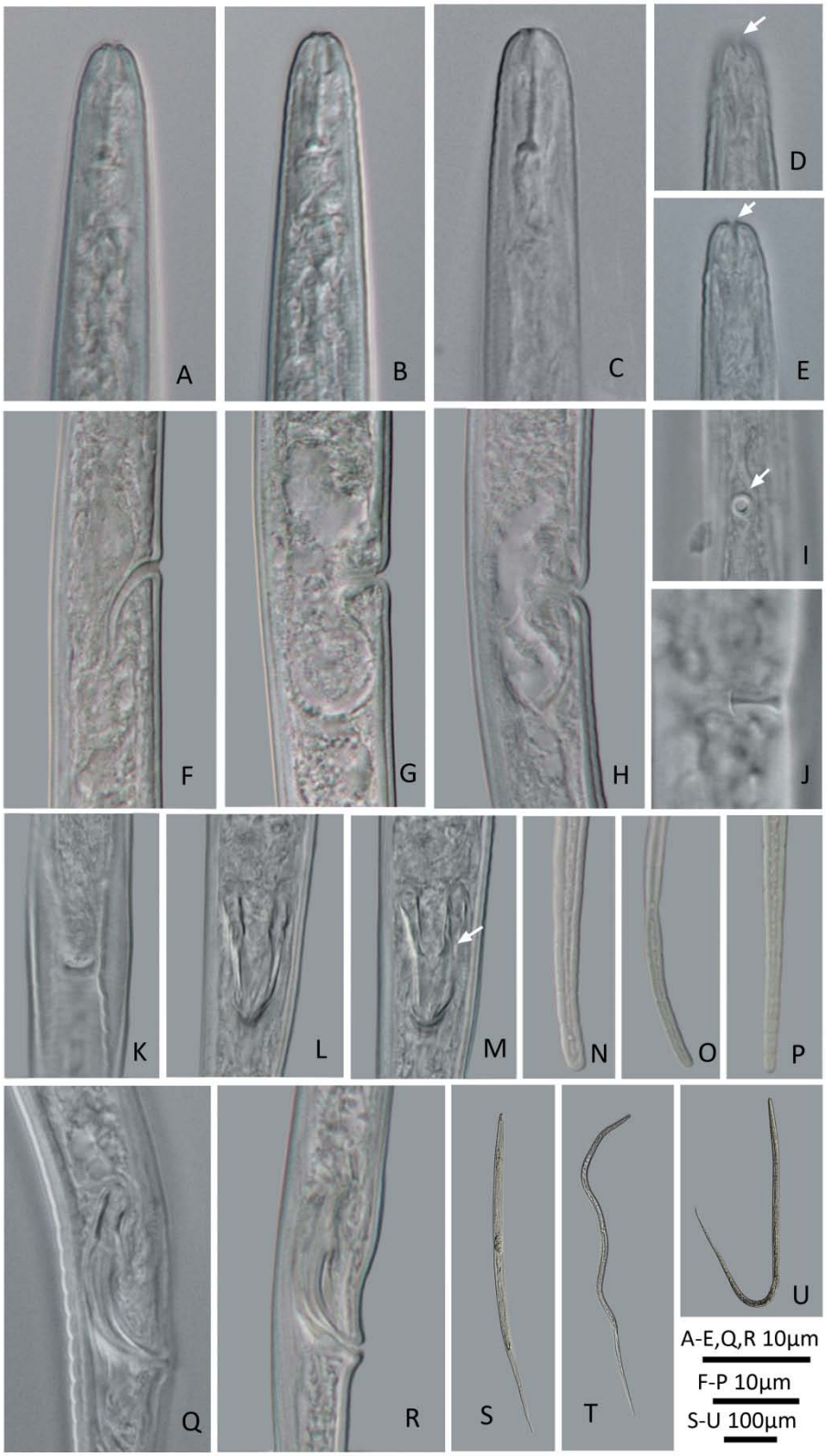

Figure 1. LM pictures of *Labrys chinensis* gen. nov., sp. nov. A-E: female cephalic region, arrow point lateral view of amphidial aperture. F: excretory pore and duct. G, H: vulva. I: ventral view of excretory pore. J: ventral view of vulva. K: ventral view of cloacal. L, M: ventral view of spicule, arrow point sharp protruding in spicule blade. N: male tail tip. O, P: female tail tip. Q, R: ventral view of spicule. S, U: female habitus. T: male habitus.

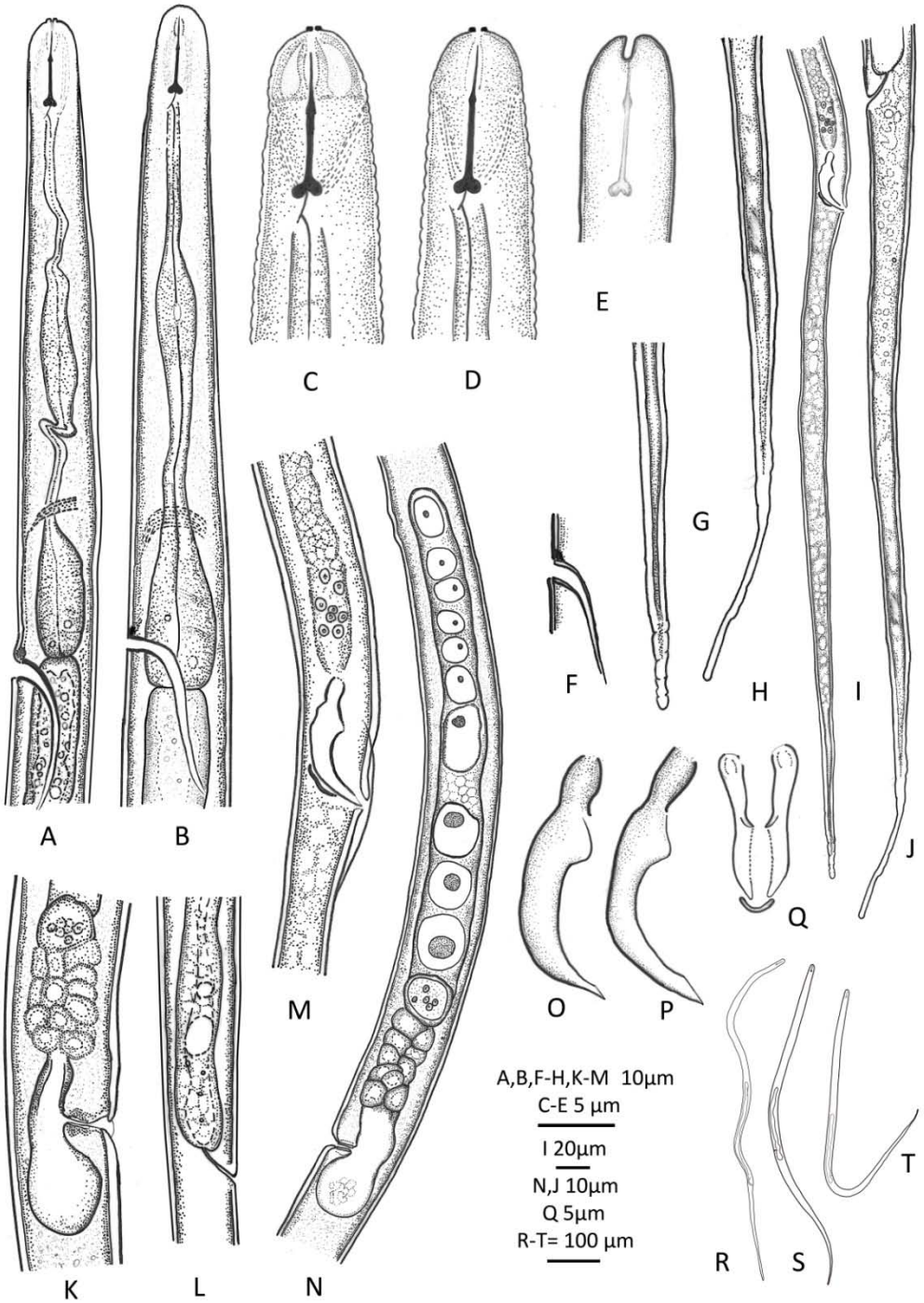

Figure 2. Line drawing of *Labrys chinensis* gen. nov., sp. nov. A, B: female anterior body. C-E: female cephalic region. F: excretory pore and sclerotized duct. G: male tail tip. H: female tail tip. I: male tail. J: female tail. K: vulva. L: anus. M: cloacal region. N: female gonad. O, P: lateral view of spicule. Q: ventral view of spicule. R: male habitus. S, T: female habitus.

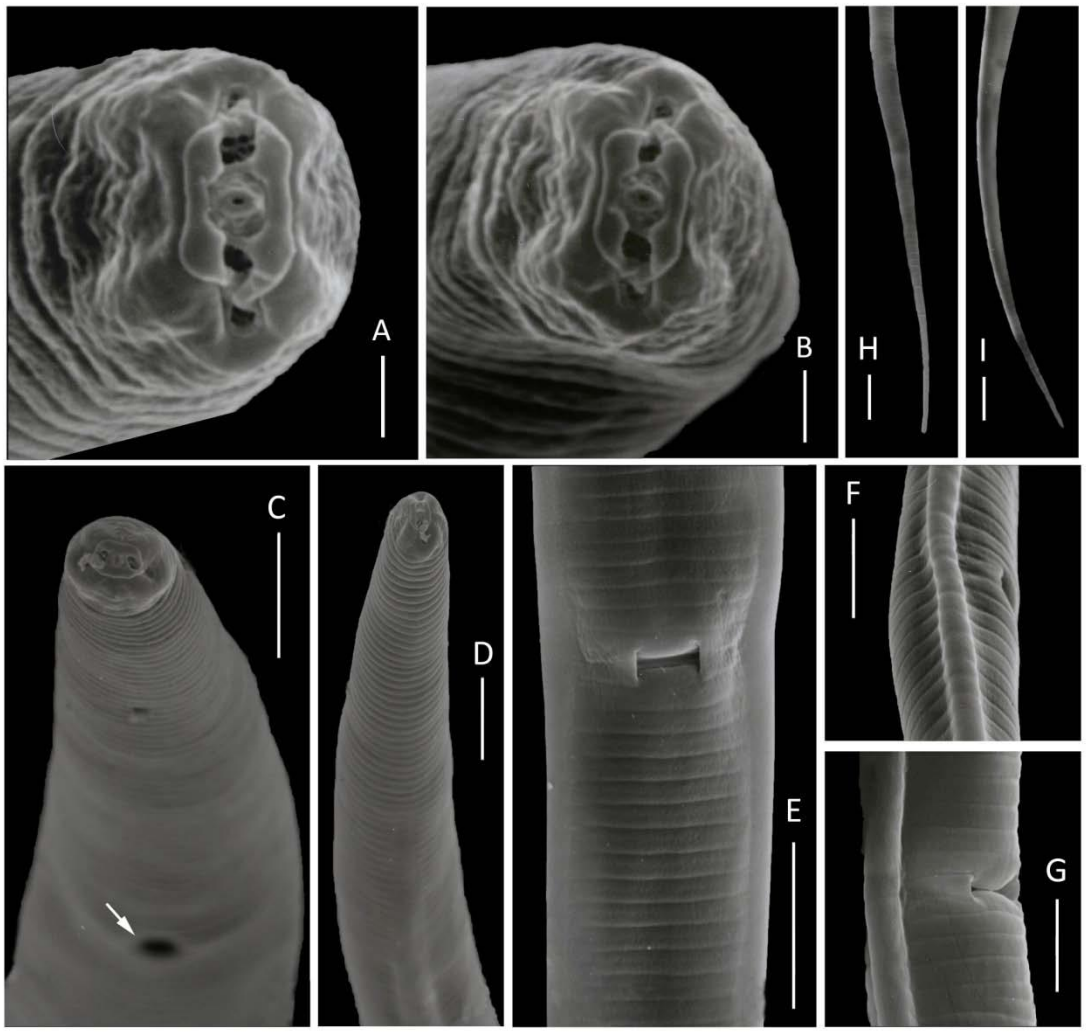

Figure 3. SEM of *Labrys chinensis* gen. nov., sp. nov.. A, B: *en face* view of cephalic region. C: ventral view of anterior body. Arrow indicates excretory pore. D: lateral view of anterior body. E: ventral view of vulva. F, G: lateral view of vulva. H, I: female tail. Scale bar: A, B=1; C, D, F, G = 5 μm; E, H, I=10 μm

Taxonomy

Labrys gen. nov.

DESCRIPTION

Same with species description.

DIAGNOSIS AND RELATIONSHIP

The new genus belongs to subfamily Tylenchinae, family Tylenchidae. It is characterized by unique labial plate that has four narrow lobes with detached tips from the adjacent cuticle, visible in LM as two small protruding lips at the anterior end (Figs 1A, B; 2C, D). Geraert and Raski (1987) attributed the Tylenchidae lip region into seven patterns and the pattern in *Labrys* gen. nov. differs from all known lip patterns in Tylenchidae and is here considered as an eighth unique pattern. Beside, the wide, laterally broad and elongated amphidial aperture (Fig. 1D), the spicule with a sharp protrusion at the blade (Fig. 1M) and round spacious PUS are also very rare in Tylenchidae. The new genus resemble genera *Allotylenchus*, *Lelenchus*, *Filenchus* and *Polenchus* in general appearances, detailed comparisons the see Table 4. Genus *Sakia* also similar to presented new genus (broad cap-like cephalic region and the sclerotized excretory duct), though its validation is still in discussions (Fortuner and Raski 1987; Geraert 2008; Husain 1972; Siddiqi 1986,2000). The type species *S. typica* Khan (1964) was described without drawings but still shows several differences: small, oval slit amphidial aperture (*vs* broad and elongated aperture, obvious from laterally view), stylet with cone equal to shaft (*vs* shaft two times more than cone), spermatheca not set-off (*vs* spermatheca set-off) and a reduced PUS (*vs* round spacious PUS). Other species belong to *Sakia* are all have the anterior end without protruding lips, spicule without sharp protrusion and different in lateral incisures (absent/four in *Sakia vs* two in *Labrys* gen. nov.).

ETYMOLOGY

The selected genus name is derived from the shape of labial plate, which resembles a symmetrical double-bitted axe, one of the famous symbols of Greek civilization.

TYPE AND ONLY SPECIES

Labrys chinensis gen. nov., sp. nov.

Labrys chinensis gen. nov., sp. nov. (Figs 1-3, Table 5).

ZooBank (zoobank.org) identifier: CE6004B6-D242-4989-9ABE-1F000FA2AEFE.

Holotype

Female, from population P1, recovered from soil underneath *Quercus aliena* from Taibai

(34° 03'40"N, 107° 41' 9.6"E), China, at an altitude of 1963 m.a.s.l. Deposited at the Ghent University Museum, Zoology Collections, collection number UGMD 104322.

Paratypes

Four females and one male paratypes collected from the same location and same sample of holotype. Deposited at the Ghent University Museum, Zoology Collection, collection number UGMD 104323, Ghent University, Belgium. Additional paratypes are available in the UGent Nematode Collection (slide UGnem-162) of the Nematology Research Unit, Department of Biology, Ghent University, Belgium.

TYPE HABITAT AND LOCALITY

Type population P1 from soil underneath *Quercus aliena* from Taibai (34° 03'40"N, 107° 41' 9.6"E), China, at an altitude of 1963 m.a.s.l. Three other populations were found in different locations in China (Table 1).

DESCRIPTION

Female: body slender, straight to ventrally arcuate. Cuticle appearing as bright lining in stereomicroscopy, smooth in LM but finely striated in SEM. Lateral field distinct, an elevated ridge forming two incisures, starts at level of metacorpus. Cephalic region rounded, continuous, framework weak, not sclerotized. Labial plate offset and constricted dorso-ventrally, forming four lobes, taping towards tip and detached from adjacent cuticle (Figs 3A, B; 4 type VIII). Labial papillae six, arranged as a circle in oral disc. Cephalic papillae invisible. Amphidial apertures broad slits, laterally elongated, confined in first annulation after labial plate, edge of apertures thicker than adjacent cuticle forming a elevated ring (Fig. 3A, B). Stylet knobbed, shaft about two times longer than cone. Dorsal pharyngeal gland orifice close to stylet base. Excretory pore wide 2.0-2.5 μm, excretory duct long, heavily sclerotized, generally at the level of pharyngo-intestinal junction. Deirids at the level of basal bulb. Hemizonid just above excretory pore. Corpus cylindroid, metacorpus elongated-fusiform, its cuticular valve weak, and gradually transiting to cylindrical isthmus. Basal bulb spindle-shaped. Vulva with small flap, one annulus wide. Vagina wall thin, perpendicular to body. Postvulval uterine sac (PUS) round, occupying full body width, one body diameter long. Female gonoduct monodelphic, prodelphic. Ovary outstretched, oocytes

in one row. Spermatheca offset, filled with spherical sperm cells. Uterus quadricolumellate, probably with five or six cells in each row.

Male: Bursa ad-cloacal, slightly crenated. Spicules with velum, proximal part of blade sharply protruding, gubernaculum simple. Tails filiform, ending in a rounded terminus.

ETYMOLOGY

Species name is given after China, where it was recovered.

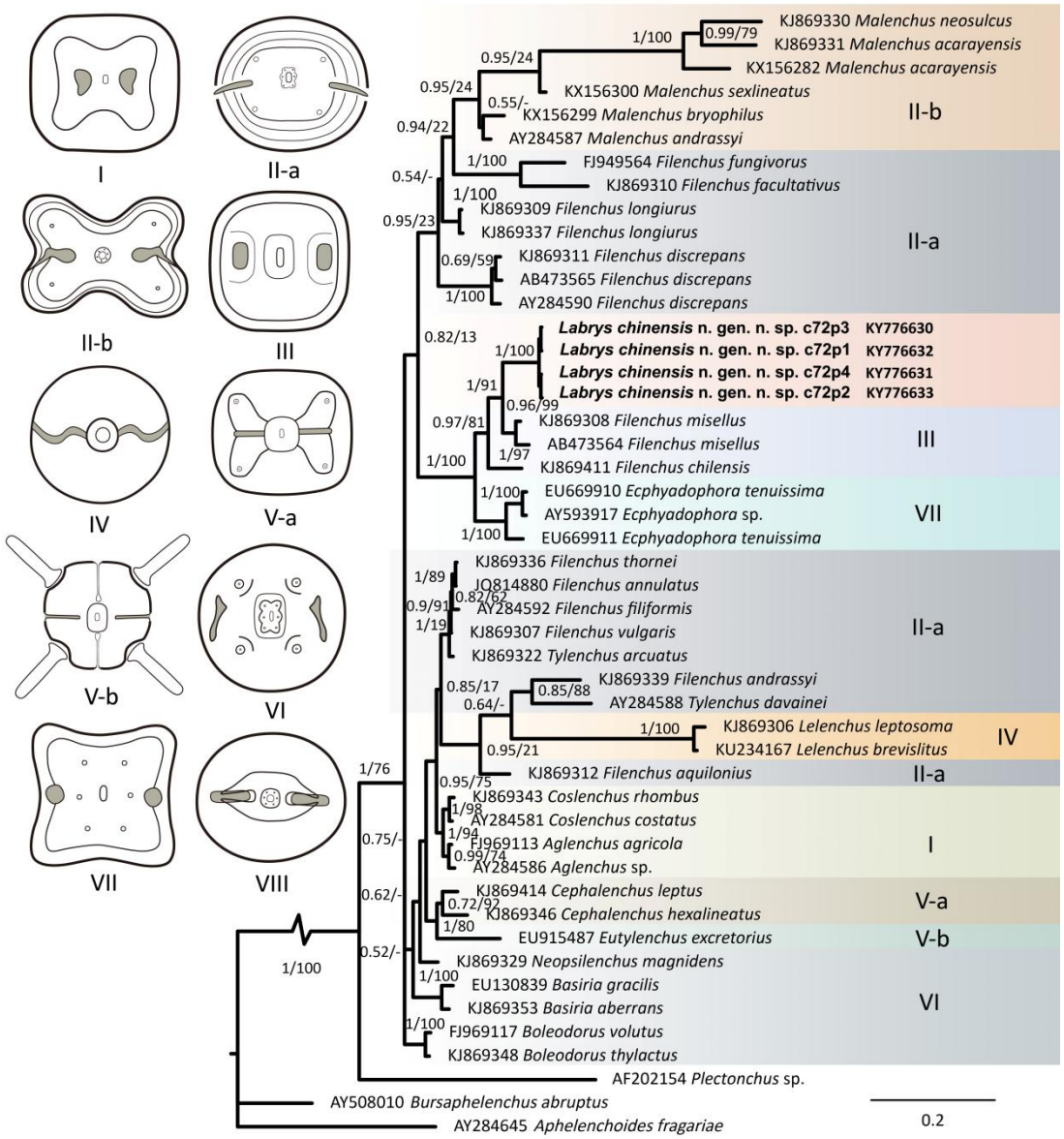

Figure 4. 50% majority rule consensus tree of Bayesian phylogeny analysis of the 18S *rRNA*. Branch supports is indicated in the following order: PP value in BI analysis/BS value from

ML analysis. Illustrations indicate lip region arrangement in each clade and codes of each type (I-VII) follows Geraert & Raski (1987). I: front plate laterally elongated, undivided, carries al1 the sensillae. The amphidial apertures are entirely within the plate; II-a: Amphidial apertures are not confined to the oral plate but continue on the lateral side as longitudinal slits. The end-on view is round to quadrangular; II-b: Similar with II-a except for a dorso-ventrally flattened end-on view; III: Slit-like amphidial apertures confined to the oral plate but the slits are dorso-ventrally directed. IV: Offset oral disc, the cephalic region is dorso-ventrally flattened. The amphidial aperture is very long and mostly sinuous, it starts at oral disc and continues longitudinally on the narrow lateral side of the cephalic region; V-a: amphidial slits start immediately at the oral disc, laterally directed but are only found on the front end of the cephalic region. The amphidial apertures are surrounded by a plate that bears the four cephalic papillae, that plate is constricted dorso-ventrally to form lobes; V-b: similar with V-a but labial plate is constricted to form a cleft and with seta; VI: Labial plate undivided, four prominent cephalic papillae dome-shaped, outside of anterior surface. Amphidial apertures start between or outside the four cephalic papillae and are simple oblique slits or have an inverted V-shape; VII: with very small pore-like amphidial apertures. The lip region of *Labrys chinensis* gen. nov., sp. nov. is different from all known type thus considered as VIII.

Table 4. Comparison of *Labrys chinensis* gen. nov. to other related genera in Tylenchidae.

Genera	Characters					
	Anterior end	Amphideal fovea	Median bulb	Flap in Vulva	Excretory duct	Spicule blade
Labrys gen. nov.	Two lips protruding	Indistinct	Elongated-fusiform	Indistinct	Sclerotized	Sharply protruding
Allotylenchus	continuous	Indistinct	Well developed	Large	Sclerotized	Less protruding
Polenchus	continuous	Indistinct	Well developed	Indistinct	Weak	Less protruding
Filenchus	continuous	Indistinct	Well developed to Elongated-fusiform	Indistinct	Weak	Less protruding
Lelenchus	continuous	Pouch-like	Elongated-fusiform	Indistinct	Weak	Less protruding

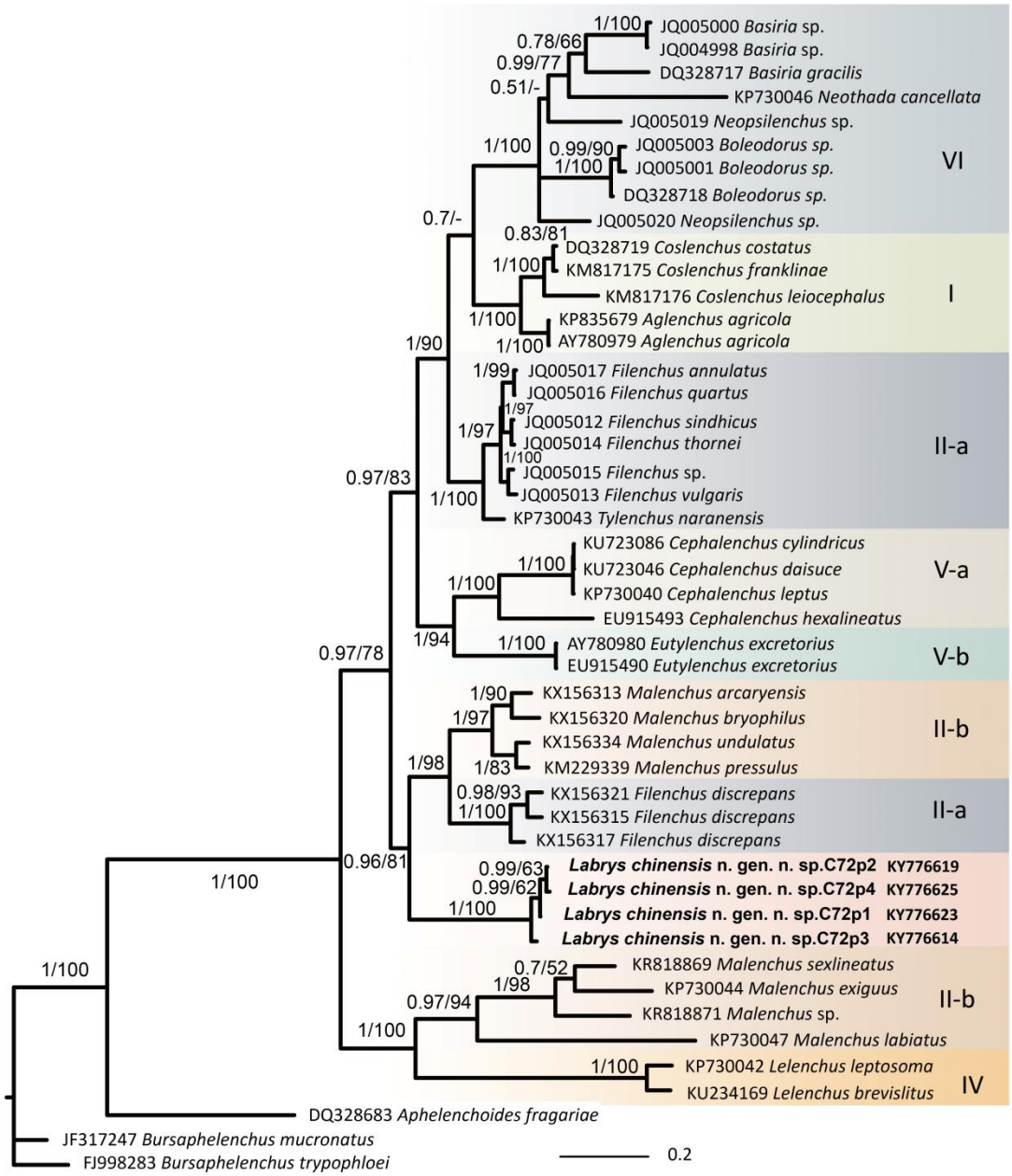

Figure 5. 50% majority rule consensus tree of Bayesian phylogeny analysis of D2D3 domain of 28S *rRNA*. Branch supports is indicated in the following order: PP value in BI analysis/BS value from ML analysis. The lip region arrangement code in each clade corresponds to Fig 4.

Table 5. Morphometric data for *Labrys chinensis* gen. nov., sp. nov. All measurements are in μm and in the form: mean ± s.d. (range).

Characters	Female		Male
	Holotype	Paratypes	Paratypes
n	-	9	4
L	636	616±24 (580-637)	611±12 (602-627)

a	41.6	44.1±3.9 (37.7-48.7)	35.1±1.6 (33.7-37.4)
b	6.9	6.6±0.6 (5.4-7.3)	6.2±0.38 (5.6-6.5)
c	3.8	3.8±0.23 (3.5-4.4)	3.6±0.20 (3.4-3.8)
c’	17.3	18.0±2.1 (13.3-20.3)	17.6±2.9 (14.8-21.5)
V	56.5	56.6±1.5 (53.9-59.8)	-
V’	76.5	77.1±1.0 (75.0-78.1)	-
Tail length/vulva-anus distance	1.5	1.6±0.12 (1.3-1.7)	-
Body diam.	15	14±1.3 (12-16)	17±0.91 (16-19)
Stylet	8.5	9.3±0.42 (8.9-10)	9.4±0.54 (8.7-10)
MB	49	43±4.2 (38-51)	44±1.2 (44-46)
Excretory pore to anterior end	90	84±4.0 (76-87)	80±3.8 (76-84)
Excretory duct length	19	20±4.1 (15-25)	22±3.3 (18-26)
Pharynx	92	95±9.0 (82-108)	99±5.0 (96-106)
Nerve ring	67	69±8.8 (58-89)	70±4.8 (64-76)
Anus width/ cloacal width	9.6	9.2±0.83 (8.3-11)	9.7±0.87 (8.6-11)
Spicule	-	-	16±1.3 (14-17)
Post-uterine sac/gubernaculum	15	15±1.2 (13-17)	4.8±0.45 (4.4-5.4)
Tail	166	164±12 (132-171)	169±13 (159-186)

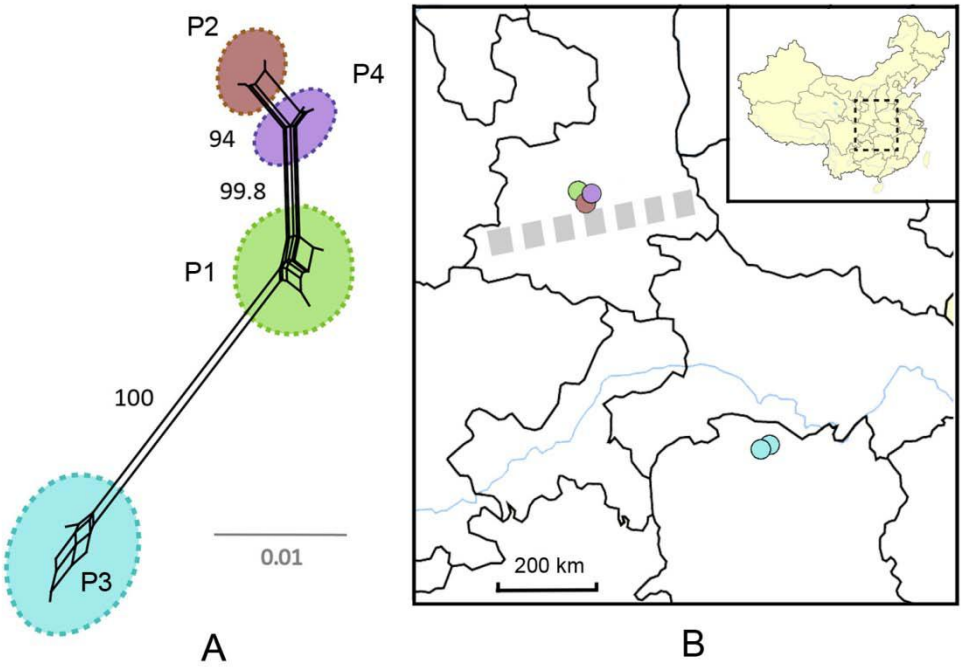

Figure 6. Phylogenetic network applied to four *Labrys chinensis* gen. nov., sp. nov. populations and their geographic distributions. A: Phylogenetic network applied to four parsimoniously informative (PI) sites using the Neighbor-net algorithm. Bootstrap values are indicated between populations. B: Geographic distribution. The black broking line upper right indicates the location in China. Wide grey dashed line in the main map represents the Qinling

Mountains.

Discussion

Currently, Tylenchidae consists of 44 genera (42 genera listed by Geraert (2008) one new recently described by Yaghoubi et al. (2016) and one by this study). In this study integrative approaches were used to describe *Labrys chinensis* gen. nov., sp. nov. Although the phylogeny of Tylenchidae remains unresolved, the results extended its diversity and highlighted the importance of detailed morphological analyses in such taxonomically difficult group. Given many of generic characters are only obvious in SEM (*e.g.* Labial patterns), the new species description should not solely based on LM.

Although 3D printing technology has been around since the 1980s, it has only recently gained real momentum as a technique as the technology matures and awareness grows. Driven by new applications, the "printable" category keeps expanding into many fields such as medicine, architecture, education, fashion, manufacturing and food (Lombardi et al. 2014; Murphy and Atala 2014; Petrick and Simpson 2013; Qing et al. 2015; Sun et al. 2015; Thomas et al. 2016). Within zoology, it has already been showing great potential in functional morphology, pest detection, anatomy and physiology (Domingue et al. 2015; Greco et al. 2014; Igic et al. 2015; Porter et al. 2015; Thomas et al. 2016). Here we extend the application of 3D printing in the field of taxonomy and describe for the first time a new taxon together with a printed model (Fig.7). Although the accuracy of our models is not comparable to 3D reconstruction based on serial TEM (Transmission Electron Microscopy) sections or electron tomography techniques, the models are useful and time-efficient complements' to pictures and drawings of species descriptions to illustrate complex 3D structures. Future taxonomical applications can also be extended to virtual reality approaches that allow observation and dissection without damaging precious specimens, which represents a promising direction for both taxonomy and education. Therefore, as we add 3D printing to the toolkit of taxonomical research, we also underline the relevance of its development as a synergic discipline link of frontier zoology research and zoological education.

In this study we also experienced the importance of selecting the optimal printing materials to achieve model quality. ABS and PLA are two effective commercial materials that combine mechanically desirable performance and low cost, whereas resin is considered a more advanced material that delivers the highest quality output but at a much higher price. Our tests based on four taxa reveal that thermoplastic polymers ABS and PLA gave similarly acceptable coarse surfaces in 8 cm scale, while all labial details were completely lost in 4cm print (see http://www.nematodes.myspecies.info). Conversely, resin provides highly resolved details, *i.e.* all papillae are clearly visible, even when model size is reduced to 4 cm (Fig. 7I-P). Therefore such high quality in small size print can compensate for the less competitive price of resin (usually 1.5-2 times that of PLA). Moreover, resin can be printed in light color, semi-opaque color or even transparent which facilities the visibility of internal structures. In conclusion, resin is highly recommended for zoological anatomy education and research while PLA/ABS is also useful but only for larger print size (8 cm or more).

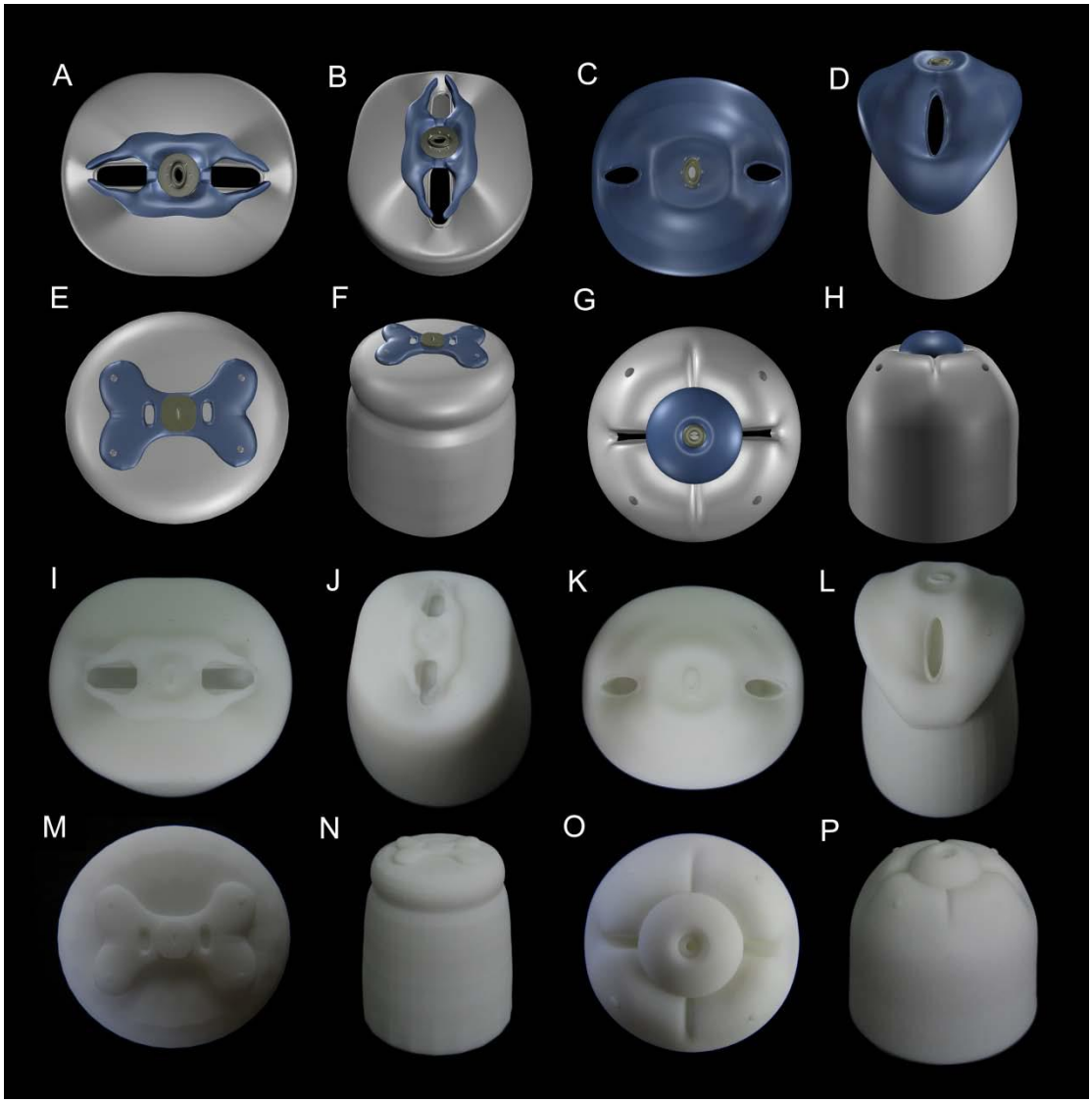

Figure 7. 3D models (A-H) and printed resin models (I-P) of representatives of other genera in Tylenchidae. A, B, I, J: *Labrys chinensis* gen. nov., sp. nov.; C, D, K, L: *Cucullitylenchus* sp.; E, F, M, N: *Cephalenchus* sp.; G, H, O, P: *Tylodorus* sp. All models were printed by white resin in a height of 4 cm, performances of other materials see: http://www.nematodes.myspecies.info. Models are freely available to download from: www.thingiverse.com.

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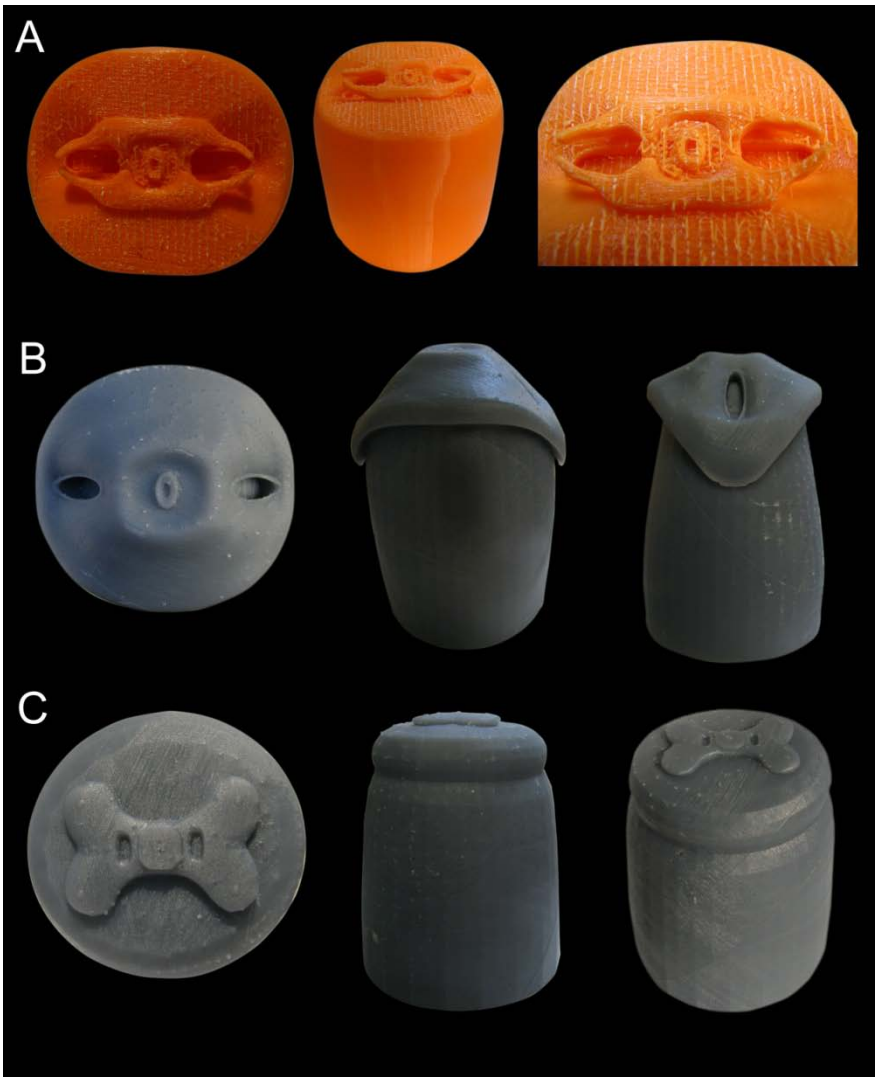

Fig. S1 The Comparsion of models printed by PLA (A) using FDM (fused deposition modeling) method and resin (B, C) using stereolithography method. A: cephalic region of

Labrys gen. nov. B: cephalic region of genus *Cucullitylenchus*. C: cephalic region of genus *Cephalenchus*.

Chapter VI

A new species of *Malenchus* (Nematoda: Tylenchomorpha) with an updated phylogeny of the family Tylenchidae

Chapter modified from:

Qing X.[1], Pereira T.[2], Slos D.[1], Couvreur M. [1], Bert W. [1] A new species of *Malenchus* (Nematoda: Tylenchomorpha) with an updated phylogeny of the family Tylenchidae. Submitted to *Invertbrate Systematics*

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Abstract

The Tylenchidae family is one of most abundant and diverse nematode groups found in soil habitats. However, little is known with respect to its diversity and phylogeny. In this study an unusual new species *Malenchus cylindricus* sp. nov. was described based on light microscopy and scanning electron microscopy and molecular data were based on 18S and 28S of rRNA. The new species is characterized by elongate-cylindrical vulva-anus body shape and a narrow annulation. We updated the phylogeny of family Tylenchidae by adding first molecular data for the rare genera *Miculenchus* and *Tenunemellus* and new morphological data for genus *Lelenchus*. Additionally, we compared the effect of alignment methods on the tree topologies and supportive values to minimize any bias introduced by problematic molecular data. The results show that Tylenchidae phylogeny remains unresolved. The rare genera are molecularly and morphologically divergent from other Tylenchidae species and thus the position of subfamily Ecphyadophorinae needed to be discussed. The comparison of alignment methods suggests phylogenies inferred from sequence-based alignment are more similar but differs from secondary structure-aided methods and supportive values that not agree with each other can be reconciled by proper selection of alignment method.

Additional Keywords: *Miculenchus*, *Tenunemellus*, *Lelenchus*, *Ecphyadophora*, *Ecphyadophoroides*, Ecphyadophorinae

Introduction

The Tylenchidae family is one of most abundant and diverse nematode groups found in soil habitats, where they may represent up to 30% of the nematode abundance in any given soil sample. However, little is known with respect to its phylogeny. Owing to their relatively short and weak stylet, they are considered as weak root feeders of higher plants or parasitizing lichens and mosses, although few species have been proven to feed on fungi. Current phylogenetic analyses based on DNA sequences of the 18S and 28S ribosomal RNA (rRNA) genes have shown that Tylenchidae is not monophyletic (Fig. 1). In particular, diverse Tylenchidae genera such as *Filenchus* and *Malenchus* might include species representing very divergent lineages in the Tylenchomorpha phylogeny. Uncertainty regarding the phylogenetic position of many Tylenchidae taxa underscores the need to reevaluate classical morphology-based systems in Tylenchomorpha.

Within Tylenchidae, the genus *Malenchus* is the second most specious (after *Filenchus*) with 36 valid species. Recently, *Malenchus* was recognized as polyphyletic and monophyletic on the basis of the 28S and 18S genes of the ribosomal RNA (rRNA), respectively. As a result, the generic definition of *Malenchus* was recently amended in light of molecular and morphological evidence. Instead of using the presence of a coarsely annulated cuticle and markedly tapering body posterior to the position of the vulva, the new definition appointed the offset lateral region with small sub-ridges, pouch-like amphidial fovea and vagina with swollen wall to be generically important.

In this study, a new species of *Malenchus* is discovered that is exemplary for the new *Malenchus* definition (Qing and Bert, 2017). This puzzling species that resembles both *Malenchus* and *Filenchus* is herein descried based on morphological and molecular data. Moreover, three rare genera of Tylenchidae *viz*. *Miculenchus*, *Tenunemellus* and *Lelenchus*, which are not commonly found in soil samples, especially in comparison with cosmopolitan genera such as *Filenchus* and *Malenchus*, were examined. These genera are presumed to be related to *Malenchus*, but molecular data are either absent or misleading. Detailed morphology of all studied nematode species were based on light microscopy (LM) and

scanning electron microscopy (SEM), and molecular data were based on 18S and 28S of rRNA. Additionally, we compared the effect of alignment methods on the tree topologies and supportive values to minimize any bias introduced by problematic molecular data in Tylenchidae (i.e. coverage limitations and/or high nucleotide substitution rate coverage).

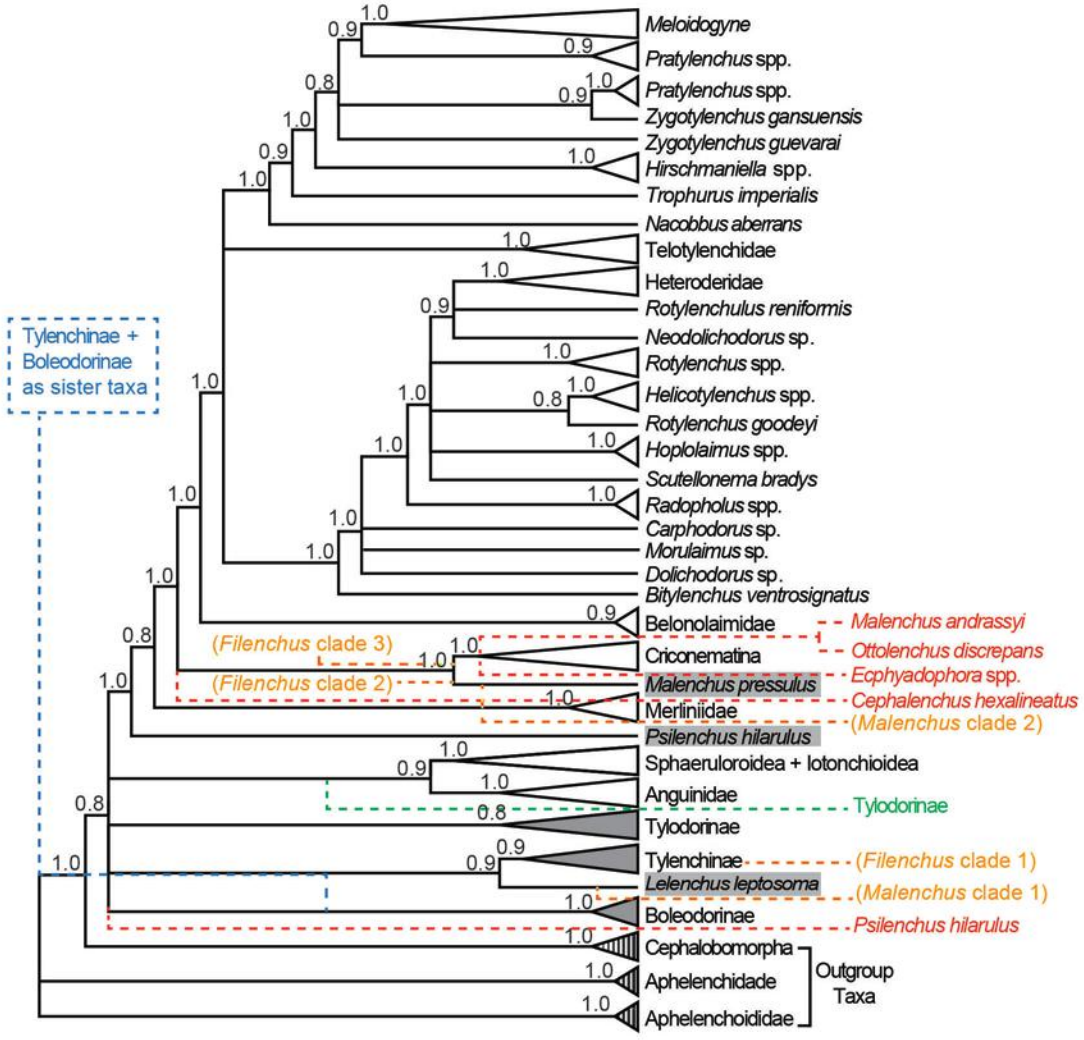

Fig 1. Overview of Tylenchomorpha phylogeny based on a concatenated analysis of the 18S and 28S rRNA genes (adapted from Pereira *et al.*, 2017). The 80% majority rule consensus tree from the Bayesian analysis is presented. BPP ≥ 80% are given for appropriate clades. Tylenchidae taxa as defined in Geraert (2008) are highlighted in grey. Colored dashed lines indicate conflicting positions of some Tylenchidae taxa according to different studies (Blue: based on 28S rRNA and supported in Subbotin *et al.* (2006), Palomares-Rius *et al.* (2009),

Atighi *et al.* (2013); Red: based on 18S rRNA and supported in Bert *et al.* (2008), Holterman *et al.* (2009), and Van Megen *et al.* (2009); Green: based on 18S and 28S rRNA genes and supported in Palomares-Rius *et al.* (2009) and Pereira *et al.* (2017); Yellow: Based on 18S and 28S rRNA genes and supported in Qing *et al.* (2017).

Materials and methods

Sampling and isolation of nematode specimens

Nematodes were extracted from soil samples using a Baermann tray and concentrated using a 400-mesh sieve (37 µm opening). Samples collected outside Belgium (i.e. China and Mexico) were divided into two parts and fixed with 4% formalin and DESS solution for morphological analyses and molecular analyses, respectively.

Morphological analyses

Formalin fixed specimens were rinsed several times with deionised water and gradually transferred to anhydrous glycerin for permanent slides, following the methods of Seinhorst (1962) as modified by Sohlenius and Sandor (1987). Observations and drawings were made with an Olympus BX51 microscope (Olympus Optical, Tokyo, Japan) equipped with differential interference contrast (DIC). Digital vouchers including LM images and multifocal videos were captured from nematode specimens used for molecular procedures with a Nikon DS-FI2 camera (Nikon Corporation, Tokyo, Japan). Digital specimen vouchers are available at http://nematodes.myspecies.info. Female reproductive system was extracted and examined based on the methods of Geraert (1973) and Bert *et al.* (2008). Illustrations were prepared using Adobe Illustrator CS3 and LM drawings.

For Scanning Electron Microscopy (SEM), fresh specimens were heated in a microwave in Trump's fixative (2% paraformaldehyde + 2.5% glutaraldehyde in a 0.1 M Sorenson buffer) for a few seconds. Specimens were subsequently washed three times in double-distilled water and dehydrated through a series of graded ethanol (*e.g.* 30, 50, 75, 95%, 20 min each) and 3x in 100% ethanol (10 min each). Specimens were critical point-dried with liquid CO_2, mounted

on stubs with carbon discs and coated with gold (25 nm) before observation with a JSM-840 EM (JEOL, Tokyo, Japan) at 15 kV.

Molecular and Phylogenetic analyses

DNA extraction, amplification, and sequencing: DNA was extracted from fresh or DESS preserved nematode specimens. Briefly, single individuals were transferred to a PCR tube with a solution containing 10 µl NaOH and 1µl Tween20, incubated for 15 min at 95°C then 40 µl of double-distilled water was added to each sample. The D2-D3 domains of 28S rRNA were amplified with primers D2A and D3B. The 18S rRNA gene was amplified using primers SSU 18A and SSU 26R. PCR reactions were carried out under following conditions: 30 s at 94 °C, 30 s at 54 °C and 2 min at 72 °C for 40 cycles.

DNA sequences representing *Tenunemellus*, *Miculenchus* and the new species of *Malenchus* were analysed together with additional Tylenchidae sequences in GenBank. Multiple alignments form both rRNA genes were made using the G-INS-i algorithm of MAFFT v. 7.205. To evaluate contradict node support values (see discussion) and minimize alignment-introduced errors, we preformed additional alignments with three methods: Muscle which is solely based the nucleotide sequences, Q-INS-i algorithm in MAFFT which use the four-way consistency objective function for incorporating secondary structure information, and RNAsalsa 1.4.2 which produce structure-based alignment taking both thermodynamic and compensatory/consistent substitutions into considerations. The consensus structure was predicted by PPfold and input structure acceptance level was set to 100% in RNAsalsa. To compare trees topology and branch length difference among different methods, we calculated Robinson-Foulds (RF) and Kuhner-Felsenstein (KF) distances in R version 3.25 (R Development Core Team) using the package Phangorn.

For phylogeny reconstruction, the best-fitting substitution model was estimated using AIC (Akaike Information Criterion) in jModelTest v. 2.1.2. Maximum Likelihood (ML) and Bayesian Inference (BI) analyses were performed on the CIPRES Science Gateway, using RAxML 8.1.11 and MrBayes 3.2.3 respectively. ML analysis included 1000 bootstrap (BS) replicates under the GTRCAT model. Bayesian phylogenetic analysis was carried out using

the GTR+I+G model with four independent chains for 1×10^7 generations in two runs. Markov chains were sampled every 100 generations and 25% of the converged runs were discarded as burnin. All alignments and phylogenetic trees are available at TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S21606)

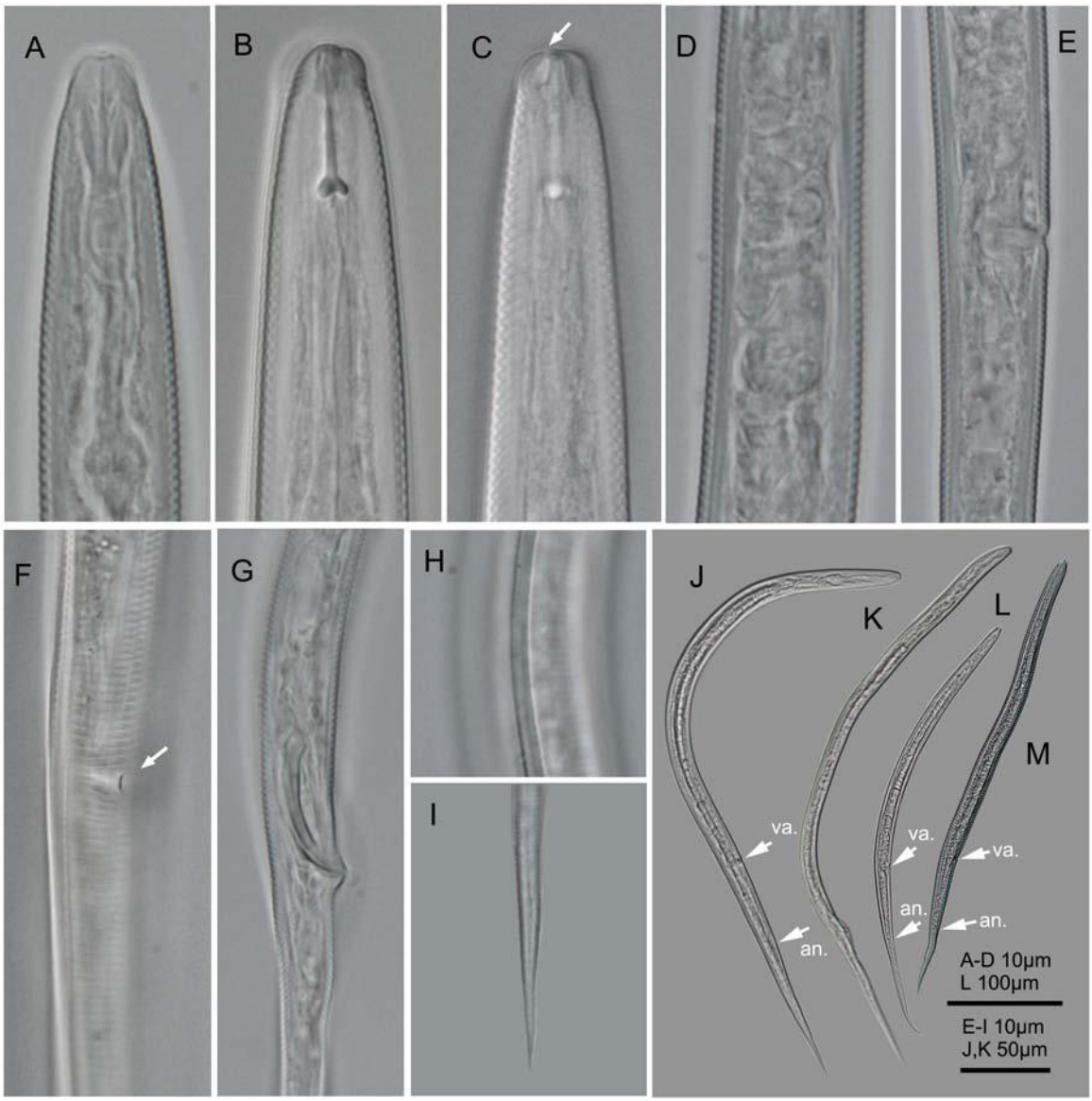

Fig. 2 LM micrographs of *Malenchus cylindricus* sp. nov. (A-K), *M. exiguus* (L) and *M. pachycephalus* (M). A, D-K: specimens in permanent glycerin slide; B, C: fresh specimens; A-C: female anterior body region. Arrow indicates amphidial fovea; D: female middle body; E, F: vulva region. Arrow indicates lateral view of vulva opening; G: spicule and gubernaculum; H: Lateral field in vulva region; I: female tail end; J: female habitus with elongate-cylindrical vulva-anus body; K: male habitus. L, M: examples of typical female

body shape in *Malenchus* markedly tapered posterior to the vulva. For additional comparisons, readers are referred to Fig.2 in Qing and Bert (2017). va.= vulva, an.=anus.

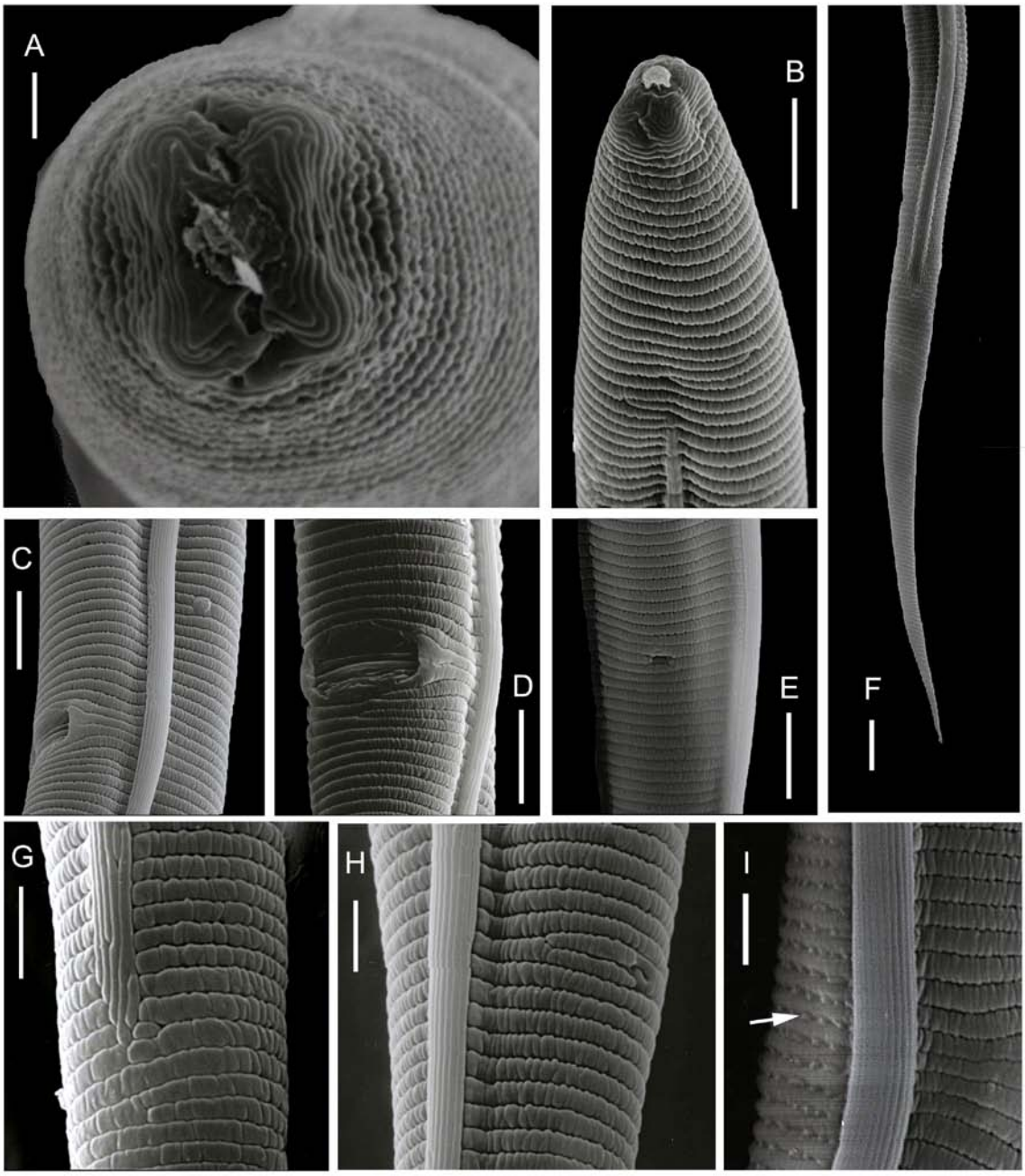

Fig. 3 SEM micrographs of female *Malenchus cylindricus* sp. nov. A: *en face* view. B: lateral view of anterior body region. C, D: vulva region. E: ventral view of anus. F: tail region. G: posterior end of lateral field. H: lateral view of anus region, showing the longitudinal striae in cuticle. I: lateral field with small ridges. Arrow indicates particles between annuli. Scale bar: A=1, B-F=5μm, G-I=2μm.

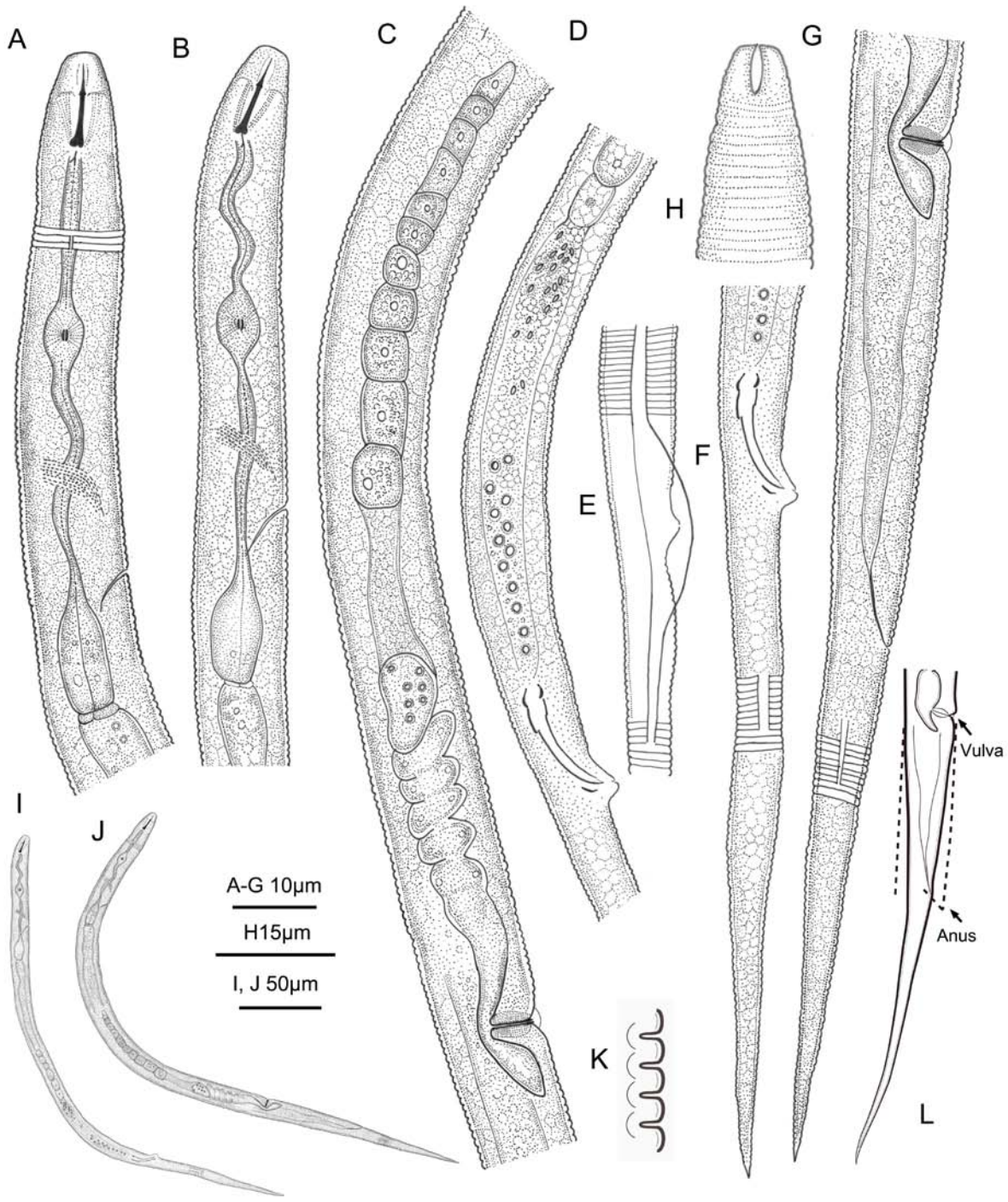

Fig. 4 Line drawing of *Malenchus cylindricus* sp. nov. A, C, G, H, J: female; B, D-F, I: male. A, B: anterior body region; C, D: reproductive system; E: bursa; F, G: tail; H: head region showing amphidial fovea; I, J: body habitus; K: diagrammatic representation of cuticle annulations; L: diagrammatic representation of an elongate-cylindrical (broken lines) *vs* markedly tapered vulva-anus body shape (solid lines), the latter was proposed by Siddiqi (1979) as a generic character for *Malenchus*.

Result and discussion

Malenchus cylindricus sp. nov. Qing and Bert, 2017
=*Malenchus* sp. C163. Qing *et al.* 2017a, 2017b

(Figs 2, 3, 4; Table 1)

http://zoobank.org/urn:lsid:zoobank.org:act:26D037F3-3977-4DEB-9C7D-55B402A703B1.

Material examined

Type habitat and locality. Recovered from moss (*Eurhynchium* sp.) rhizosphere in the bank of several small streams, Poeke, Belgium. GPS coordinates: N 51°02'35.4" E 3°26'56.3". The cuticle surface is associated with particles as observed on LM and confirmed by SEM (Fig. 3I), which may be unknown bacteria or fungi, a similar association was also reported from *M. pachycephalus*.

Type materials. Holotype female, four female paratypes and one male paratype were deposited at the Ghent University Museum, Zoology Collections (Collection numbers UGMD104320 and UGMD104321), Ghent University, Ghent, Belgium. Additional paratypes are available in the UGent Nematode Collection (slide UGnem144) of the Nematology Research Unit, Department of Biology, Ghent University, Ghent, Belgium.

Description

Measurements. See Table 1

Female. Body size small and mostly ventrally arcuate. Vulva-anus body shape elongate-cylindrical (EC). Cuticle slightly folded between annuli as seen in permanent mount (Fig. 2A), although it appears smoother (and similar to the annulation pattern in *Filenchus*) in fresh specimen (Fig. 2B, 2C). Longitudinal striae in the cuticle can only be observed with SEM (Fig. 3A, 3B, 3G, 3H) but not in LM. Annulations narrow (0.85-1.1µm), groove (the depression between two annuli) hardly visible in LM, with indistinct folded part (type 1, Qing and Bert (2017)). Lateral field prominent, starting at half of procorpus and ending at middle of tail, with 12 incisures (or less at the most anterior and posterior body regions) in an

elevated ridge with smooth margin (Figs. 2H, 3C, 3G-I). Lip region elevated, dorso-ventrally compressed. Amphidial apertures S-shaped, starting at the labial plate with wide hole and extend until the third annuli. Labial framework weak. Stylet slender and delicate, cone about one third of total stylet length and much narrower (except for the cone base which slightly wider) than adjacent shaft. Stylet knobs oblong, posteriorly directed. Dorsal pharyngeal gland orifice (DGO) at base of stylet knob. Median bulb oval and weakly developed, sclerotized, and valve present. Isthmus long and slender. Terminal bulb short and pyriform. Excretory pore located at the level of anterior end of the pharyngeal bulb. Hemizonid not visible. Deirids at the level of excretory pore. Reproductive system monodelphic, prodelphic, ovary outstretched with oocytes arranged in a single row. Spermatheca rounded to elongated, offset, sperm globular. Crustaformeria with five cells in each row. Uterus sac spacious with thickened wall, egg not observed (not gravid). Vulva sunken in body contour, vulval lateral flaps present, two to three annuli long. Epiptygmata present. Vagina perpendicular to body with swollen vaginal wall. Prophasmid prominent, 9-13 annuli anterior to vulva (Fig. 3C). Tail tapering gradually to more or less pointed hook-shaped tip.

Male. Less frequent than females. General morphology similar to that of female except genitals and a more slender body. Testis single, outstretched, located along ventral side of the body. Spermatogonia in one row, spermatids few, hardly visible, spermatozoa round, filling proximal part of vesicula seminalis. Vas deferens clearly differentiated. Tail straight, ventrally directed. Cloacal opening on prominent cone with small lips. Bursa short but prominent, adanal, starting at the level of spicules' capitulum and ending at 1/5 to 1/4 of the tail. Spicules slightly bent ventrally but more straight in distal part, capitulum part rounded, shaft and blade slightly tapering. Gubernaculum short and very thin.

Etymology

The new species is name after its typical EC vulva-anus body shape.

Diagnosis and relationships

Recent studies have suggested that ridging of the lateral field, presence of distinct

amphidial fovea and vaginal wall swelling are informative traits that distinguish *Malenchus* (Qing *et al*,. 2017; Qing and Bert, 2017). Therefore, the new species is assigned to the genus *Malenchus* based on a combination of these morphological characters.

The morphological and morphometric differential traits of *M. cylindricus* sp. nov. and related species (species with smooth annulations or with EC vulva-anus body shape) are given in Table 2. This new species is unique in *Malenchus* by the fact that it resembles *Filenchus* spp. and *Ottolenchus* spp., including a similar body shape, a cuticle with relatively smooth annulations, an EC vulva-anus body shape (instead of being markedly tapered, which is a generic character for *Malenchus* as proposed by Siddiqi (1979) (Fig. 4L). Along with the presence of relatively smooth annulations and EC vulva-anus body shape, the new species also differs from other morphologically similar *Malenchus* species based on morphometrical data (Table 2). The annulation and body shape can be misleading when identifying new species as *Malenchus*, especially under LM. However, *Malenchus* can be well separated (with or without SEM) by the revised generic delimitation characters proposed by Qing *et al.* (2017), *i.e.* the aforementioned distinct amphidial fovea, lateral region with small ridges and swollen vagina.

Duosulcius and *Zanenchus* are either considered valid genera within Tylenchidae or synonymies of *Filenchu*s. Based on their general body appearance, intermediate cuticle annulation (*i.e.*, coarser than most of *Filenchus* spp. but weaker than *Malenchus* spp.), EC vulva-anus body shape and two lateral incisures as observed under LM, *M. cylindricus* sp. nov. is similar to the former genera. However, a prominent S-shaped amphidial aperture (*vs* indistinct in *Duosulcius* and *Zanenchus*) and the presence of a developed (*vs* absent or reduced) post uterus sac (PUS) differentiate the new species from those in *Duosulcius* and *Zanenchus* (Table 2).

Phylogenetic placements

In this study, phylogenetic analyses included only taxa traditionally (i.e. based on morphology) classified as Tylenchidae. Although Tylenchidae is herein recognized as not monophyletic (Fig.2), this strategy of using solely Tylenchidae taxa allows for a faster

comparison among the diverse tested alignment methods. In addition, molecular divergence among Tylenchidae representatives can be further and more straightforwardly quantified.

Tree topologies inferred by ML and BI were largely congruent with Qing *et al*. (2017). Bootstrap values (BS) and posterior probabilities (PP) are summarized on the Bayesian consensus tree reconstructed from G-INS-I alignment (Figs 5, 6). In all analyses, based on different alignment methods, the genus *Malenchus* is either monophyletic (18S, PP=99, BS=30) or split into two well supported clades: *Malenchus* clade 1 (28S, PP=1, BS=99) and *Malenchus* clade 2 (28S, PP=0.9, BS=87). Similarly, *M. cylindricus* sp. nov. is either sister to *M. undulatus* (18S, PP=0.75, BS=64) or to *M. acarayensis* and *M. bryophilus* in clade 1 (28S, PP=1, BS=87).

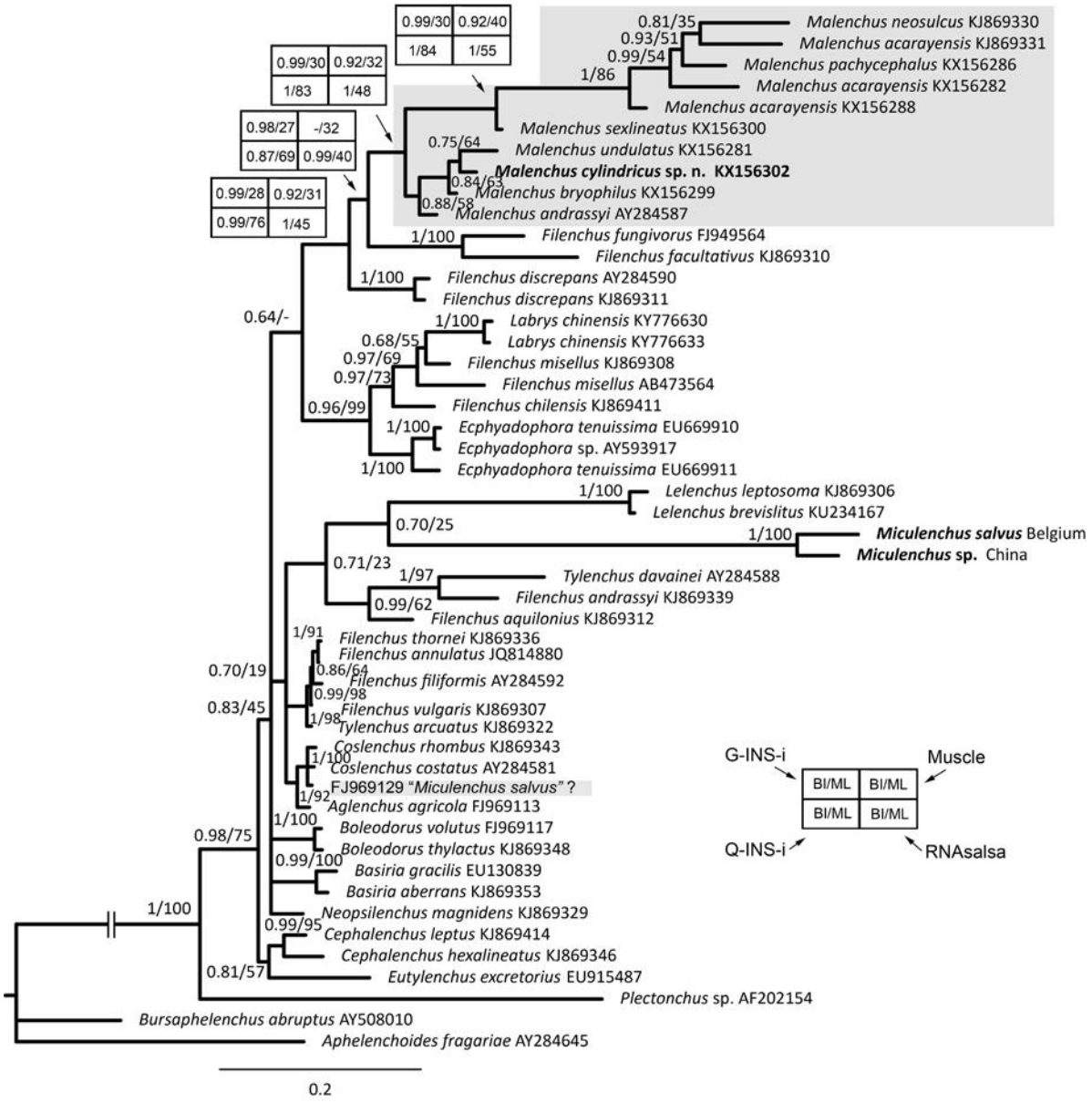

Fig 5. Bayesian 50% majority-rule consensus tree interfered on 18S rRNA aligned with sequence-based method G-INS-I implemented in MAFFT. The section's order of support value: PP/BS. The new species and new sequences original to this study are indicated in bold. Alternative support values from different alignment methods are listed in boxes at the node when the support values of ML and BI analysis are not in agreement (PP>0.98, BS<50 or PP<0.7, BS>80).

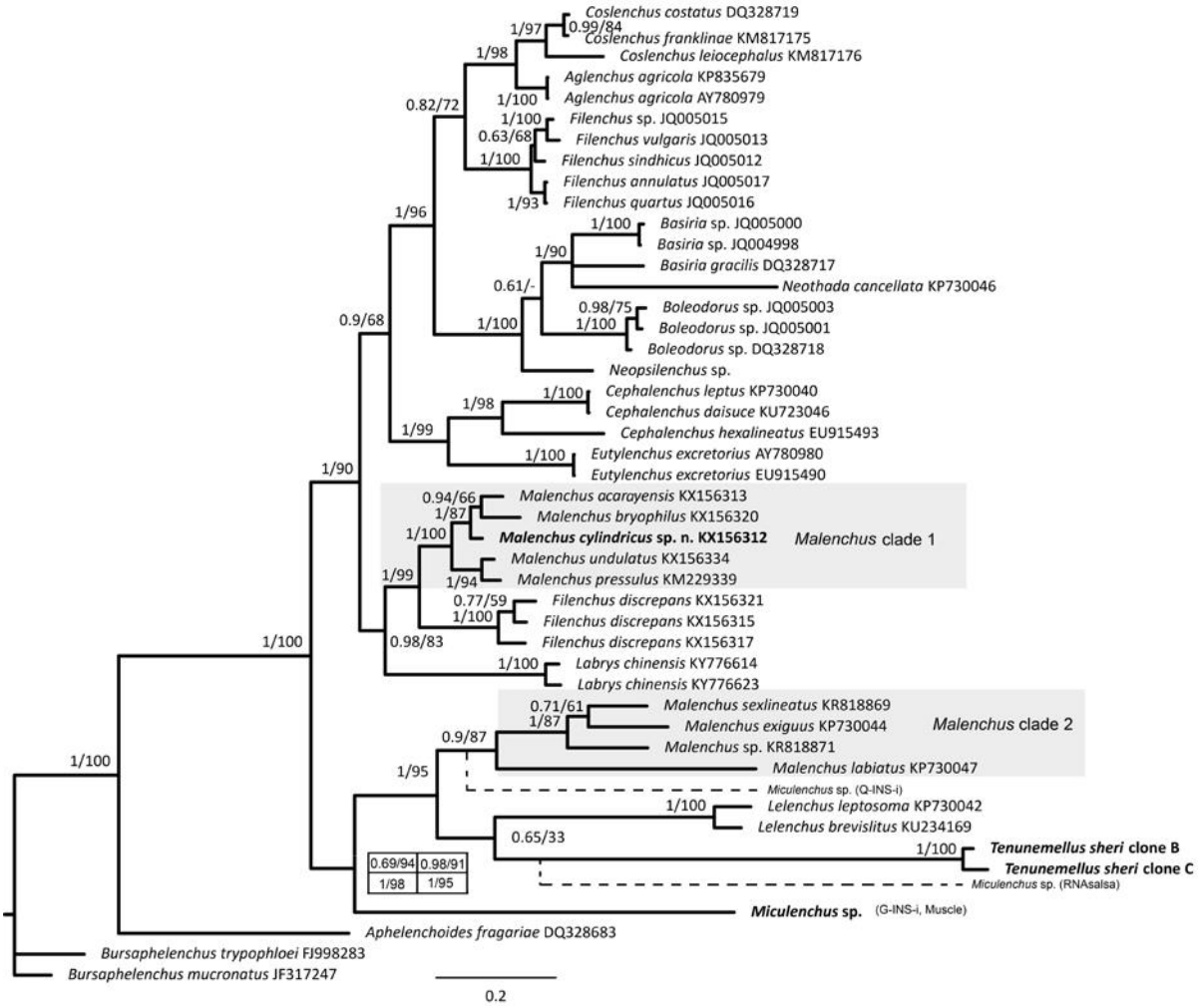

Fig 6. Bayesian 50% majority-rule consensus tree interfered on 28S rRNA aligned with sequence-based method G-INS-I implemented in MAFFT. The section's order of support value: PP/BS. The new species and new sequences original to this study are indicated in bold. Alternative support values from different alignment methods are list in boxes at the node when the support values of ML and BI analysis are not in agreement (PP>0.98, BS<50 or PP<0.7, BS>80). Dashed lines show alternative placement of *Miculenchus* from different alignment methods (methods indicated in brackets).

Table 1. Morphometric data for *Tenunemellus sheri* and *Malenchus cylindricus* sp. n. All measurements are in μm and in the form: mean ± s.d. (range).

	Tenunemellus sheri	*Malenchus cylindricus* sp. n.		
	Female	Holotype	Paratype	
n	7♀♀	1♀	7♀♀	3♂
L	808±37 (750-851)	386	381±26 (353-427)	341±5.8 (334-344)
a	94±9.0 (79-103)	27	29±2.9 (26-34)	34±2.9 (31-37)

b	6.3±0.27 (5.9-6.8)	4.7	4.6±0.19 (4.4-4.9)	4.3±0.11 (4.1-4.4)
c	3.6±0.17 (3.0-3.8)	5.6	4.9±0.43 (4.4-5.6)	4±0.02 (4.0-4.1)
c’	45±3.9 (38-49)	8.9	10±1.3 (8.9-12)	10±0.16 (10-10)
V	58±0.73 (57-59)	66	65±1.2(63-66)	-
V’	81±3.3 (78 -87)	80	79±6.4(66-83)	-
Tail /vulva-anus	1.9±0.33 (1.7-2.5)	1.1	1.2±0.19 (1.0-1.4)	-
Body diam.	8.6±0.50 (8.1-9.5)	14	13±1.5 (11-15)	10±0.71(9.3-11)
Stylet	8.7±0.22 (8.5-9.1)	9.8	10±0.42 (9.8-11)	10±0.11 (10-10)
MB	50±3.0 (47-55)	46	47±1.1 (46-49)	49±1.4 (48-50)
Excretory pore to anterior end	93±7.2 (81-100)	67	66±2.4 (62 -69)	54±4 (51-59)
Pharynx	127±3.6 (123-134)	83	82±2.8 (78-86)	80±1.1 (79-81)
Nerve ring	71±4.9 (67-81)	57	56±2.4 (54-61)	48±1.3 (47-49)
Annuli width	1.1±0.3(0.6-1.5)	1.0	0.98±0.1 (0.85-1.1)	0.90±0.06 (0.83-0.95)
Vulva/ spicule	470±12 (458-489)	255	248±19 (222-279)	17±0.15 (16-17)
Anus/ cloacal	5.0±0.51 (4.4-6.0)	7.7	7.6±0.54 (6.9-8.2)	8.3±0.12 (8.2-8.4)
PUS/gubernaculum	8.0±0.83 (6.4-8.7)	7.3	7.3±1.1 (6.11-8.2)	5.7±0.74 (4.9-6.2)
Tail	222±15 (205-247)	69	79±7.9 (69-91)	84±1.9 (82-86)

Table 2. Comparison of *Malenchus cylindricus* sp. n. with other morphologically similar species. Shape=vulva-anus body shape. T/VA=Tail /vulva-anus. All measurements are in µm.

	Shape	Annuli	Stylet	PUS	Tail	T/VA	a	c
M. cylindricus sp. n.	EC	0.8-1.1	9.8-11	present	69-91	1.0-1.4	26-34	4.4-5.6
*M. andrassyi**	MT	1.0-1.2	10-11	present	88-106	1.1-1.5	24-34	4.1-5.2
M. acarayensis	MT	1.0-1.7	8.0-8.5	present	70-76	1.1-1.5	19-26	3.8-5.5
M. nanellus	MT	0.8-1.1	7.5-8.5	present	80-90	1.6-1.8	23-30	3.5-3.9
M. sexlineatus	MT	0.7-0.9	6.2-7.5	present	63-67	1.4-1.8	21-26	4.1-4.3
Duosulcius acutus	EC	0.8-0.9	6.0-7.0	absent	89-122	1.0-1.2	39-46	5.0-6.0
Zanenchus zanclus	EC	0.8-0.9	8.0-9.0	absent	74-92	0.7-0.8	36-40	5.7-6.1

* The new species genus is assigned to *M. andrassyi* by the key of Geraert (2008).

Tenunemellus sheri Raski *et al.*, 1982

(Figs. 7, 8, 9)

Material examined

The description is based on Mexican samples recovered from moist soil with a high organic matter, on the edge of a creek at the Cañon de Doña Petra (GPS coordinates: 31° 54' 03"N, 116° 36' 32"W), Ensenada, Baja California, Mexico.

Description

Measurements. See Table 1

Female. Body very slender, straight, ventrally curved or S-shaped. Cuticle finely striated. Lateral field obscure but two incisures are visible in SEM (Fig. 7F). Head dorso-ventrally compressed, thus giving a laterally offset impression. Amphidial aperture distinct, long, slender-ovate shaped (Figs 7A-7C, 8H). Amphidial fovea exceptional spacious, appearing as a prominent chamber oval (laterally) or hemispherical (dorso-ventrally) shaped chamber. Labial framework weak. Stylet slender and delicate, cone about 1/3 of total length. Stylet knobs oblong, thin, perpendicular to the shaft (Figs 8C-8F, 9A-9D). In few specimens, dorsal knob sloping anteriorly while ventro-submedian ones sloping posteriorly (Fig. 9A). Dorsal pharyngeal gland orifice at base of stylet knobs. Median bulb weak, no valve present. Excretory pore at the level of anterior part of pharyngeal bulb. Hemizonid not visible. Deirids at the level of excretory pore. Reproductive system monodelphic, prodelphic, ovary outstretched with oocytes arranged in a single row. Spermatheca small, not filled with sperm cell, 1/2-1/3 to adjacent body width, offset, elongated. Uterus sac present, narrow, about one body diameter. Body contour around vulva slightly elevated, but sunken to form a vulva cave (Fig. 8I). Lateral flaps present, four annuli wide. Epiptygmata absent. Vagina perpendicular to body. Prophasmid not observed. Tail exceptional long, tapering gradually to a straight tail tip.

Male. Not found. Probably not present in examined population, as sperm cells were not observed in spermatheca.

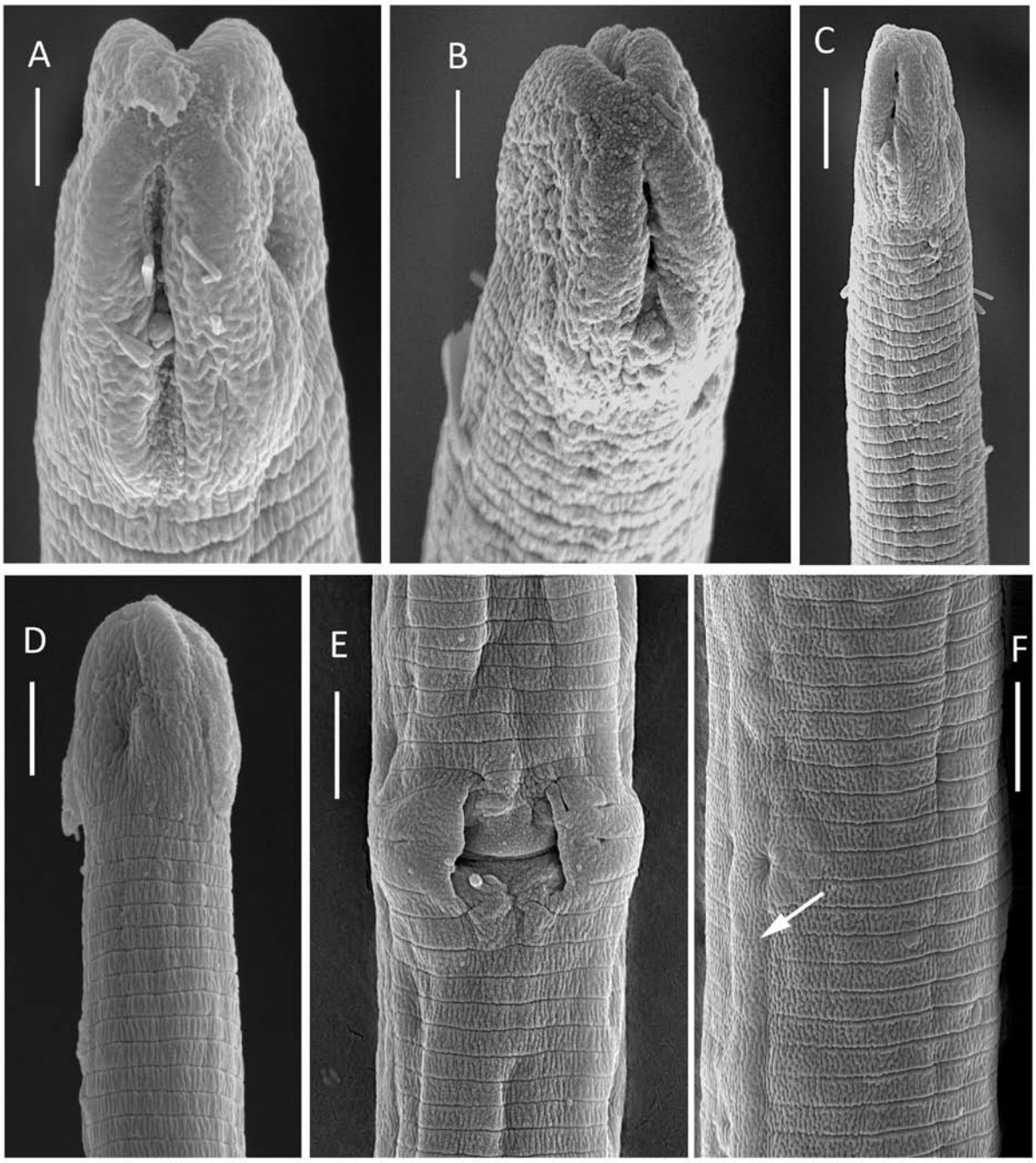

Fig. 7 SEM micrographs of female *Tenunemellus sheri*. A-C: lateral view of head region; D: ventral view of head region; E: vulva; E: middle body, arrow indicates two indistinct lateral incisures. Scale bar: A,B=1μm, C-F=2μm.

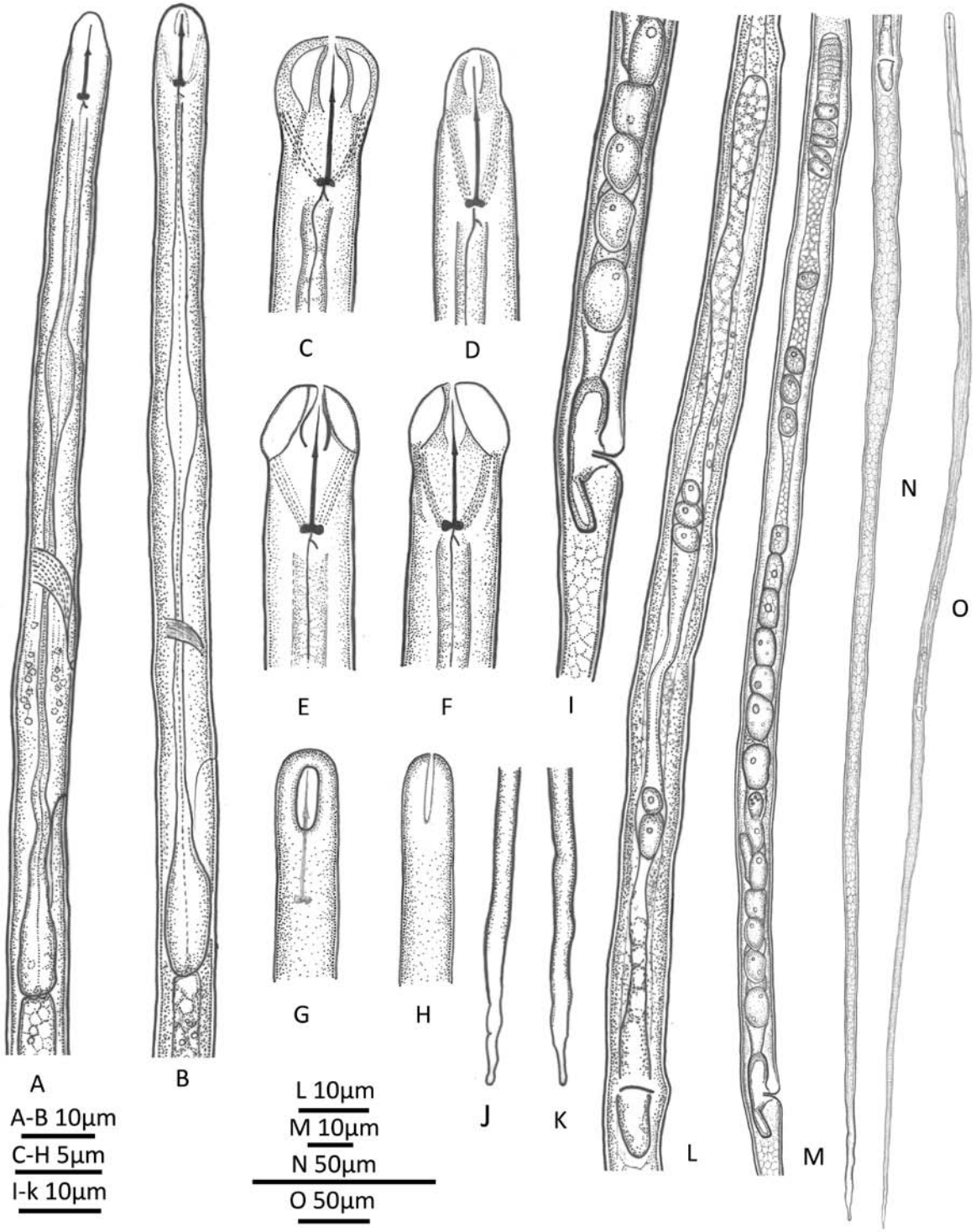

Fig 8. Line drawing of female *Tenunemellus sheri*. A, B: anterior part of body. C-F: ventral view of head region in different image plans. G: lateral view of head region showing pouch-like amphidial fovea. H: lateral view of head region showing slender-ovate amphidial aperture. I: lateral view of vulva region. J, K: tail tips. L: ventral view of reproductive system. M: lateral view of reproductive system. N: posterior part of body. O: body habitus.

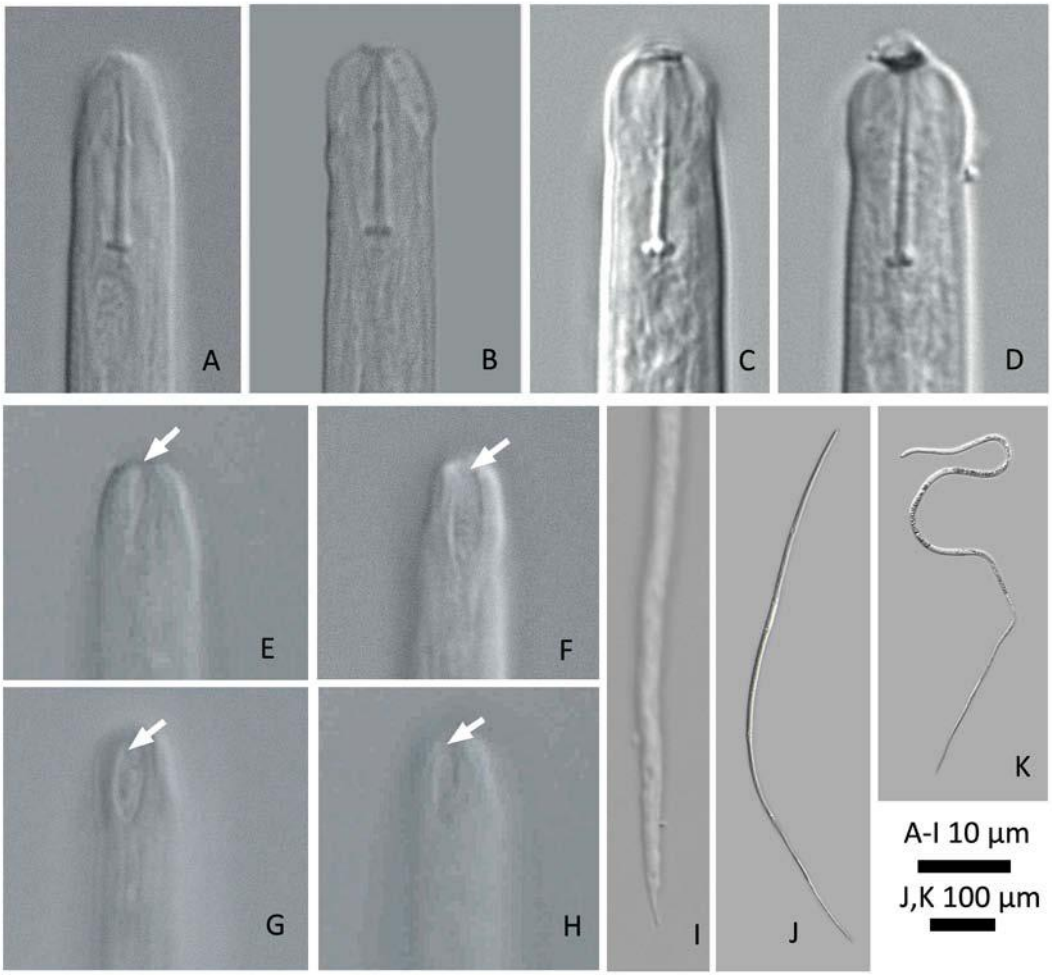

Fig. 9 LM pictures of female *Tenunemellus sheri*. A-H: head region. Arrows indicate pouch-like amphidial fovea. I: tail. J, K: body habitus

Position and classification of Ecphyadophorinae

Species belonging to Ecphyadophorinae are among the most remarkable of all Tylenchidae. Their long and extremely slender forms render their detection, handling and identification extremely difficult. The first genus in this group, *Ecphyadophora*, was proposed by de Man (1921), and Corbett (1964) later added the new genus Ecphyadophoroides, separated from the former by a dorso-ventrally flattened head (*vs* round head) and the gradually tapering female body (*vs* abruptly narrowing). Subsequently, Raski *et al*. (1982) used the long slit amphidial aperture as a generic character for *Ecphyadophoroides* (*vs* small oval amphidial aperture in *Ecphyadophora*). The most specious genus *Tenunemellus* was added by Siddiqi (1986) to accommodate *Ecphyadophoroides* species that lacked longitudinal

striae in the cuticle and did not have clear incisures in lateral field. The genus *Lelenchus* also belongs to Ecphyadophorinae, but differs significantly from all the above by the absence of a lobbed bursa. It was first proposed by Andrássy (1954) as a subgenus of *Tylenchus* with *Tylenchus* (*Lelenchus*) *leptosoma* de Man, 1880 designated as the type species and *Lelenchus* was subsequently raised to generic level by Mely (1961).

Given the high variation in head shape, amphidial aperture, vulva, and bursa shape, Ecphyadophorinae is a heterogeneous group and no clear delimitation character has been found, except for the extremely slender body. In this study, molecular phylogenetic analyses support *Lelenchus* as being a separate clade rather than a genus or subgenus in Tylenchinae. It also suggests that the ecphyadophorid-like slender body shape may have evolved independently, as *Ecphyadophora* and *Lelenchus* are not sister taxa: in our analyses the former genus is nested in a robust clade (18S, PP=96, BS=99) together with *Filenchus misellus*, *F. chilensis* and *Labrys chinensis* while the latter is positioned in a clade together with *Miculenchus* or *Tenunemellus* and/or *Miculenchus* (depending on the analyses) (Figs 5, 6).

These findings contradict the current taxonomic classifications, which consider, on the basis of general body shape, *Ecphyadophora*, *Ecphyadophoroides*, *Lelenchus* and *Tenunemellus* as closely related genera within Ecphyadophorinae. Current molecular phylogenies further support the pouch-like amphidial fovea as an informative character to define clades in Tylenchidae. *Ecphyadophora*, embedded within Tylenchinae, has a small pore-like amphidial aperture lacking a prominent fovea, different from other ecphyadophorids such as *Ecphyadophoroides*, *Lelenchus* and *Tenunemellus*, which share a long slit-like aperture with pouch-like fovea. Hence, according to molecular data and shape of the fovea, *Ecphyadophora* should be transferred to Tylenchinae whereas *Malenchus*, *Lelenchus* and all other ecphyadophorids bearing a pouch-like fovea may represent a natural clade, and as such may be grouped in a separate family or subfamily. However, the unresolved phylogeny and limited availability of molecular data of ecphyadophorid genera reduce the conclusive power of this assumption. For the time being, therefore, no taxonomic actions will be taken to accommodate the ecphyadophorid genera, pending additional molecular and morphological

data so that a more complete and conclusive analysis can be provided.

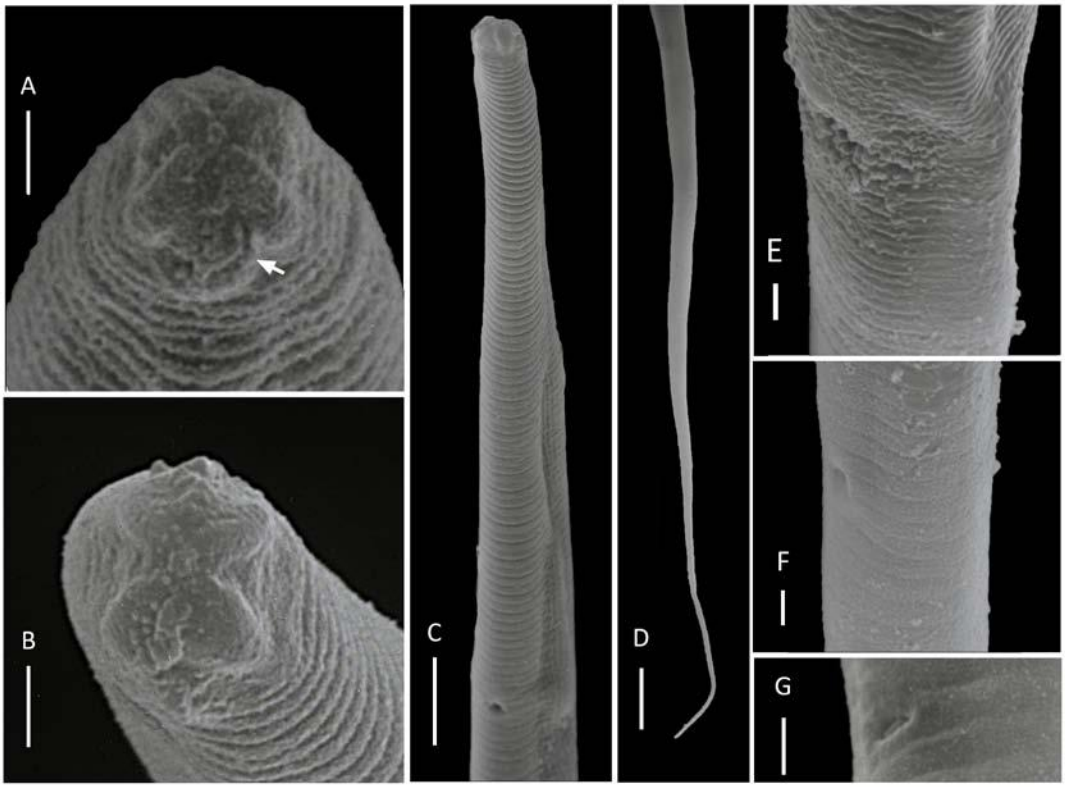

Fig 10. SEM micrographs of female *Lelenchus leptosoma* C114 isolated from Belgium. A, B: *en face* view. Arrow indicates S-shaped amphidial aperture; C: anterior body; D: tail; E: ventral-lateral view of vulva region; F, G: anus. Scale bar: A, B, E- G=1μm; C=5μm; D=10μm.

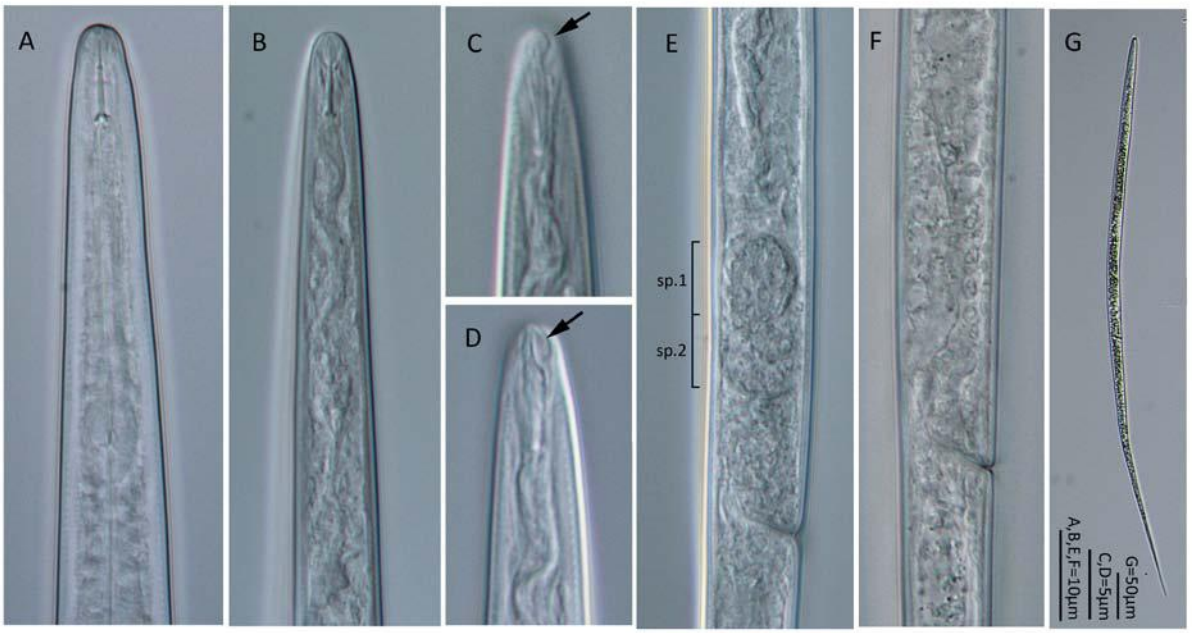

Fig 11. LM micrographs of two *Lelenchus leptosoma* populations isolated from Belgium C219 (A) and C114 (B-G). A: anterior of female. B: anterior of male. C: head region of female, arrow indicating S-shaped amphidial aperture. D: head region of female, arrow indicating pouch-like amphidial fovea. E, F: vulva region. sp.1=first spermatheca; sp.2=second spermatheca. Sperm cells present in both spermathecae. G: female body habitus.

Morphology and taxonomy of Lelenchus

In this study we examined two *Lelenchus leptosoma* populations from Belgium, both agreeing with the original description in terms of morphology and morphometrics. The SEM micrograph shows that population C114 has an S-shaped amphidial aperture: this is similar to Brzeski and Sauer (1982) and the Chilean population from Raski and Geraert (1985),but differs from the American population, which has a broader amphidial opening.

The spermatheca from the examined populations is offset with two parts (three cells in spermatheca 1 and nine in spermatheca 2, Figs 11E, 12A). Based on LM *in toto* observation, *Lelenchus* is either bilobed as in *L. brevislitus* and *L. leptosoma* or single as in *L. filicaudatus* and *L. schmitti*. In the present study, based on careful dissections, it is confirmed that the spermatheca is bilobed. The bilobed spermatheca observed in LM can be the result either of the presence of two sacs (as in this study) or of an unusual sperm positioning (sperm in anterior part of uterus). Remarkably, sperms were only visible in the second part of the expelled spermatheca, although they were clearly present in both parts before dissecting. It is possible that that the second part of spermatheca offer higher internal pressure and the sperms of the first part are compressed into the second part after dissection. A possible evolutionary advantage of a bilobed spermatheca with differences in pressure is to facilitate a more diverse fertilization.

Geraert and Raski (1987) proposed a dorso-ventrally flattened head together with a long slit amphidial aperture as identifying generic characters. However, these characters are problematic: given the slender body and small size, an accurate assessment of head dorso-ventral flatness is difficult in LM; a dorso-ventral flattened head is inconsistent among species; and a similar long slit amphidial aperture can also be present in *Filenchus* spp. Hence,

Bernard (2005) considered the above diagnostic characters not to be informative while a pouch-like amphidial fovea was put forward as being of greater utility.

In addition to the fovea shape, we propose that the vulval region be used to supplement the current generic definition of *Lelenchus*, including the following three traits: (1) the body contours in the vulva region are straight (*i.e.* not sunken or protruding); (2) vaginal muscles are obscure and walls prominent with the same thickness (or slightly thinner) as the cuticle, comparable to the adjacent body cuticle in LM (similar light refraction); (3) the vagina is anterior directed, PUS extremely reduced.

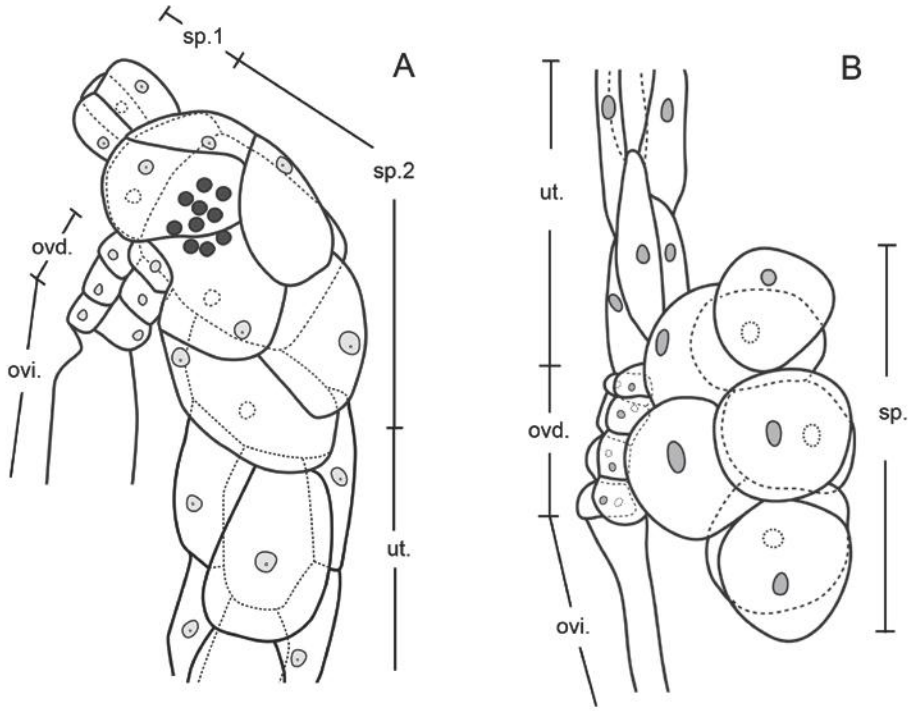

Fig 12. Line drawing based on dissected specimen of the cellular composition of oviduct, spermatheca and distal part of uterus of *Lelenchus leptosoma* (A), and *Miculenchus salvus* (B) from dissected specimen. Abbreviations: ovi.=ovary; ovd.=oviduct; sp.=spermatheca; sp.1=first part of spermatheca; sp.2=second part of spermatheca, in examined specimen sperm cell can present in both part or only in second spermatheca, only the latter case is illustrated.

Morphology and phylogeny of Miculenchus

In this study, *Miculenchus salvus* was recovered from moss rhizosphere soil in Belgium;

the general morphology and morphometrics are similar to the original description. One additional *Miculenchus* individual was found in fern rhizosphere soil in China but not identified to species due to inadequate morphology of the specimen. Molecular analyses inferred from 18S and 28S rRNA suggested that these two populations are closely related but divergent from known *M. salvus* sequences in GenBank (accession: FJ969129, KY119705 and KY119922). The phylogenic position of *Miculenchus* is not straightforward: 28S phylogeny suggests a sister relationship to *Malenchus* clade 2 or *Tenunemellus sheri* (depending on the different alignment methods), while in the 18S phylogeny, *Miculenchus* is sister to *Lelenchus* and thus divergent from *Malenchus* (Figs 5, 6). Therefore, the phylogenetic position of *Miculenchus* remains uncertain and the effects of long branch attraction cannot be disregarded.

The spermatheca of *M. salvus* comprises only eight cells and this cellular architecture is unusual compared to other Tylenchomorpha, which usually have ten or more spermatheca cells. The uterus is comprised of a quadricolumella with five cells in each rows and an oviduct with four cells in two rows, in agreement with its classification within Tylenchidae.

Miculenchus is a rare genus and usually co-exists with *Malenchus*, albeit in much lower density. It is morphologically unique owing to its zigzag transverse cuticle, male without bursa and a round amphidial aperture in the labial plate (Fig 13). These morphological features suggest *Miculenchus* to be a unique lineage within Tylenchidae. Surprisingly, molecular analyses appointed *Miculenchus* to be nested within a well-supported *Coslenchus* clade and sister to *C. franklinae*. The sequence divergence between *M. salvus* (GenBank accession: FJ969129) and *C. franklinae* (GenBank accession: AY284583) is only 10 bp (about 0.58%). Two additional 18S rRNA sequences deposited in GenBank were also assigned to *M. salvus*, although nematode ID assignment was only based on the highest sequence identity match from a BLAST search. Moreover, these two sequences (GenBank accessions: KY119705 and KY119922) are much shorter (about half or less) than the 18S multiple alignment, thus confining possible interpretations. The fact that our *M. salvus* sequences represent populations that clearly agree more with the morphological data (*i.e. Miculenchus* is clearly different from *Coslenchus*) suggests that previous DNA sequences were incorrectly

assigned to *Miculenchus*. Thus, we must consider the new *Miculenchus* sequences from this study as the first and only valid representatives of the genus for molecular analyses.

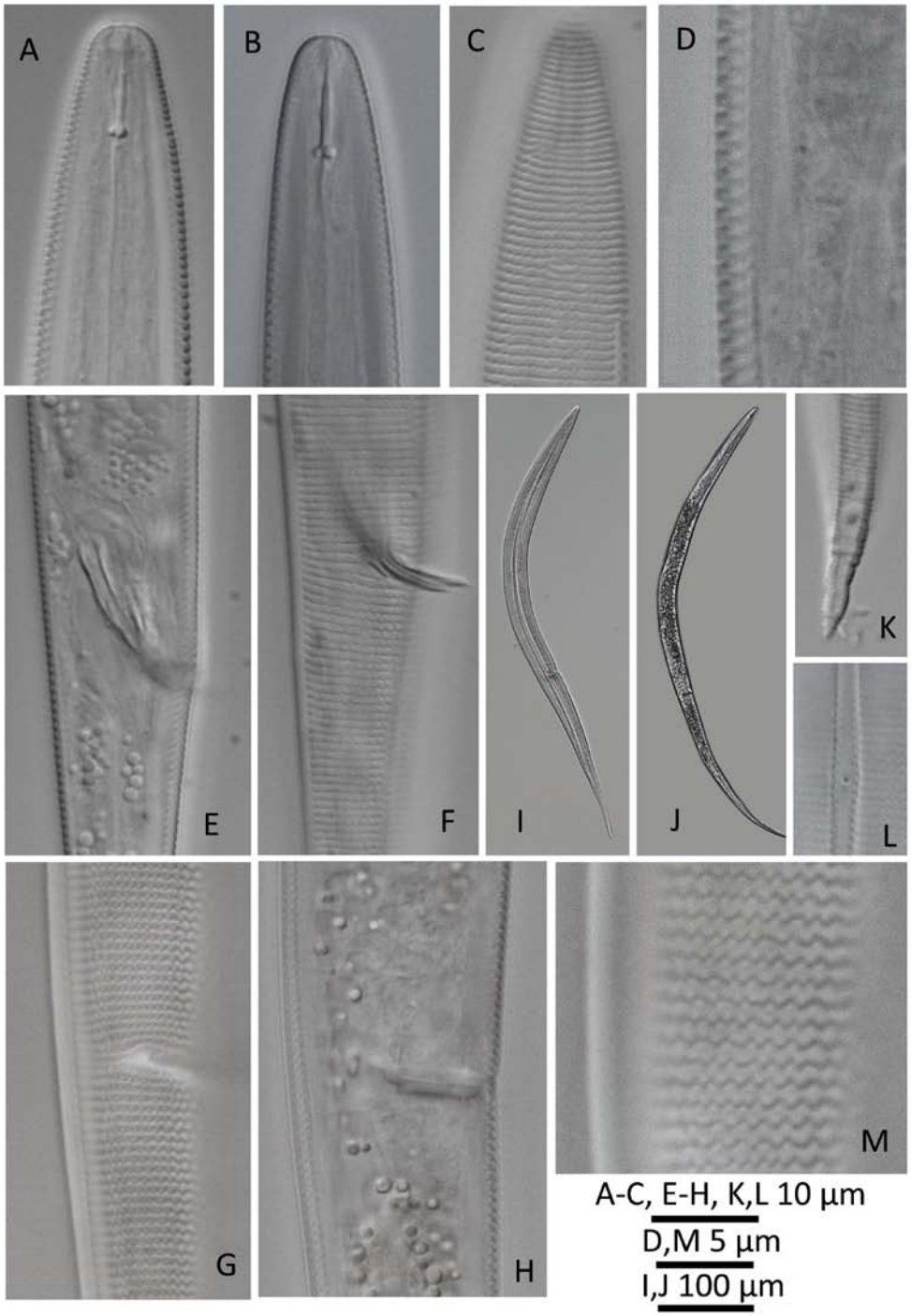

Fig 13. LM micrographs of *Miculenchus salvus*. A, C-I, K-M: specimens in permanent glycerin slide; B, J: fresh specimens. A-C: female anterior part; D: lateral view of cuticle annulations; E, F: spicule without bursa; I, J: female body habitus; K, female tail tip; L: lateral region showing deirid; G, H: vulva region; M: middle body ventral view showing zigzag

transverse annulations.

Comparison of alignment methods

DNA sequences used for current Tylenchidae phylogenies are divergent, due to the scarcity of homologous sites (coverage limitations) and/or high nucleotide substitution rates. As a result, the chosen alignment method has a greater impact on the alignment quality of the sequence, and has a further effect on the phylogenetic power.

In the present study, we experienced support values not in agreement (PP>0.98, BS<70 or PP<0.7, BS>80) both in the 18S and in the 28S phylogenies (Figs 5, 6). Four alignment methods were used, in order to assess their effects, including two sequence-based (G-INS-I and Muscle) and two structure-aided methods (Q-INS, RNAsalsa). A total of 16 trees were built and compared by RF and KF distances. These distance methods offer a straightforward comparison between multiple trees, taking into account variations in topologies and branch lengths. The resulting trees are generally congruent both in topology and node support values. Among these trees, a lower RF and KF distance (indicating that two trees are more similar) suggest the Muscle alignment is more similar to G-INS-I compared with structure-aided methods (Table 3). Interestingly, Q-INS has relatively high PP and BS values in comparison with some poorly supported clades based on other methods (Figs 5, 6; Table 3). Since the secondary structural information has been forwarded to improve the alignment of rRNA sequences, the phylogeny based on these alignments may perform better during tree reconstruction.

Table 3. Comparison of tree topologies generated from alternative alignment methods with G-INS-i measured by Robinson-Foulds (RF) and Kuhner-Felsenstein distance (KF). Section's order: RF/KF.

Construct methods	Genes	Muscle	Q-ins-i	RNAsalsa
ML	18S	7.0/0.13	23/0.55	15/0.29
	28S	7/0.43	17/0.46	15/0.53
BI	18S	10/0.11	28/0.59	16/0.27
	28S	10/0.43	16/0.49	14/0.56

Acknowledgements

The first author thanks the China Scholarship Council (CSC) for providing a grant. T.J.P. is supported by CAPES Foundation, Ministry of Education from Brazil. This work was also supported by a special research fund UGent 01N02312.

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Chapter VII

Tylenchidae (Nematoda) in China: first checklist with 17 new records

Chapter modified from:

Qing X.[1], Liu G. [2], Bert W.[1] Tylenchidae (Nematoda) In China: First Checklist with 17 New Records. Manuscript in prepare

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Abstract

A country-wide sampling from terrestrial natural ecosystem in China revealed 25 species that belong to Tylenchidae, 17 species and 5 genera are new records for China. The detailed morphometric data are provided for these recovered populations. The first Chinese checklist of Tylenchidae is presented, including the habitat of each species, based on current study and a literature review. The Chinese Tylenchidae comprise 56 species representing 11 genera.

Key words: Nematodes, nematofauna, terrestrial, natural ecosystem

Introduction

Tylenchidae are common soil dwelling species. They may constitute up to 30% of the nematode individuals in any given soil sample (Yeates and Brid, 1994; Ferris and Bongers, 2006). Tylenchidae bear a hollow stylet similarly to what plant-parasitic nematodes use to puncture plant cells, however, this group do not comprise economically important plant-parasites and their exact feeding behavior is not fully understood. Compared with plant-parasitic nematodes, the Tylenchidae fauna is relatively poorly studied in China. Although 39 species have been reported (See Table 2), they were poorly described and published in national journals with a limited access. The majority of these reports majority originated from agro-ecosystems. However, Bert and Geraert (2000) demonstrated that more natural habitats and non-conventional crops contain an unknown diversity of tylenchid nematofauna. Therefore, in present study we mainly sample in natural ecosystem to explore the actual diversity of Tylenchidae. Sixty five samples from 30 locations were collected. Morphological and morphometric information of the retrieved nematodes was carefully examined and presented. The newly generated data together with other published data were compiled as a checklist in order to provide an improved insight into the diversity and distribution of the Tylenchidae in China.

Materials and methods

Nematodes were extracted from soil samples using a Baermann tray, concentrated using a 500 mesh sieve (25μm opening) and fixed with 4% formalin at 60℃ for the morphological analyses. The fixed specimens were rinsed with deionised water and gradually transferred to anhydrous glycerin for permanent slides. Slides were examined and photographed using an Olympus BX51 DIC Microscope (Olympus Optical, Tokyo, Japan), equipped with an Olympus C5060Wz camera. Morphological vouchers for all examined specimen were made using a combination of movies and photomicrographs (De Ley and Bert, 2002) and are available upon request from the first author. Specimens were identified to species level based on available keys (Raski and Geraert, 1986; Geraert, 2008) and confirmed with original

descriptions. The classification and species validation followed Geraert (2008), except for genus *Psilenchus* which was not included as it has been shown to be outside the Tylenchidae according to molecular data (Holterman *et al.*, 2008; Bert *at al.*, 2008).

Results and discussion

A list of Tylenchidae recorded in China, together with locations, corresponding habitats is presented in Table 1. The morphometric data are listed in table S2 in appendix.

The Chinese samples from natural ecosystem reveal a high diversity of Tylenchidae. Twenty five different species belong to 10 genera were recovered, among which 5 genera (*Boleodorus*, *Coslenchus*, *Lelenchus*, *Miculenchus* and *Neopsilenchus*) and 17 species (*Basiria duplexa*, *Boleodorus thylactus, Cephalenchus cephalodiscus*, *Coslenchus costatus, C. oligogyrus, Filenchus afghanicus, F. balcarceanus, F. discrepans, F. hamuliger, F. magnus, F. misellus, F. tenuis, Lelenchus leptosoma, Malenchus acarayensis, Miculenchus salvus, Neopsilenchus longicaudatus* and *N. magnidens*) are new to the Chinese nematofauna. *Filenchus* is the most common genus and the most widespread species appeared to be *F. vulgaris, F. heterocephalus* and *F. ditissimus.* All newly-recovered genera and most newly-recorded non-*Filenchus* species are from natural ecosystems, concurring with Bert and Geraert (2000) that natural ecosystems are rich in tylench diversity. Therefore, further studies on Tylenchidae diversity should also include natural ecosystems rather than agro-ecosystem.

Although we have list 56 species of Tylenchidae, the actual diversity is most likely still severely underestimated given the wide geographic area, diverse ecosystems and disparate vegetation in China. Further intensive sampling covering more locations need to be done to have a better understanding of the nematode diversity in China.

Table 1. The checklist of species from Tylenchidae reported from China. Numbers refer to the studies where the taxa are mentioned; 1: Luo *et al.*, 2008; 2: Teng *et al.*, 2012; 3: Yin, 1995; 4: Xie *et al.*, 1994; 5: Zhang *et al.*, 2012; 6: Wu & Qin, 1999; 7: Jiang & Liu, 1999; 8: Xie & Feng, 1994; 9: Jin *et al.*, 2010; 10: Xie & Feng, 1996b; 11: Huai *et al.*, 2010; 12: Yan *et al.*, 2005; 13: Zhao *et al.*, 2004; 14: Zhou *et al.*, 2005; 15: Qi *et al.*, 2014; 16: Jiang & Liu, 2000; 17: Wu *et al.*, 1994; 18: Xie & Feng, 1996c; 19: Guliasiman *et al.*, 2007; 20: Lin *et al.*, 2008; 21: Li *et al.*, 2012; 22: Ding *et al.*, 2015; 23: Xie & Feng, 1996a; 24: Duan *et al.*, 1995; 25: Hu *et al.*, 2012; 26: Li *et al.*, 2009; 27: Zhang *et al.*, 2009; 28: Zhang *et al.*, 2013; 29: Qing *et al.*, 2015; 30: Li, 1996; 31: Xie *et al.*, 2007; 32: Xie & Feng, 2001; 33: Xie & Feng, 1997; 34: Xie & Feng, 1995; 35: this study.

Genera/Species	Habitats	Location	Ref.
Genus *Aglenchus*			
A. agricola (de Man, 1884) Andrássy, 1954	Rhizosphere of *Lycopersicon esculentum*	Benxi, Liaoning	1
A. muktii Phukan et Sanwal, 1980	Soil of nursery garden	Nanjing, Jiangsu	2
Genus *Basiria*			
B. duplexa (Hagemeyer et Allen, 1952) Geraert, 1968	Soil from soybean field	Baishui, Shaanxi	35
B. graminophila Siddiqi, 1959	Rhizosphere of mango tree	Guangzhou, Dongguan and Shenzhen, Guangdong	3
B. guangdongensis (Xie, *et al.* 1994) Siddiqi, 2000*	Rhizosphere of *Momordica charantia* and *Arachis hypogaea*	Shenzhen, Guangdong and Fuzhou Fujian Longyan, Fujian	4, 5
B. kashmirensis Jairajpuri, 1965	Rhizosphere of *Platycodon grandiflorus*	Bozhou, Anhui	6
B. parvamphidia Andrássy, 1963	Rhizosphere of *Platycodon grandiflorus*	Bozhou, Anhui	6
B. tumida (Colbran, 1960) Geraert, 1968	Soil from *Solanum tuberosum* field	Dalian, Liaoning	7
Genus *Boleodorus*			
B. thylactus Thorne,1941	Soil from *Abies* sp. forest	Qinling, Shaanxi	35
Genus *Cephalenchus*			
C. cephalodiscus (Sultan et Jairajpuri, 1982)	Soil from *Betula* sp. forest	Qinling, Shaanxi	35
C. concavus Xie et Feng, 1994	Soil around *Pyrus* sp.	Shenzhen, Guangdong	8
C. leptus Siddiqi, 1963	Soil around *Pyrus bretschneideri*; *Abies* sp. from forest	Bayizhen, Tibet; Qinling, Shaanxi	9, 35

Genus *Coslenchus*			
C. costatus (de Man, 1921) Siddiqi, 1978	Primary forest soil	Zhangjiajie, Hunan	35
C. oligogyrus Brzeski, 1987	Soil from soybean field	Baishui, Shaanxi	35
Genus *Filenchus*			
F. afghanicus (Khan et Khan, 1978) Siddiqi, 1986	Soil from *Abies* sp. forest	Qinling, Shaanxi	35
F. australis Xie et Feng, 1996	Rhizosphere of *Capsicum frutescens*; *Populus deltoids*; banana plantation	Dalong farm, Hong Kong; Sihong, Jiangsu; Nanning, Guangxi; Xuwen, Guangdong	10, 11, 12
F. balcarceanus Torres et Geraert, 1996	Soil from apple garden	Baishui, Shaanxi	35
F. butteus (Thorne et Malek, 1968) Raski et Geraert, 1987 **	Soil from vegetable field	Lanzhou, Gansu	13
F. capsici Xie et Feng, 1996	Rhizosphere of *Capsicum frutescens*	Dalong farm, Hong Kong	10
F. cylindricus (Thorne et Malek, 1968) Niblack et Bernard, 1985	Soil from Solanaceae field; apple garden	Dalian, Liaoning; Baishui, Shaanxi	7, 35
F. discrepans (Andrássy, 1954) Andrássy, 1972	Soil from forest with mixed tree species	Yangling, Shaanxi	35
F. ditissimus (Brzeski, 1963) Siddiqi, 1986 ***	Rhizosphere of mango tree; vegetable field; flowers plantations of *Spathiphyllum floribundum* and *Philodendron ensation*	Guangzhou, Dongguan and Shenzhen, Guangdong; Lintao and Lanzhou, Gansu; Xinyang, Henan; Shenyang, Liaoning Guangzhou, Guangdong; Shiyan, Hubei	3, 13, 14
F. equisetus (Husain et Khan, 1967) Raski et Geraert, 1987	Soil from vegetable plantation	Lianyungang and Yancheng, Jiangsu	15
F. facultativus (Szczygiel, 1970) Raski et Geraert, 1987	Rhizosphere of *Capsicum frutescens* and *Solanum tuberosum*	Pulandian, Liaoning	16
F. hamatus (Thorne et Malek, 1968) Raski et Geraert, 1987	Rhizosphere of *Panax ginseng* and *Panax quinquefolium*	Zuojia and Fusong, Jiling; Benxi and Shenyang, Liaoning; Laiyang, Shandong	17
F. hamuliger Brzeski, 1998	*Quercus aliena* forest soil	Qinling, Shaanxi	35
F. heterocephalus Xie et Feng, 1996	Soil from *Solanum melongena*, pomegranate, grape, apricot, *Pyrus* sp farm; *Erigeron*	Dalong farm, Hong Kong; Shule, Awati and Akesu; Luxi, Yunan; Shiyan and Xianfeng,	18,19, 20, 21,

	breviscapus; tobacco farm; vegetable plantation	Hubei; Haikou, Hainan	22
F. hongkongensis Xie et Feng, 1996	Rhizosphere of *Ipomoea aquatica*	Dalong farm, Hong Kong	18
F. magnus (Husain et Khan, 1977) Siddiqi, 1986	Primary forest soil and litter	Jinping, Yunan	35
F. misellus (Andrássy, 1958) Raski et Geraert, 1987	Primary forest soil	Zhangjiajie, Hunan	35
F. montanus Xie et Feng, 1996	Soil from sugarcane plantation	Dayushan, Hong Kong	23
F. neonanus Raski et Geraert, 1987	Rhizosphere of *Capsicum frutescens* and *Solanum tuberosum*	Pulandian, Liaoning	16
F. orientalis Xie et Feng, 1996	Rhizosphere of *Ipomoea aquatica*	Jintian, Hong Kong	23
F. orbus Andrássy, 1954	Rhizosphere of *Phaseolus calcaratus*; maize; *Populus deltoids*	Changchun, Jilin; Shenyang, Liaoning; Sihong, Jiangsu	11, 24
F. sheri (Khan et Khan, 1978) Siddiqi, 1986	Soil from *Pyrus bretschneideri, Helianthus annuus* and *Triticum aestivum*; primary forest soil and litter	Bayizhen, Lulangzhen, Linzhizhen, Tibet; Qinling, Shanxi	9, 35
F. tenuis (Siddique et Khan, 1983) Siddiqi, 1986	Soil from soybean field	Baishui, Shaanxi	35
F. uliginosus (Brzeski, 1977) Siddiqi, 1986	Soil from soybean field; garden plant seedlings	Gongzhuling, Jilin; Jintan, Jiangsu	24, 25
F. vulgaris (Brzeski, 1963) Lownsbery et Lownsbery, 1985 ****	Soil from mango tree, vegetable field; banana plantation; vegetable garden; maize field; nursery garden; *Phalaenopsis amabilis*; primary forest	Guangzhou, Dongguan and Shenzhen, Guangdong; Lanzhou, Gansu; Nanning, Guangxi and Xuwen, Guangdong; Taigu, Shanxi; Nanjing, Jiangsu; Xiamen, Quanzhou and Longyan, Fujian; Zhangjiajie, Hunan; Yangling, Shaanxi	2,3,12,13,26, 27, 28, 35
Genus *Lelenchus*			
L. leptosoma (de Man, 1880) Andrássy, 1958	Soil from moss and fern in forest	Shimen, Hunan	35
Genus *Malenchus*			
M. acarayensis Andrássy, 1968	Soil from *Quercus aliena* forest	Qinling, Shaanxi	35
M. exiguus (Massey, 1969) Andrássy, 1981	Soil from deciduous forest around the roots of	Mount Taibai, Shaanxi	29

	Betula sp.		
M. pachycephalus Andrássy, 1981	Soil from deciduous forest	Shimen, Hunan	29
M. platycephalus (Thorne et Malek, 1968) Andrássy, 1981	Soil from sugarcane	Fanyu, Guangdong	30
M. nanellus Siddiqi, 1979	Soil around roots of fern and moss in forest	Pingwu, Sichuan	29
Genus *Miculenchus*			
M. salvus Andrássy, 1959	Soil from *Abies* sp. forest in snow mountain	Qinling, Shaanxi	35
Genus *Neopsilenchus*			
N. longicaudatus Sultan, Singh et Sakhuja, 1988	Soil around roots of grass and moss in forest	Qinling, Shaanxi	35
N. magnidens (Thorne, 1949) Thorne et Malek, 1968	Soil around roots of fern	Shimen, Hunan	35
Genus *Tylenchus*			
T. bhitai (Maqbool et Shahina, 1987)	Soil from pomegranate, grape, apricot and *Pyrus* sp farm	Shule, Awati and Akesu, Xinjiang	19
T. davainei Bastian, 1865	Soil from bamboo root	Huzhou, Zhejiang	31
T. elegans de Man. 1876	Soil from *Anthurium andraeanum*	Yanling, Henan	14
T. exiguus de Man, 1876	Rhizosphere of mango tree	Guangzhou, Dongguan and Shenzhen, Guangdong	3
T. guangdongensis Xie et Feng, 2001	Rhizosphere of *Allium fistulosum*	Baoan, Guangdong	32
T. luci Xie et Feng, 2001	Soil in maize farm	Shenzhen. Guangdong	32
T. minor Xie et Feng, 1997	Rhizosphere of *Allium fistulosum*	Baoan, Guangdong	33
T. stylolus Xie et Feng, 1995	Rhizosphere of *Ananas comosus*	Daguling, Hong Kong	34
T. paraminor Xie et Feng, 1997	Rhizosphere of *Citrus reticulata*	Baoan, Guangdong	33

* Reported as *Rhabdotylenchus guangdongensis*

** Reported as *Tylenchus cylindricollis*

*** Reported as *Tylenchus paravissimus* in Yin (1995)

**** Reported as *Tylenchus fusiformis*in Yin (1995)

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Chapter VIII

Ultrastructural, phylogenetic and rRNA secondary structural analysis of a new mycophagous nematode with recovery of intestinal crystals

Chapter published as:

Qing X., Slos D., Claeys M. and Bert W. (2017) Ultrastructural, phylogenetic and rRNA secondary structural analysis of a new nematode from mushroom with recovery of intestinal crystals. *Zoologischer Anzeiger* 269: 13-25.

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Abstract

Abursanema quadrilineatum n. sp. was described both morphologically from light microscopy, scanning electron microscopy and transmission electron microscopy and molecularly based on 18S and 28S rRNA. The mycophagous stage of the new species recovered from fruiting body of *Trametes* sp. growing on decaying wood. The new species is unique in *Abursanema*, indicated by the presence of four lateral lines. The secondary structures of the D2 and D3 domain of 28S rRNA were predicted for the new species and a general model for the superfamily Sphaerularioidea was built for comparative analysis. The ultrastructure of the cuticle, sperm cells and oocytes was examined and cuticle layers were defined, providing the first known information on cuticle ultrastructure in Sphaerularioidea. Needle-shaped crystals were recovered in female mycophagous female intestines and spermatheca, and chemical tests revealed that they are not constituted of calcium oxalate or proteins.

Keywords: *Abursanema*, Hexatylina, Paurodontidae, phylogeny, Sphaerulariidae, Sphaerularioidea, taxonomy, ultrastructure

Introduction

Species belonging to Sphaerularioidea (*sensu* Siddiqi 2000) are taxonomically diverse, displaying a fascinating array of lifestyles: most species are primarily parasitic, feeding on insect or mite haemocoel but may have also a free-living mycophagous or plant-parasitic generation. They have widespread plasticity in morphology, exhibiting dimorphism or even tri- or tetramorphic females in different life stages (Siddiqi, 2000). Regardless of this diversity, their taxonomy and phylogeny are problematic: most species were only described based on light microscopy (LM) and the molecular backbone is missing. This situation continues even today for most species published recently (Golhasan et al., 2016; Nasira et al., 2013; Yu et al., 2013; Yu et al., 2014) and consequently increases taxonomic confusion and hampers our understanding of their biology and ecology. Therefore, a detailed morphology combined with molecular data is essential for new species descriptions.

Abursanema was recently described (Yaghoubi et al., 2014) in the family Paurodontidae (Sphaerularioidea). It has been characterized by its knob-less stylet, stem-like projection in intestine and by the absence of bursa in the male. Here we describe *Abursanema quadrilineatum* sp. n. as a second species of this genus, by combining both morphological and molecular analyses. Information about the secondary rRNA structure was included in our study, and comparative analysis was conducted for Sphaerularioidea. The ultrastructure of cuticle and sperm cells in the female was also examined, providing the first recorded knowledge on cuticle ultrastructure in Sphaerularioidea. Furthermore, needle-shaped crystals were found in the mycophagous female intestine and spermatheca, and their chemical and physical properties have been analysed and discussed for the first time.

Materials and methods

Sampling and isolation

Nematodes from fungus-living stage were isolated from fruiting bodies of *Trametes* sp.

using the modified Baermann method (Hooper, 1986). Parasitic stage specimens were directly picked up from dissected Mycetophildae pupae collected from same sample. Nematodes were immediately used for molecular analyses or fixed with 4% formalin for the morphological analyses.

Morphological studies

Formalin fixed specimens were rinsed several times with deionised water and gradually transferred to anhydrous glycerin for permanent slides, following the protocol of Seinhorst (1962) as modified by Sohlenius and Sandor (1987). Observations and drawings were made with an Olympus BX51 (Olympus Optical, Tokyo, Japan) equipped with differential interference contrast (DIC). Light microscopic images and multifocal videos (De Ley and Bert, 2002) were taken with a Nikon DS-FI2 camera (Nikon Corporation, Tokyo, Japan). The resulting digital specimen vouchers are available at http://nematodes.myspecies.info. Female reproductive system was extracted and examined based on the method of Geraert (1973) and Bert et al. (2008). Illustrations were prepared using Adobe Illustrator CS5 and light microscope drawings.

For Scanning Electron Microscopy (SEM), live animals were fixed in a microwave in Trump's fixative (2% paraformaldehyde + 2.5% glutaraldehyde in a 0.1 M Sorenson buffer) for a few seconds. Specimens were subsequently washed three times in double-distilled water. The specimens were dehydrated by passing them through a graded ethanol concentration series of 30, 50, 75, 95% (20 min each) and 3x 100% (10 min each). The specimens were critical point-dried with liquid CO_2, mounted on stubs with carbon discs and coated with gold (25 nm) before observation with a JSM-840 EM (JEOL, Tokyo, Japan) at 15 kV. To determine the ultrastructural morphology, specimens are prepared for observation by transmission electron microscopy (TEM) using JEOL JEM 1010, following the method detailed in Qing et al. (2017).

Intestinal crystal analysis

To identify if the crystals contain protein the Bradford protein assay (Bradford, 1976) was applied. To test if the crystals consists of calcium oxalate we used a 5 % solution of sodium hypochlorite to dissolve the nematodes, followed by a treatment of 5% acetic acid to dissolve the calcium oxalate.

DNA extraction, PCR and sequencing

DNA was extracted from fresh specimens. The nematode was then transferred to a PCR tube with a solution containing 10 µl NaOH and 1µl Tween20, heated for 15 min at 95°C, and 40 µl of double-distilled water was added. PCR reaction was done following the protocol of Qing et al. (2017) and Bert et al. (2008) respectively. The D2/D3 domains of 28S rRNA were amplified with primers D2A and D3B (De Ley et al., 2005). The 18S rRNA gene was amplified using SSU 18A and SSU 26R (Blaxter et al., 1998).

Secondary structure analysis

Secondary structures were predicted separately for the D2 and D3 domain of 28S rRNA. The secondary structure of our new species was built by RNAstructure 5.8 (Reuter and Mathews, 2010) using the energy minimization approach and the variation sites of *Abursanema iranicum* (KF885742) were mapped. For a comparative analysis of the Sphaerularioidea superfamily, we used the same sequences data as for the phylogenetic analysis except for *Paurodontella parapitica* (KU522237) due to its incomplete available D2 domain. A Sankoff algorithm was used and simultaneous aligned and fold using LocaRNA (Smith et al., 2010) and RNAalifold (Bernhart et al., 2008) was used to build consensus structure. Structures were visualized using RnaViz (De Rijk et al., 2003) and drawn using Adobe Illustrator CS5.

Phylogenetic analysis

The obtained sequences were analysed with other relevant available sequences in

GenBank. Multiple alignments of the different genes were made using the E-INS-i algorithm of MAFFT v. 7.205 (Katoh and Standley, 2013). The best-fitting substitution model was estimated using AIC in jModelTest v. 2.1.2 (Darriba et al., 2012). Maximum Likelihood (ML) and Bayesian (BI) analysis were performed at the CIPRES Science Gateway (Miller et al., 2010), using RAxML 8.1.11 (Stamatakis et al., 2008) and MrBayes 3.2.3 (Ronquist and Huelsenbeck, 2003) respectively. ML analysis included 1000 bootstrap (BS) replicates under the GTRCAT model. Bayesian phylogenetic analysis was carried out using the GTR+I+G model for 1×10^7 generations and Markov chains were sampled every 100 generations and 25% of the converged runs were regarded as burnin. Gaps were treated as missing data for all phylogenetic analysis.

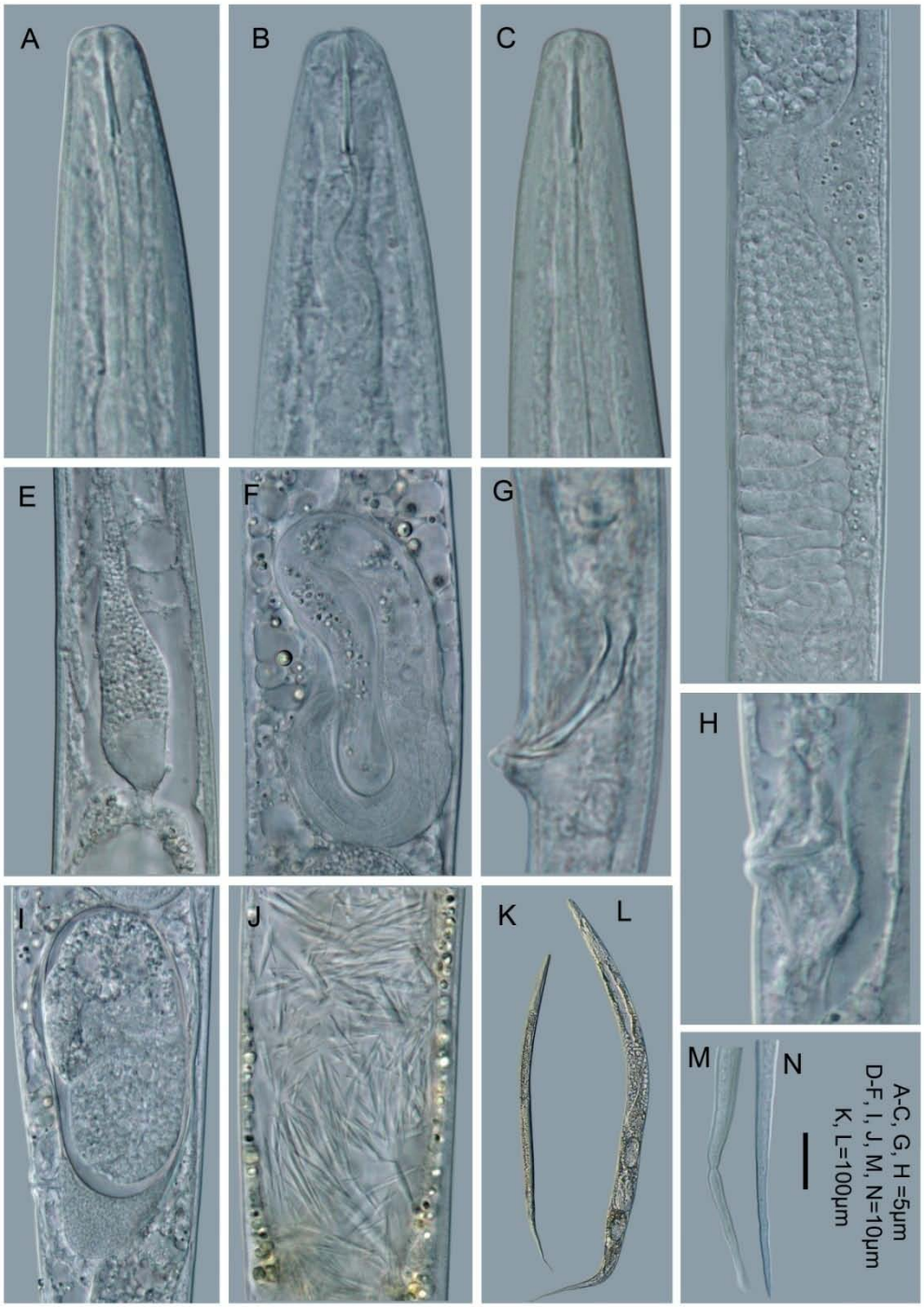

Figure 1. LM pictures of *Abursanema quadrilineatum* n. sp.. (A, D, E, H, J, M, N) early stage female. (B, F, I) ovoviviparous female. (C, G, K) male. (A-C) head region. (D) partial gonoduct showing spermatheca filled with sperm cells arranged in straight lines. E: posterior pharynx showing stem-like extension penetrating into intestine. F: hatched juvenile. (G) spicule. (H, I) lateral view of vulva. (J) anterior part of pharynx filled with needle-shaped crystals. (K, L) body habitus. (M, N) tail tip.

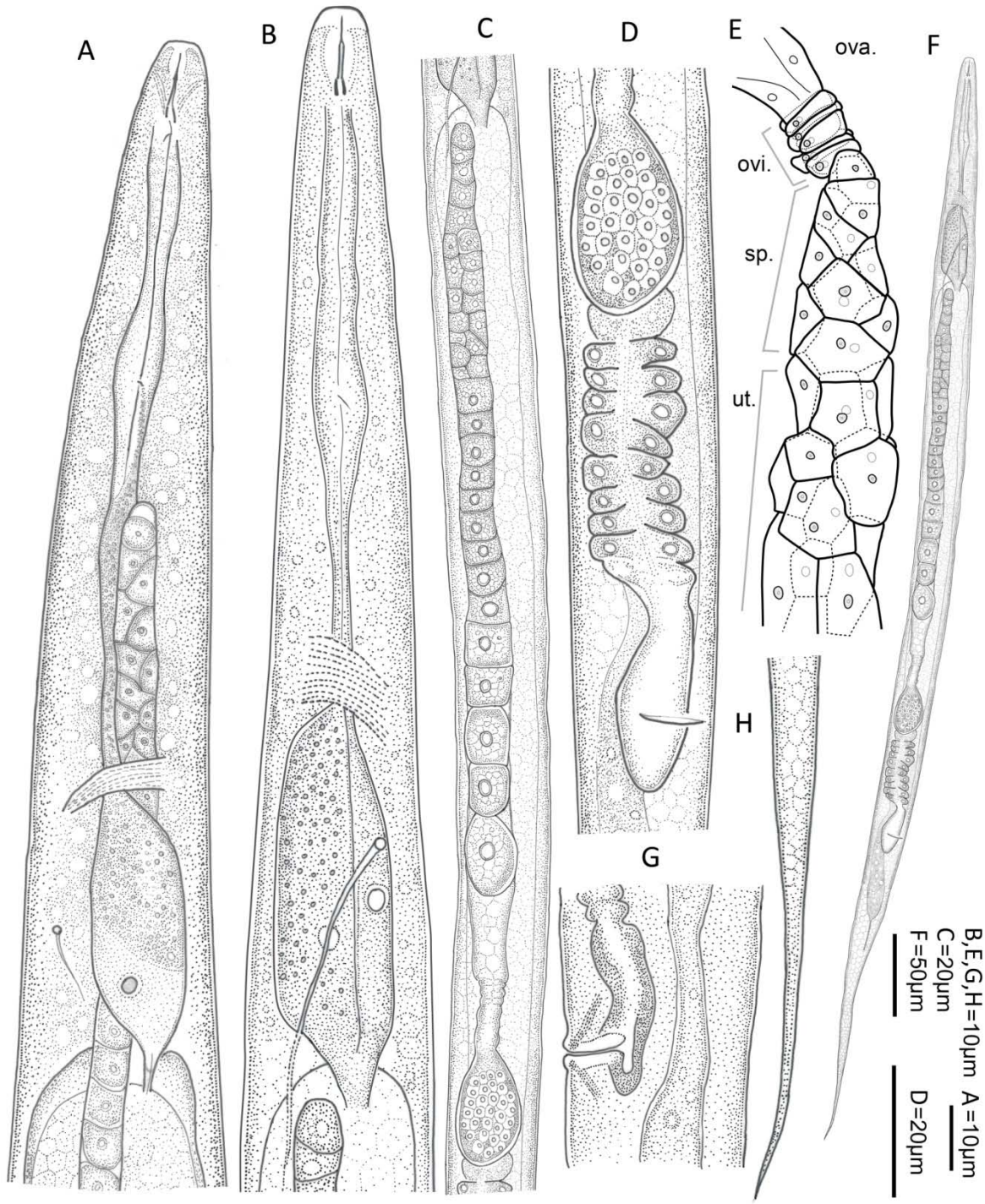

Figure 2. Mycophagous early stage female of *Abursanema quadrilineatum* n. sp. with empty uterus. (A, B) anterior body. (C) ovary. (D) ventral view of gonad distal part. (E) illustration of expelled gonoduct. (F) body habitus. (G) lateral view of vulva. (H) tail. Abbreviations: ova.=ovary; ovi.=oviduct; sp.=spermatheca; ut.=uterus

Result and discussion

Taxonomy

Abursanema quadrilineatum sp. nov.

(Figs 1-6, Table 1)

Specimen depositories

Holotype: Female, collected in 16. Feb. 2016, from Blaarmeersen, Sport- and Recreation park, 51°02'26.6"N 3°41'16.0"E, Deposited in Zoology Collections, Ghent University Museum, Belgium. Slide number: UGMD 104318.

Paratypes: 4 mycophagous stage ♀, 1 ♂, same collecting data as holotype, UGMD 104319. 2 ♀, 2 ♂, same collecting data as holotype, Nematode Collection of the Nematology Research Unit, Department of Biology, Deposited in Ghent University, Ghent, Belgium. Slide number: UGnem-161.

Registered in Zoobank with identifier: urn:lsid:zoobank.org:act:64FB846D-2DE1-431B-9C00-29E73C96EBEA

Type habitat and locality.

Mycophagous stage found in old fruiting body of *Trametes* sp. from decaying wood. Parasitic stage found in early pupa of Mycetophilidae recovered from the same old fruiting body of *Trametes* sp.

Description

Mycophagous early stage female (not gravid) (Figs. 1A, D, E, H, J, M, N; 2): Body slender, straight to ventrally arcuate in mycophagous stage. Cuticle finely striated. Lateral fields distinct, each with four incisures. Cephalic region low, continuous, framework slightly sclerotized, head 4.9-6.9 µm wide. Amphideal apertures indistinct in LM but slit visible in SEM (Fig. 3). Stylet short, shaft part longer than cone part, knobs absent or modified as rods, symmetrical or slightly asymmetrical (Figs. 1A-C; 2A, B; 4A; 5A, B). Dorsal pharyngeal gland orifice close to stylet base. Excretory pore generally at or near middle of basal bulb. Deirids at level of excretory pore. Pharynx non-muscular, corpus, isthmus, and spindle-shaped basal bulb well differentiated, the latter containing glands and with a

stem-like/tubular extension penetrating into intestine (Figs. 1E, 2A, B). Corpus cylindroid, metacarpus slightly swollen, cuticular valve absent but subventral gland duct orifice distinct. Vulva posterior, lips slightly protruding, not modified. Ovary monodelphic, prodelphic, usually outstretched until pharynx level, occasionally reflexed at anterior end. Oviduct two rows with five cells in each row (Fig. 2E). Spermatheca 15.7-26µm long and 9.7-14µm wide, arranged in line (axial). In few females filled with crystals (Fig. 4F). Sperm cells round, well arranged in line or randomly distributed. Uterus quadricolumella with nine cells in each row, empty (Fig. 2E). Postvulval uterine sac short, 1/3-1/2 body diameter. Rectum distinct, 6.9-10µm long. Tail filiform.

Mycophagous ovoviviparous female (Fig. 4): similar to early stage female but with slightly larger body size, indistinct spermatheca and uterus filled with 2-4 eggs and 1-2 hatched juveniles (Figs.1F, I; 4C, D).

Mycophagous male (Figs. 1C, G, K; 5): Male only observed in mycophagous stage, generally similar to female except for smaller body size. Bursa reduced or absent. Testis outstretched. Spicules and gubernaculum simple.

Entomoparasitic adult not recovered.

Etymology

The species name refers to the primary distinguishing trait: four incisures on the lateral field of the mycophagous female.

Diagnosis and relationship

Abursanema quadrilineatum n. sp. belongs to the family Paurodontidae (Sphaerularioidea) because of the stem-like/tubular extension in pharynx which penetrates into intestine. It belongs to *Abursanema* because of the reduced or absent bursa in the male. It can be differentiated from *A. iranicum*, the only species of genus, by four incisures *vs* two incisures. This morphological difference also support by both 28S and 18S rRNA (Figs. 8, 9).

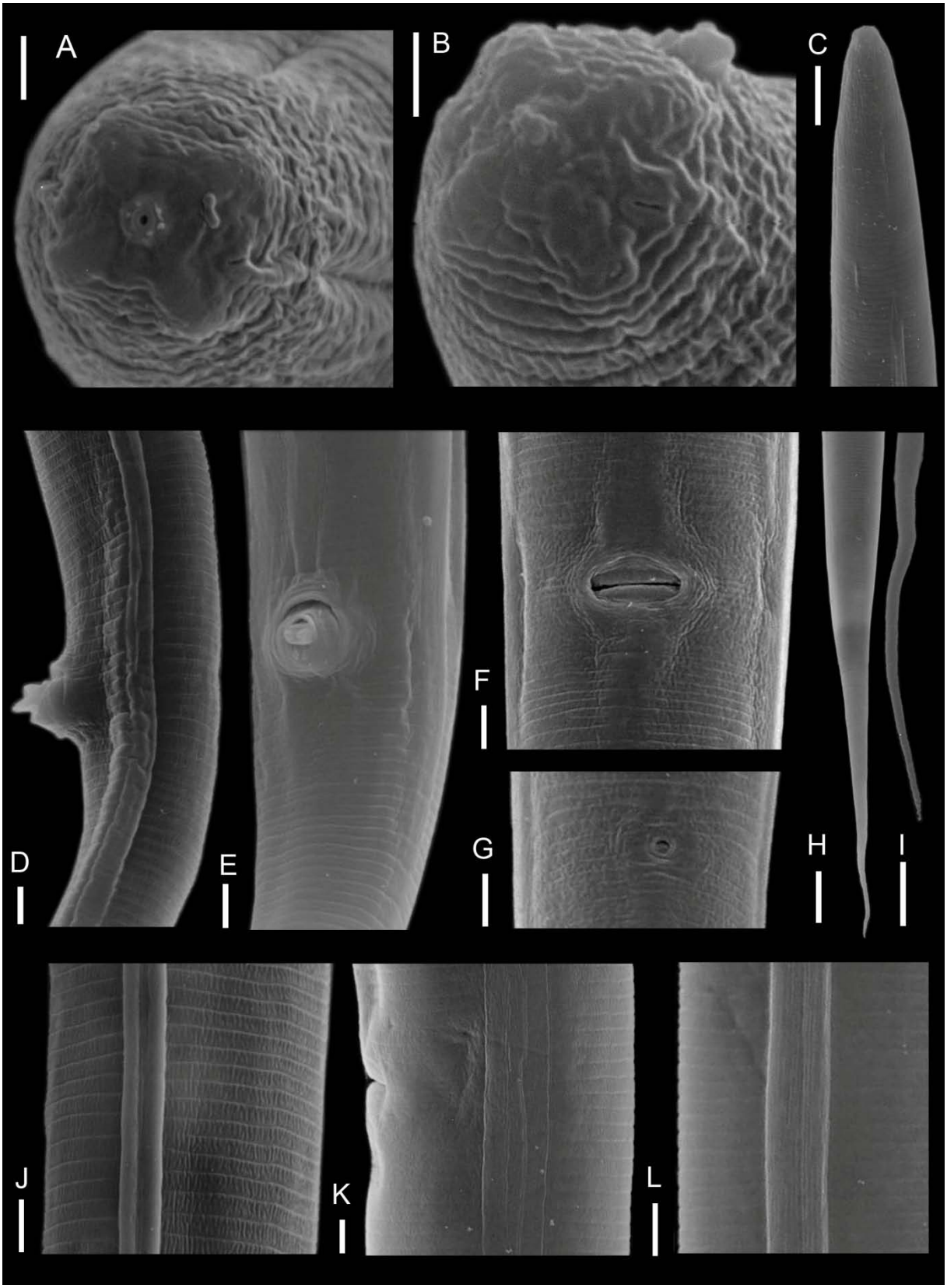

Figure 3. SEM of mycophagous early stage *Abursanema quadrilineatum* n. sp.. (A, B) female *en face* view. (C) female anterior end. (D) lateral view of cloacal region. (E) ventral view of cloacal region. (F) ventral view of vulva. (G) anus. (H, I) female tail. (J) Male lateral lines. (K)

Female lateral lines in vulva region. (L) Male lateral lines near cloaca. Scale bars: A, B=1µm; C, I =5µm; H=10µm; D-G, J-L=2µm.

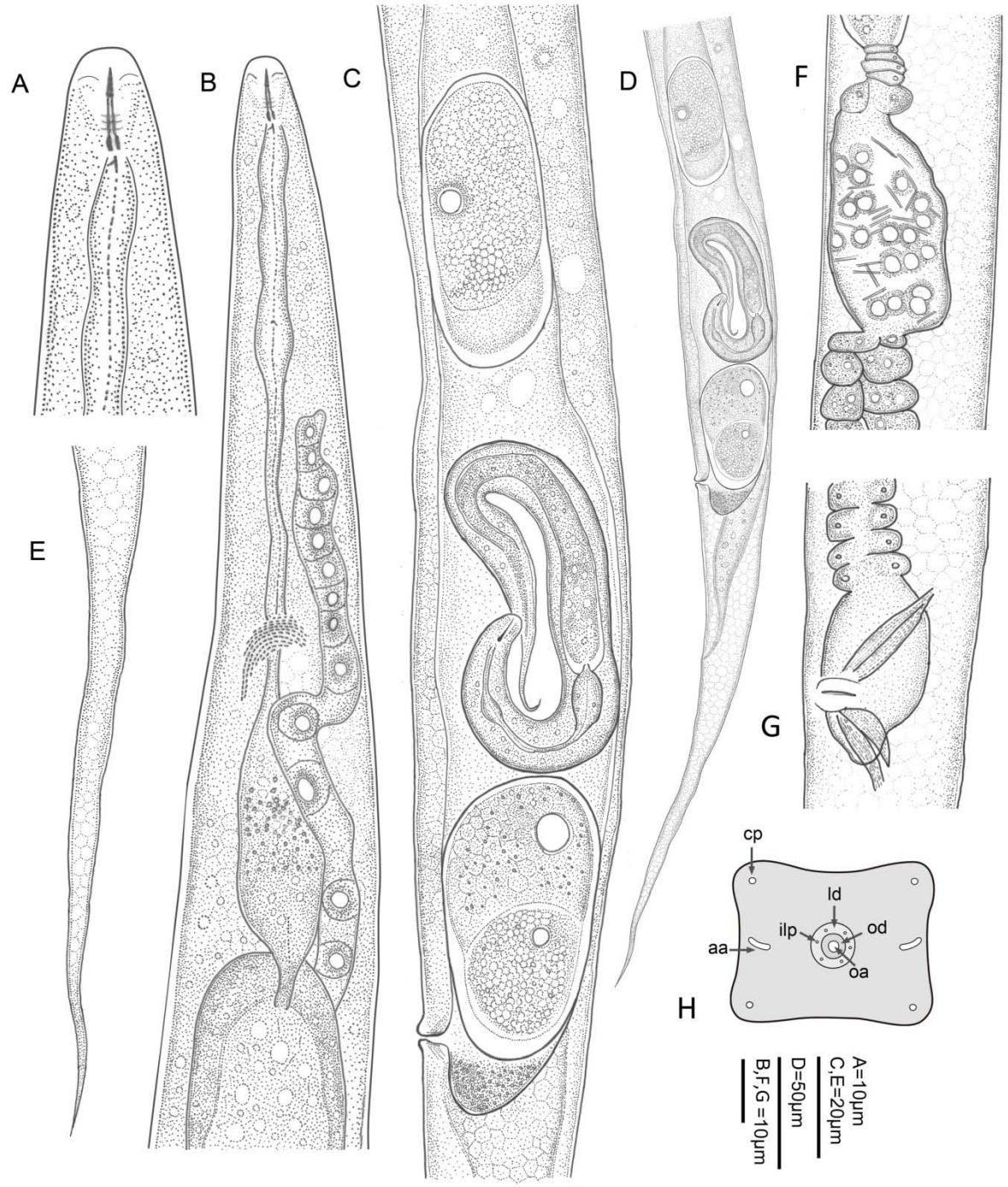

Figure 4. Mycophagous ovoviviparous (A-E) and early stage (F, G) female *Abursanema quadrilineatum* n. sp. with hatched juvenile. (A, B) anterior end of body. (C) gonad showing eggs and hatched juvenile. (D) posterior body. (E) tail. (F) spermatheca filled with sperm cells and short needle-shaped crystals. (G) ventral view of vulva. (H) illustration of *en face* view.

Abbreviation: cp=Cephalic papillae; aa=amphidial aperture; ld=labial disc; od=oral disc; oa=oral aperture; ilp=inner labial papillae.

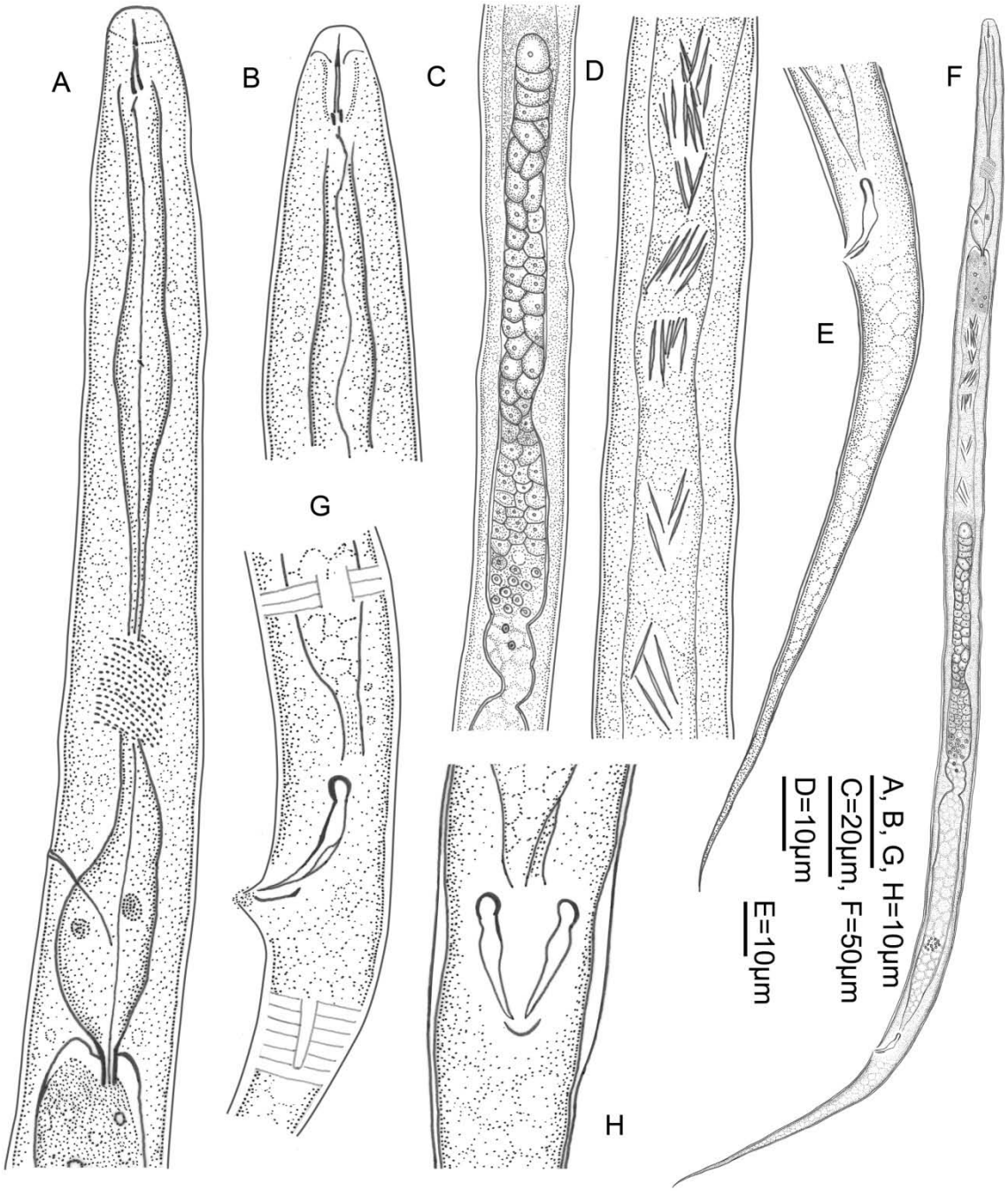

Figure 5. Male of *Abursanema quadrilineatum* n. sp. (A, B) anterior part. (C) testis. (D) intestine with needle-shaped crystals. (E) ventral view of tail region. (F) body habitus. (G) lateral view of spicule. (H) ventral view of spicule.

Cuticle and sperm ultrastructure

The ultrastructure of the body cuticle differentiates into four distinct zones (Decraemer et al., 2003), described from outer most to inner most: (1) epicuticle, (2) cortical zone, (3) median zone and (4) basal zone bordered by the basal lamina. In *Abursanema quadrilineatum* n. sp., these four layers (Fig. 6I) are all present and generally resemble those of other species in Tylenchomorpha (Mounport et al., 1993a; Mounport et al., 1993b; Mounport et al., 1997; Valette et al., 1997) but differ from *Malenchus* (Qing et al., 2017) by the presence of radial striae at the basal zone.

The spermatozoon in female spermatheca consists of amoeboid bipolar cells subdivided into a pseudopod and a main cell body (Fig. 6A, B). The main cell body consists of a centrally located nucleus, many spherical mitochondria and membranous organelles (MO) (Fig. 6C). The nucleus is round, lacks a nuclear envelope and has highly condensed nuclear chromatin. MO has an irregular shape and has finger-like invaginations of the outer membrane. The pseudopod is devoid of organelles and consists of fibrous elements. This assembly is similar to with other known Sphaerularioidea (*Contortylenchus genitalicola*, *Deladenus* sp.) (Yushin et al., 2006, 2007) and Anguinidae (*Ditylenchus arachis* and *D. dipsaci*) (Slos et al., 2015) but different from Hoplolaimina *sensu* Siddiqi (2000) (Yushin et al., 2011), which lack MO. Such observations also concur with our phylogeny that the new species is more closely related to Sphaerularioidea than Tylenchoidea.

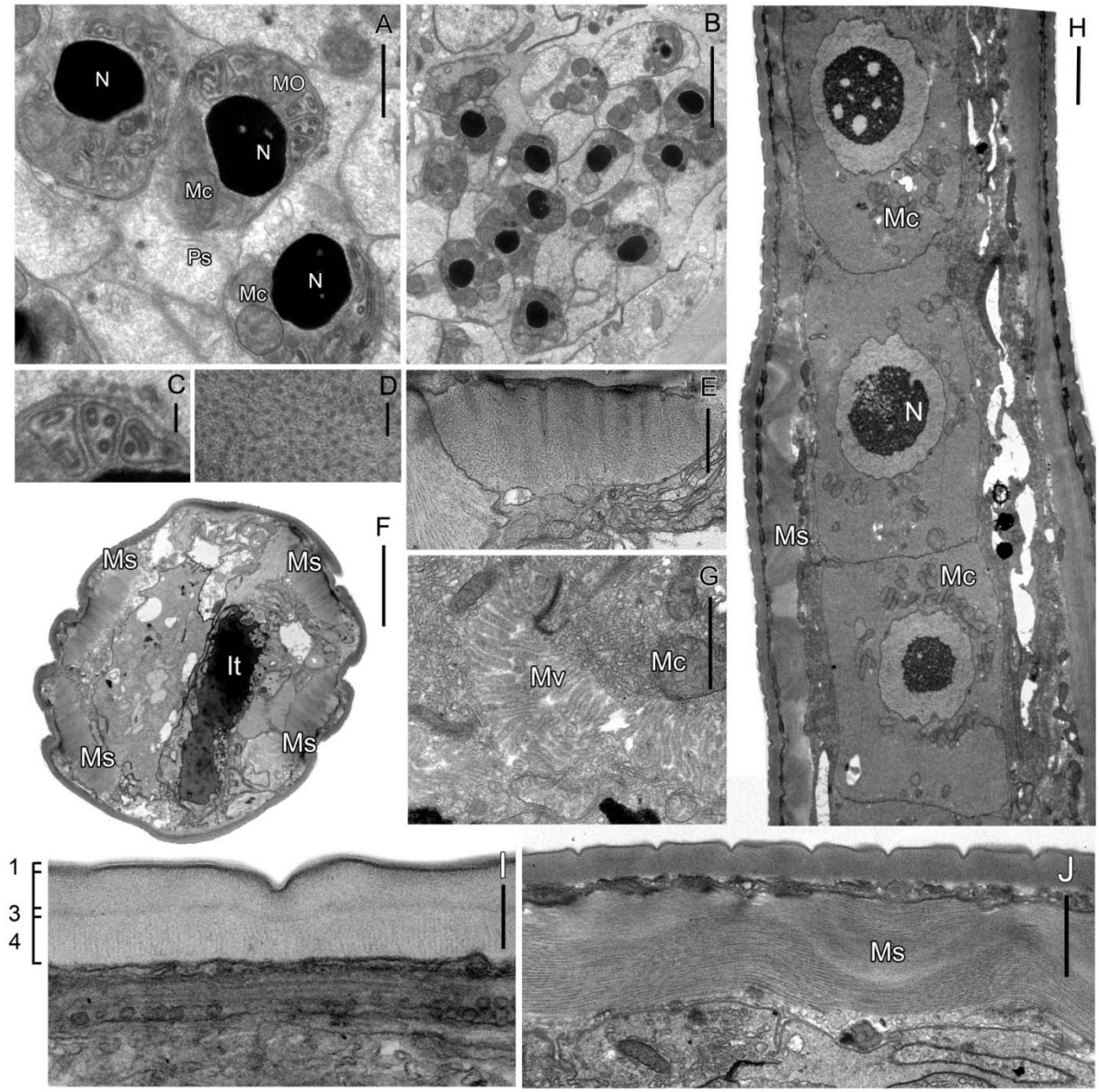

Figure 6. Ultrastructure of female mycophagous *Abursanema quadrilineatum* n. sp. (A) matured sperm cells. (B) spermatheca. (C) membranous organelles. (D, E) cross view of somatic muscles. (F) cross view of middle body. (G) longitudinal view of intestine. (H) chain of oocytes in posterior part of ovary. (I, J) longitudinal view of cuticle. Cuticular layers: (1) epicuticle. (2) cortical zone. (3) median zone. (4) basal zone. Abbreviations: MO=membranous organelles; Ps=pseudopod; N=nucleus; Mc=mitochondria; Ms=muscles; Mv= microvilli; It=intestine. Scale bars: A = 0.5μm; B, H = 2μm; C, D = 0.1μm; E, G, J = 1μm; F = 4μm; I = 0.3μm.

Intestinal crystals

The needle-shaped crystals were recovered from the intestine of mycophagous males or females (Figs. 1J, 5D). In a few specimens, they were also found to be present in the spermatheca (Fig. 4F). Similar crystals were also located in the intestine of females of *Praecocilenchus rhaphidophorus* Poinar 1969 (Aphelenchoidea) parasitizing the palm weevil's haemocoel and in the male genital tract from the free-living stage of *Rhabditis pseudoteres* (Rhabditidae) (Schulte, 1989). Remarkably, although belonging to diverged lineages, these nematodes share similar insect parasitic/associated life stage, indicating that these crystals may play an important role in nematode-insect interaction.

Poinar (1969) assumed that these crystals are probably formed during development within the digestive system and may represent waste products stored in a non-toxic state, but this was not further analysed. Urea and proteins are the two most important crystal-forming products that are involved in the animals' digestive metabolism. However, both were excluded as the crystals are insoluble in water at room temperature (*ca.* 20°C) and they do not stain blue in a Bradford protein assay. Another candidate chemical is calcium oxalate. This can form needle-shaped crystals, which are widely presented in plant and fungi tissue and versatile agents in calcium regulation, plant protection, detoxification (e.g., removal of heavy metals or oxalic acid) and ion balance (Franceschi and Nakata, 2005; Gadd et al., 2014; Whitney and Arnott, 1987). However, the idea of calcium oxalate acting as the main component was also excluded, since the crystal can be dissolved in a 5 % solution of sodium hypochlorite. A further attempt at using TEM for crystal ultrastructure analysis was made, but unfortunately the crystals could not be recovered after TEM fixation.

Rao and Reddy (1980) use the absence of crystals as one of the taxonomic characters to differentiate species in the genus *Praecocilenchus*. However, our observations indicate that crystals were not always present in mature mycophagous adults, meaning that this character is more likely to be related to metabolic products in a certain life stage rather than being a stable morphological character, and therefore should not be used to differentiate species.

ribsomal RNA Secondary structure

The secondary structure model for *Abursanema quadrilineatum* n. sp. and Sphaerularioidea fit the universal model of the D2 and D3 fragments of 28 rRNA for eukaryotic organisms (Wuyts et al., 2001) and other reported models in nematodes (Bae et al., 2010; Douda et al., 2013; Subbotin et al., 2007). For the new species, a total of 372bp for D2 and 168bp for D3 domain were folded into five helices (Fig. 7A, B) and are named as C1-C1/e4 and D2-D6 respectively following Wuyts et al. (2001). The helices base pair compositions of D2/D3 domains are as follows: Watson-Crick pairs = 100/43 (68.5/79.6%), wobble guanine-uracil pairs = 43/11 (29.4/20.4%) and other non-canonical pairs = 3/0 (2.1/0%). The *A. iranicum* has a similar length in both D2 and D3 domains but with substitution, insertions and deletions appearing both in loops and helices. Conversely, sequence lengths vary greatly in Sphaerularioidea from 371bp (*Deladenus* sp., JX104317) to 580bp (*Skarbilovinema laumondi*, JX291136) and from 163bp (*Contortylenchus* sp., DQ328731) to 236bp (*Wachekitylenchus bovieni*, DQ328732) in the D2 and D3 domains respectively. Further variability mapping suggests these unusually long fragments resulting from insertions, mostly in multi-branch loops or internal loops (Fig 7C, D), in contrast to helices with relatively conserved lengths.

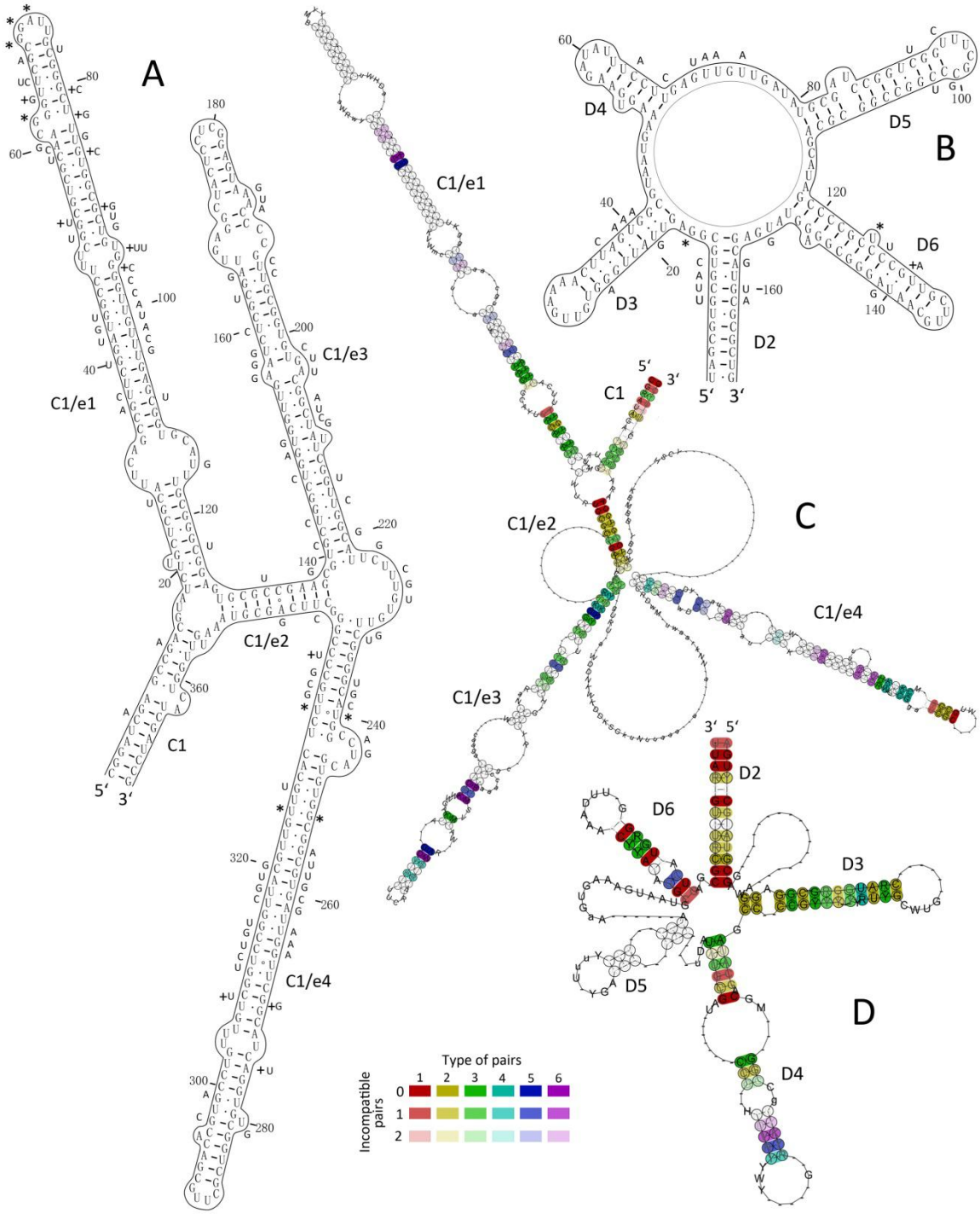

Figure 7. Predicted secondary structures and variability maps of the D2 (A, C) and D3 (B, D) domain of 28S rRNA in *Abursanema quadrilineatum* n. sp. (A, B) and Sphaerularioidea (C, D). *Abursanema iranicum* (KF885742) is compared with the new species and means insertion (plus) and indels (asterisk) are mapped next to the corresponding postion. Watson-Crick base pairs are indicated by dashes, wobble guanin-uracil pairs are represented by a solid dot, all

other non-canonical interactions by a hollow circle. In the variability maps of Sphaerularioidea (C, D), compatible base pairs are coloured in order to show sequence conservation of the base pairs, where the hue shows the number of different base pairs types presented in each site and saturation of colour decreases with the number of incompatible base pairs (*eg.* If G-C, A-U and A-G present in one site, then type of pairs counts as three and incompatible pairs count as one).

Phylogenetic relationship

The phylogenetic outcome inferred by 28S and 18S rRNA suggests that all studied families in Sphaerularioidea are polyphyletic, except for Sphaerulariidae. Both genes are congruent in placing *Abursanema quadrilineatum* n. sp. (Figs. 8, 9) as sister to *A. iranicum* but differ in support value: strongly supported in 28S rRNA (BI= 100, BS=99) while only weakly supported by 18S rRNA (BI = 75, and not supported by BS).

The phylogenetic relationships among the early diverging (= with supposedly ancestral characters) taxa Tylenchidae, Anguinidae and Sphaerularioidea is subject to discussion. Anguinidae are considered by some to be placed inside Sphaerularioidea (De Ley and Blaxter, 2002; Ryss, 1993; Siddiqi, 1986), while others situate them close to the Tylenchidae (Brzeski, 1998; Maggenti et al., 1987; Siddiqi, 2000). Our phylogeny results can not reject nor support the above assumptions, but do show evidence that insect-parasitic Sphaerularioidea and fungal-feeding or plant-parasitic Anguinidae are separate evolutionary lineages (*Nothotylenchus acris* is a single exception, but since no further information is available on that sequence, its identity cannot be confirmed). Thus, feeding habits and life cycle may be phylogenetically informative, information that is especially valuable for these morphologically similar and taxonomically vague groups.

Siddiqi (2000) considered the family Paurodontidae as *familia dubia* and pointed out that it could be a junior synonym of Sphaerulariidae due to the similarity in morphology (stem-like/tubular extension in pharynx which penetrating into intestine), and this opinion has been shared by several authors (Andrássy, 2007; Esmaeili et al., 2016; Handoo et al., 2010).

Our result confirms the closely-related nature of most Paurodontidae and Sphaerularioidea, but the exact phylogenetic relationships remain unclear. Based on the few currently available data, Paurodontidae is polyphyletic, as is the combination of Paurodontidae and Sphaerulariidae (Sphaerulariidae sensu Andrássy, 2007), in keeping with most other Sphaerularioidea families. Such a problematic taxonomical status is to be expected, as these families are characterised by both low observational resolution in LM and polymorphic morphology in different life stages. Therefore further efforts in detailed morphology, including the parasitic stage, as well as host information is needed to clarify this, and new species should only be described when sufficient and informative data have been accrued.

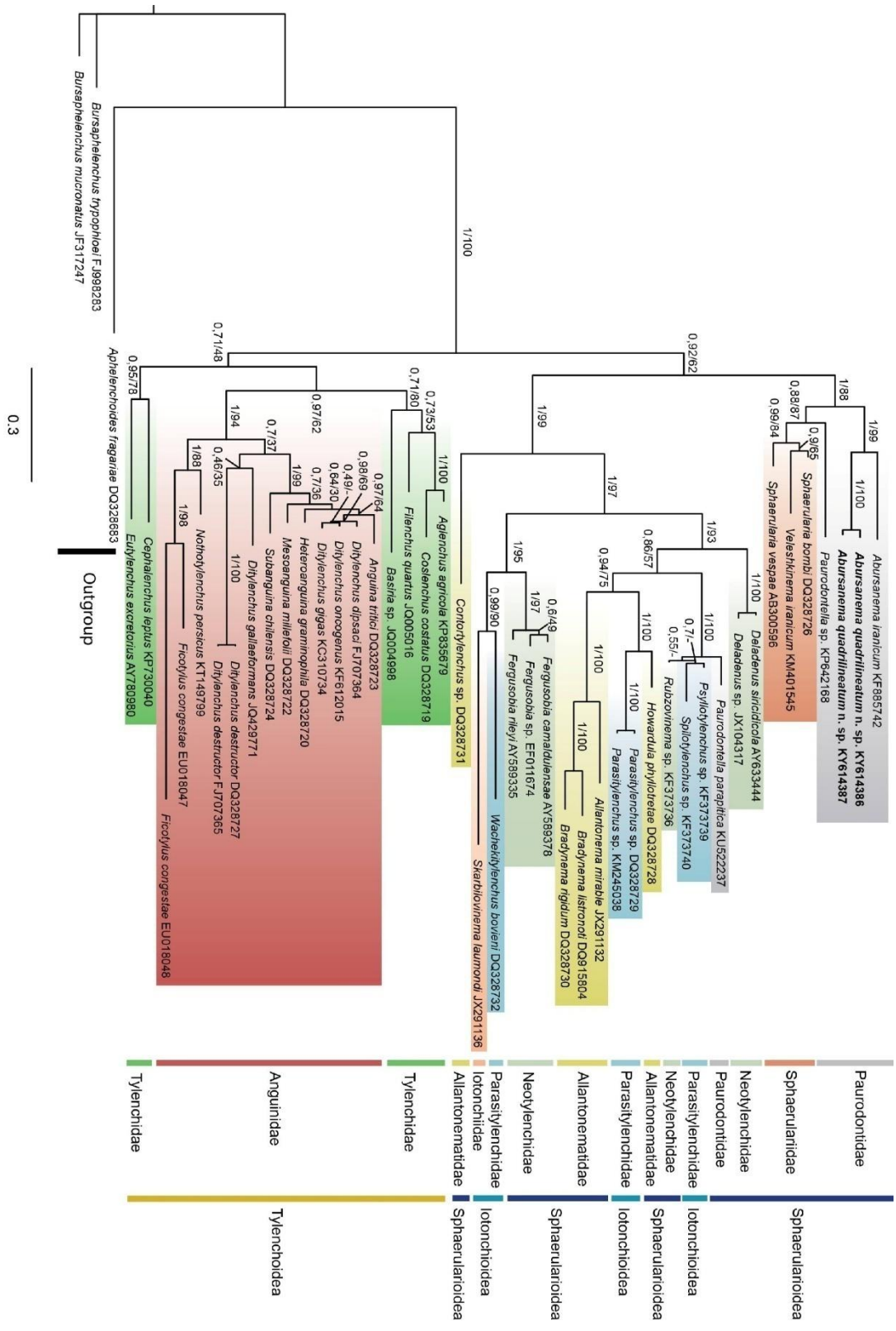

Figure 8. Bayesian 50% majority rule consensus tree interfered on 28S rRNA. The new species is indicated in bold. Branch support is indicated in following order: PP value in BI analysis/BS value from ML analysis. The family level taxonomy follows Siddiqi (2000).

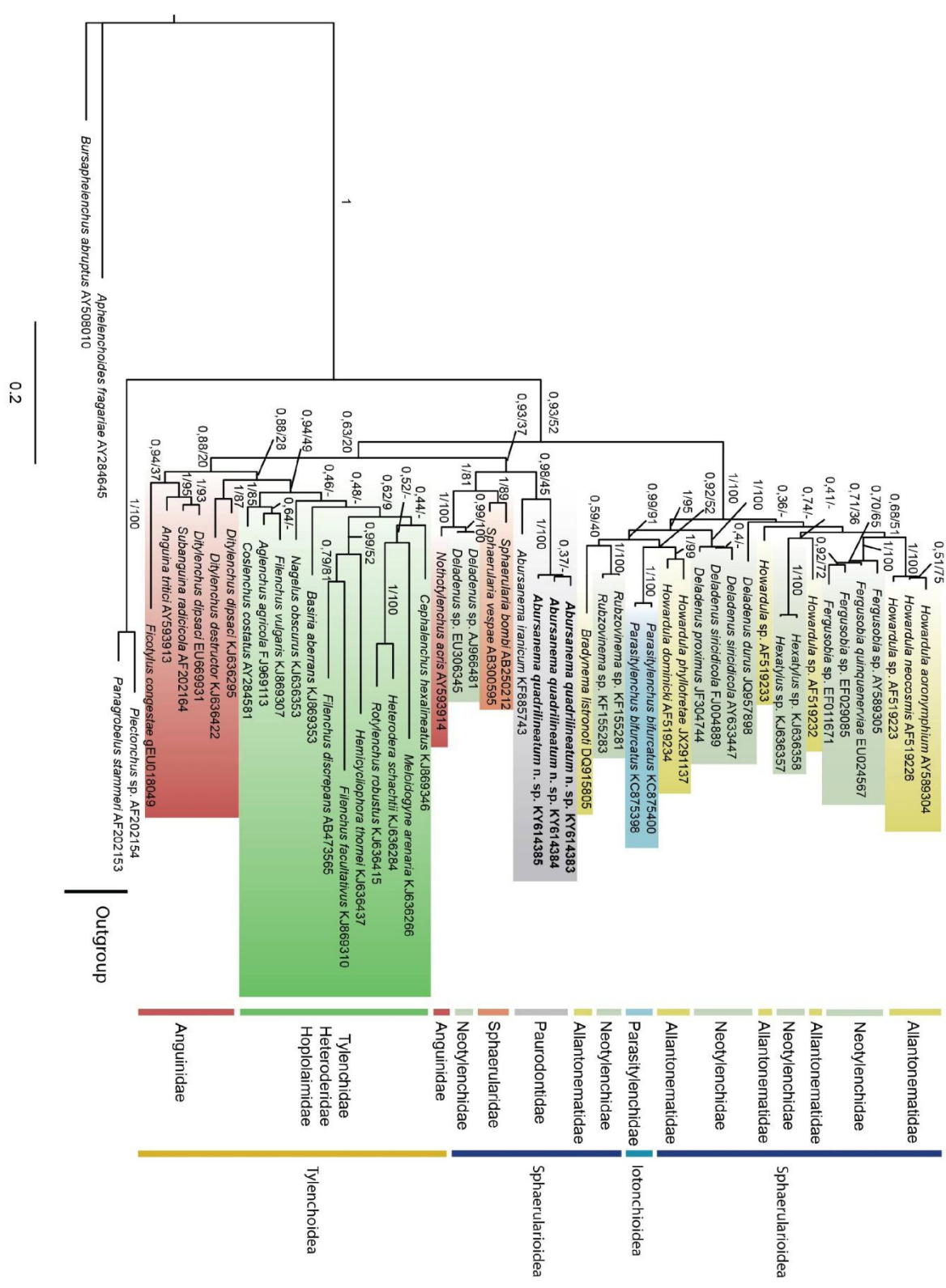

Figure 9. Bayesian 50% majority rule consensus tree interfered on 18 S rRNA. The new species is indicated in bold. Branch support is indicated in following order: PP value in BI analysis/BS value from ML analysis. The family level taxonomy follows Siddiqi (2000).

Acknowledgements

We thank Prof. Harry Horner, Prof. Bart Braeckman and Dr. Ineke Dhondt for their valuable suggestions and help during crystal analysis. We also thank Lieke Moereels for her observations on this species in the framework of her bachelor thesis. We are grateful to Marjolein Couvreur for SEM analysis. The first author thanks the China Scholarship Council (CSC) for providing a Ph.D. grant. This work was supported by a special research fund [grant number: UGent 01N02312].

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Chapter IX

Three-dimensional modeling and printing as tools to enhance education and research in Nematology

Chapter published as:

Qing X[1]., Sanchez Monge GA.[2], Bert W.[1] (2015) 1Three-dimensional modelling and printing as tools to enhance education and research in Nematology. Nematology, 17: 1245-1248.

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Introduction

Three-dimensional (3D) modeling has shown an increasing number of applications in different fields as it eases the understanding and enhances the representation of complex 3D structures and objects (Murakawa *et al.,* 2006). Within biological sciences, several tools and techniques have been used to build 3D representations of organisms, *e.g.* serial images acquired from transmission electron microscopy (TEM), confocal laser scanning microscopy (CSLM), scanning electron microscopy (SEM), digital single-lens reflex camera (DSLR), µ-CT or light microscopy (LM) reconstructions (Hall, 1995; Bumbarger *et al.*, 2006; Beutel *et al.*, 2008; Ragsdale *et al.*, 2008; Bumbarger *et al.*, 2009; Ragsdale *et al.*, 2009, 2011; Apolonio Silva De Oliveira *et al.*, 2012; Wipfler *et al.*, 2012; Handschuh *et al.*, 2013; Nguyen *et al.*, 2014). However, these techniques require multiple focal planes images, different objective angles, or rotation of the specimen. Furthermore, these techniques are not only time-consuming but often difficult for nematodes given their minute size and high transparency.

Here we propose a relatively simple time-saving method using Autodesk® Maya®, a widely used software in animation and industrial design (Derakhshani, 2012). With this method a 3D model can be created based only on the combination of LM and SEM images, LM serves as a reference for the modeling and the position of internal structures and SEM images are incorporated as a reference for general body shape and surface details. The presented method uses the default tools of the program and this program is three years freely available for students and educators (http://www.autodesk.com/education/free-software/maya).

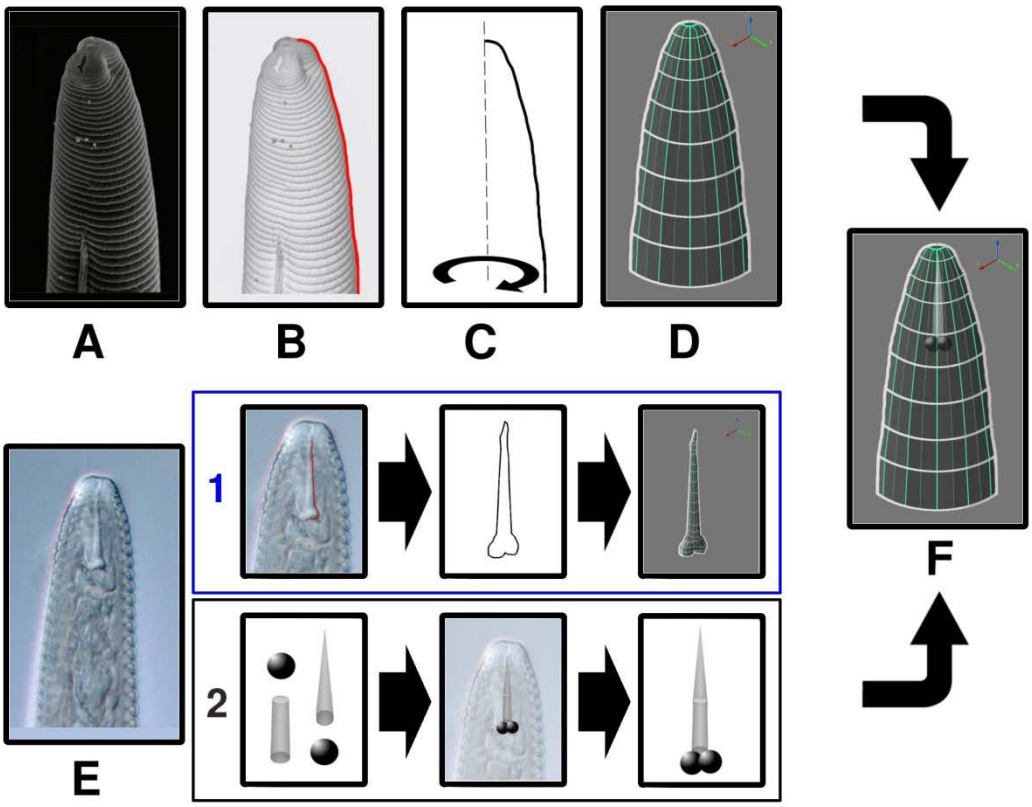

Fig. 1. Schematic representation of the process to create a 3D model of nematodes' structures using Autodesk® Maya®. Scanning electron microscopy (SEM) images (A) provide the base for the surface structure and the construction of the body shape; Light microscopy (LM) images (E) provide information to construct and design the inner structures; A border line (B, E1) is used as a guide to create a 3D object after revolving (C, D); Polygons (E2) can be added and modified to resemble inner and outer structures to obtain a better representation; The final object (F) can be edited as needed.

Result and discussion

In the first step of this method, a SEM image is imported as reference for the exterior [View>Image Plane>Select reference image] (Fig.1 A), then a line is drawn along the body contour [Creat>CV Curve Tool] (Fig. 1B). A 3D image is created by rotating the created outline around a central axis [Surface>Revolve, output as polygons] (Fig. 1 C, D). This image is modified using the "Attribute editor", by adjusting the "V" and "U" values to increase or

decrease the number of lines in the same axis (Fig.1 D, F), allowing a more detailed reconstruction. After shaping the basic design, a more realistic view is achieved by adding details provided by additional SEM images using the appropriate program tools (e.g. Move/Scale/Rotate). Internal structures are reconstructed based on imported LM images that work as reference (Fig.1 E). Structures are created following the outline (Fig.1 E1) or by importing and modifying default polygons that resemble the structures, via the program tools (Fig.1 E2). For an optimal combination of both reconstructions, the structures and the 3D representation of the body need to be set to the same scale. As an example, a representative mononchid head is presented in Fig. 2 (A-E), such image file can be rotated and observed in the program from any angle.

From the final 3D reconstruction, an anaglyph image (a stereoscopic 3D effect) can be easily created by combining two separate views of the same object in a slightly-tilted position (Fig. 2F). Hereby, the red color channel is suppressed in one of the views and the green and blue channels in the other. When both images are merged only the cyan and red channels are visible to the eye and a stereoscopic 3D effect is achieved with 3D red-cyan glasses. Such composition can be made in on-line websites or in an image edition program within few minutes. The prepared 3D model can be also exported as a ".stl" file (File > Export All or File > Export Selection) in Autodesk® Maya® and printed in a 3D printer (Fig. 2, G-K). The executable 3D printing file, the video during printing, additional high resolution 3D images and the anaglyph file of the mononchids' head are available at: http://nematodes.myspecies.info

Although there is an inherent learning curve regardless of the modeling program (Murakawa *et al.*, 2006), the presented method allows the reconstructing of a 3D model within few days. Several other freeware options are available, e.g. Blender (https://www.blender.org). There are many discussions on advantages and disadvantages, but in general both programs are similar, users can learn one within a short time if they have experience of another one. Therefore choice depends on user's personal preference.

Evidently, the accuracy of the final reconstruction is not comparable to 3D reconstruction

of serial TEM sections or electron tomography techniques. This technique is not meant to provide a completely realistic image, but rather to present anatomical aspects in a more comprehensible way. In a scientific context, this method has already been shown to be valuable in other taxa (Klaus *et al.*, 2003; Nguyen *et al.*, 2014) and it can be incorporated as a complement to pictures and drawings of (new) nematode descriptions and to illustrate complex 3D structures. The wide spectrum of applications in nematological teaching includes 3D representations, with or without 3D glasses, and 3D printed models in the classroom.

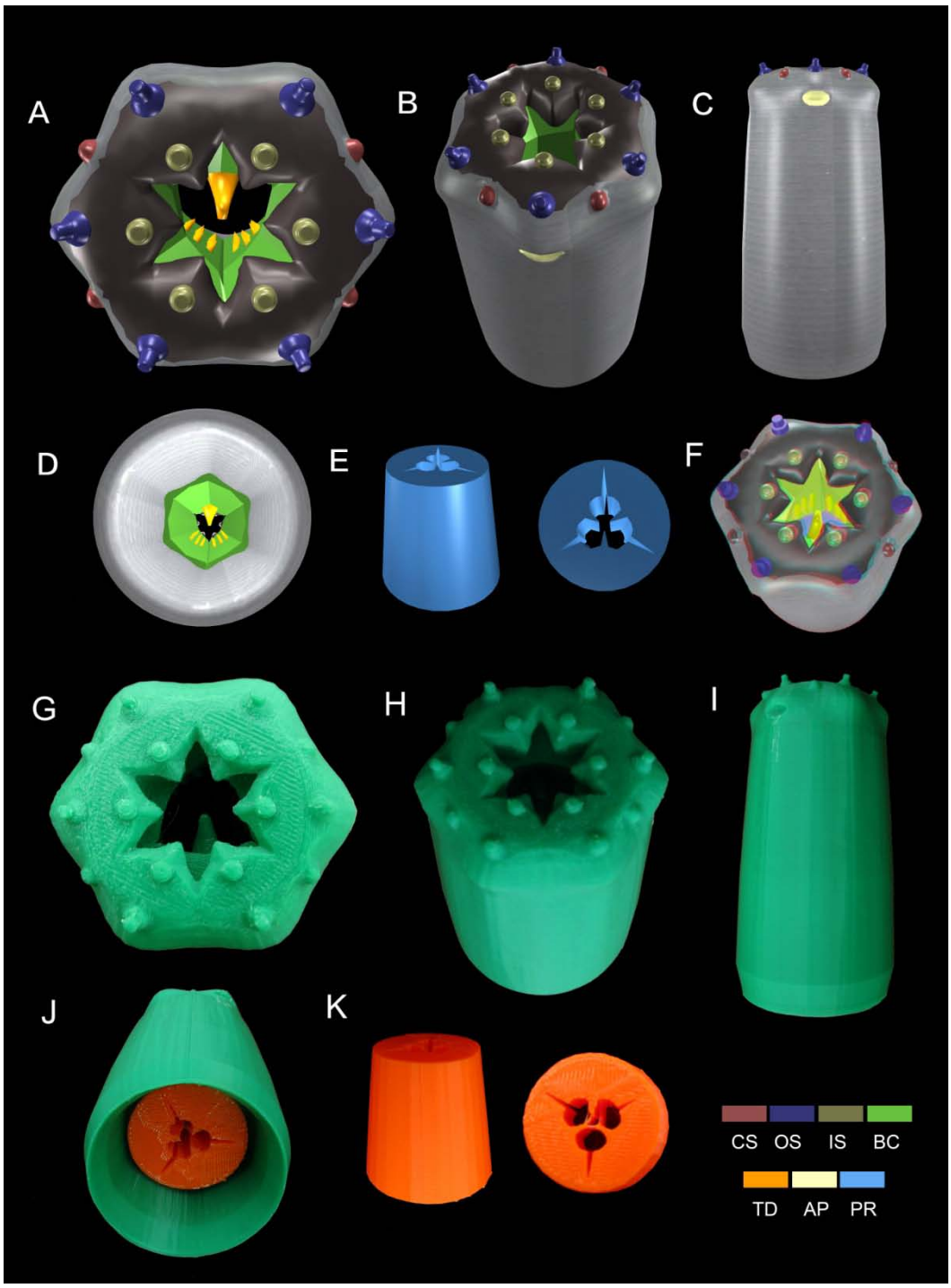

Fig.2. 3D models of typical mononchid head region. A-E: 3D images rendering from models built by Autodesk® Maya® software. A, B: *en face* view showing six inner and outer labial sensilla, and four cephalic sensilla; C: Lateral view; D: Cross view of head shows buccal cavity; E: Different views of anterior pharynx; F: Anaglyph image of head (need red/blue glasses to see the image in 3D); G-K: 3D prints of the models built by Autodesk® Maya® software (Printer: Makerbot® Replicator® 2, Model: 13cm high by 6 cm wide); G, H: *en face*

view; I: Lateral view; J: Cross view of head showing position of anterior pharynx; K: Different views of anterior pharynx. Legend for color bars: CS: Cephalic sensilla; OS: Outer labial sensilla; IS: Inner labial sensilla; BC: Buccal cavity; TD: Teeth and denticles; AP: Amphidial aperture; PR: Pharynx.

Acknowledgments

We thank Prof. Wilfrida Decraemer for her valuable suggestions on the head structure. Sandra Vangestel and Pieter De Clercq (Department for Educational Innovation, Faculty Education Services, Faculty of Sciences, Ghent University) are acknowledged for their innovative ideas and continuous support to improve nematological education.

The first author would like to acknowledge the China Scholarship Council (CSC) for his PhD grant. The second author acknowledges the MICITT, CONICIT, University of Costa Rica and PEACE Project (Erasmus Mundus) for financial support.

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Chapter X

General discussion and conclusions

Diversity and ecology of Tylenchidae

Family Tylenchidae is an important group in Tylenchomorpha. Currently, this family comprises a total of 44 genera and 411 nominal species. The most cosmopolitan genus is *Filenchus* and this genus has been reported from all continents except Antarctica, while 17 genera are monotypic and endemic to very limited locations. Species numbers in each of genera vary greatly: *Aglenchus* 8 spp., *Antarctenchus* 1 sp., *Atylenchus* 1 sp., *Coslenchus* 38 spp., *Pleurotylenchus* 2 spp., *Atetylenchus* 4 spp., *Basiria* 43 spp., *Boleodorus* 30 spp., *Neopsilenchus* 9 spp., *Neothada* 6 spp., *Psilenchus* 21 spp., *Ridgellus* 1 sp., *Thada* 1 sp., *Chilenchus* 1 sp., *Ecphyadophora* 8 spp., *Ecphyadophoroides* 2 spp., *Epicharinema* 1 sp., *Lelenchus* 4 spp., *Mitranema* 2 spp., *Tenunemellus* 6 spp., *Tremonema* 1 sp., *Ultratenella* 1 sp., *Allotylenchus* 1 sp., *Cervoannulatus* 1 sp., *Cucullitylenchus* 1 sp., *Discotylenchus* 7 spp., *Fraglenchus* 1 sp., *Gracilancea* 1 sp., *Irantylenchus* 1 sp., *Malenchus* 38 spp., *Miculenchus* 4 spp., *Polenchus* 3 spp., *Sakia*, 7 spp., *Silenchus* 1 sp., *Tanzanius* 1 sp., *Tylenchus* 28 spp., *Filenchus*: 96 spp. *Arborityncbus* 1 sp., *Campbellenchus* 2 sp., *Cephalenchus* 20 spp., *Eutylenchus* 6 spp., *Tylodorus* 2 spp., *Labrys* 1 sp., *Discopersicus* 1 sp. (based on valid species listed in Geraert (2008) and other recent described species in Bert *et al.*, (2010), Mundo-Ocampo *et al.*, (2015), Yaghoubi *et al.*, (2015), Alvani *et al.*, (2016), Soleymanzadeh *et al.*, (2016), Qing *et al.*, (2016), Yaghoubi *et al.*, (2016), Mehrabian *et al.*(2017), Mehrabian *et al.*, (2017), and Qing *et al*. (Chapter VI).

Although the family Tylenchidae is a very common group and represented in various habitats, the actual diversity of this group is far from settled. Despite very few taxonomist worldwide are working on this group, two new genera have been reported (Chapter V, Yaghoubi *et al.*, 2016) in only the last two years. Furthermore, although the genus *Malenchus* is already one of the most specious genus in Tylenchidae and only limited samples were examined during this study, yet two new species (Chapter II, VI) and three putative new species were discovered (unpublished data). It is very likely that only a fraction of the species of Tylenchidae is known: (1) most were described from the rhizosphere of economically important crops while natural ecosystems (*e.g.* forest, meadow and swamp) harbour a

significantly higher diversity of Tylenchidae compared to agro-ecosystem (Chapter IV); (2) nearly all Tylenchidae species are described from soil habitats, while both morphological and metagenomics studies (Porazinska *et al.*, 2010; Qing *et al.*, 2015) found a high diversity of Tylenchidae in litter and/or canopy (*e.g.* 80% of the species in the temperate rainforest resided in the soil, whereas only 20% in the tropics); (3) metagenomics studies suggested that tropic nematode diversity is significantly higher compared to the much better sampled temperate environments (Porazinska *et al.*, 2010; Porazinska *et al.*, 2012); and (4) the family Tylenchidae comprises cryptic species (*e.g. M. pachycephalus*, *M. acarayensis*, Chapter III) and therefore some of the nominal species are actually species complexes.

Several approaches have been developed to estimate species diversity: *e.g.* based on body size frequency distributions (May, 1988), host-specificity and spatial ratios (Erwin, 1982), time-species accumulation curves (Bebber *et al.*, 2007), patterns of higher taxonomic classification (Mora *et al.*, 2011; Bartels *et al.*, 2016), and based on metagenomic data (Ni *et al.*, 2013). However, these methods either require massive data collection and subsequent analyses (which is beyond the main scope of this thesis), or are based on assumptions that doesn't hold for Tylenchidae. Although a founded estimation is not possible from the data obtained in this study, we attempt to provide a rough estimation of Tylenchidae species number based on the ratios-between-taxa method follow Hawksworth (1991). Based on the samples examined during this study (from the tropical region, temperate Europe, and temperate and subtropical Asia), we observed that from a given sample Tylenchidae species are 1-5 times more diverse compared to obligate plant-parasitic Tylenchomorpha (PPT). Since infraorder Tylenchomorpha contains 2240 (Andrássy, 1992) or 2828 species (Siddiqi, 2000) while *ca.* 400 and 300 species are from the family Tylenchidae and superfamily Sphaerularioidea (insect parasitic and/or mycophagous nematodes) respectively, PPT comprise 1500-2100 species. Given the economic importance for crops and the higher plants, PPT are relatively well studied. Assuming that PPT species have mostly been described and giving that, based on our observations, Tylenchidae are equal to five times more diverse compared to PPT, around 2000-10,000 Tylenchidae species can be estimated . However, this estimation is very conservative, given the fact that several new PPT can be expected from

under-investigated natural habitats and the presence of cryptic species in PPT (Palomares-Rius *et al.*, 2014).

The diversity of Tylenchidae is best known in arable land of Europe, while the diversity in natural ecosystems in tropical and subtropical largely remains to be discovered. We expect, although this is not more than a wild guess, that 70% of the undiscovered species are from "neglected" regions, 20% from well studied regions and 10% are cryptic species.

With the growing application of metagenomics and integrative taxonomy approaches, putative new species will be discovered at an increasing speed. However, giving the lack of specialists/taxonomist and the minor economic impact of Tylenchidae, most of these species will have to wait for their formal description. Therefore, most likely not more than 2-3 species can be expected to be described each year and the majority of Tylenchidae species will remain completely undescribed for a long time period.

Feeding habitats in Tylenchidae is one of the most important discussion points amongst nematologists (Bongers & Bongers, 1998). They do not cause economic losses to crops and, although without experimental support, they were treated as root hair feeders (Bongers & Bongers, 1998) or algal, moss feeders (Siddiqi, 2000) due to weakly developed stylet. Some *Filenchus* species can be cultured on fungi (Okada, 2002), but this is not the case for other species/genera (*e.g. Malenchus*). Therefore, feeding behavior may be highly diversified in Tylenchidae and more researches are needed to clarify this.

Taxonomy and phylogeny in Tylenchidae, overview of the genera

Tylenchinae

Filenchus

Filenchus is clearly a polyphyletic genus, as shown by several molecular evidences (Bert *et al.*, 2008; Holterman *et al.*, 2008; Atighi *et al.*, 2013; Pereira *et al.*, 2017; Qing *et al.*, 2017) and supported by the fact that *Filenchus* is more characterised by the absence of characters than the presence of clear apomorphic characters.

Current molecular phylogeny suggests that all four-incisures species forms a well-supported clade (Qing *et al.,* 2017). Since the type species (*F.vulgaris*) is nested inside this clade, it makes the most sense that in case of a review this clade retains the genus name. The two-incisures species are more complex as they form several clades and probably they need to be split into several genera. Future studies should pay more attention to detailed morphology, for example the newly described two-incisures genus *Labrys* (Chapter V) is morphologically clearly different from *Filenchus*, but based on superficial observations this genus could be misidentified as *Filenchus*. Those *Filenchus* with multiple sub-ridges are more close to *Malenchus* and should consequently be transferred to this genus. Also, several species are reported with three incisures, these species may have three genuine incisures or observations may be the result of four incisures but with two inner incisures very closed to each other. In such case, the former may present a separate clade and the later may similar to four-incisures clade.

Tylenchus

Tylenchus is also a polyphyletic genus. The conventionally used tail shape and stylet cone/shaft proportion do not define a natural clade. Those large sized (above 700 µm) *Tylenchus* (*T. davainei*) are related to some of *Filenchus* (*F. aquilonius* and *F. andrassyi*) with similar large size (such size is rare in *Filenchus*). Similarly, small sized *Tylenchus* spp. (*T. arcuatus*), are placed in a *Filenchus* clade, indicating size may be relatively important.

Malenchus

This genus represents a divergent linage from other genera in Tylenchinae (Fig. 1) and should be removed from Tylenchinae. This genus is very likely related to some of the Ecphyadophorinae, supported by a shared pouch-like amphidial fovea. However, molecular phylogeny provides different or even contradictory conclusions and the actual placement remains unknown (Chapter III).

Miculenchus

This genus should also be removed from Tylenchinae giving its divergent position.

Although phylogeny suggested a sibling relationship with *Malenchus*, *Lelenchus* and *Tenunemellus*. However, tree reconstruction errors are possible because of long branch attraction (Chapter VI).

Tanzanius

This genus is unique in Tylenchomorpha in the shape and structure of the stylet and pharynx. The pharynx and the short tail of *Tanzanius* are divergent from other Tylenchinae or Tylenchidae, and probably related to Paratylenchidae as suggested by Andrássy (2007). However, molecular data for this genus are currently unavailable and therefore a taxonomic assignment is not yet possible.

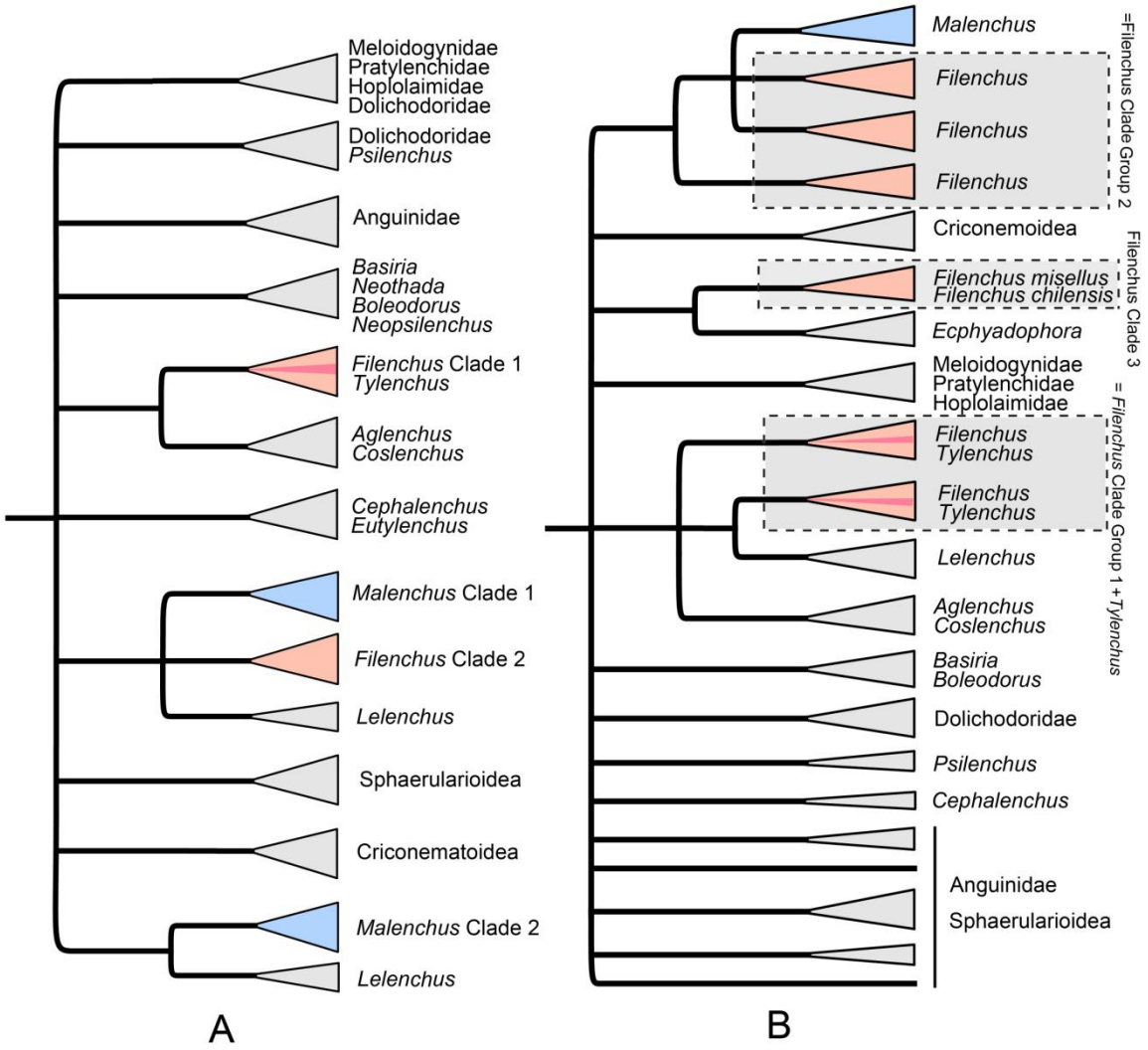

Figure 1 Summary of phylogenetic position of the Tylenchidae in Tylenchomorpha inferred from 28S rRNA (A) and 18S rRNA (B). Node with low support values (BS<80,

PP<98) are collapsed (Qing *et al.*, 2017).

Boleodorinae

This subfamily is relatively well-defined by its unique slit-like amphidial aperture and also each of the genera has an obvious genus-specific trait. However, *Psilenchus* and *Atetylenchus* have a didelphic reproductive system and are separate from other Tylenchidae based on a molecular phylogeny (Holterman *et al.*, 2006; Bert *et al.*, 2008). Therefore, they should be removed from the family Tylenchidae. This is in agreement with Bert *et al.* (2008) that the presence of a didelphic or monodelphic reproductive system is relatively important in tylenchid phylogeny.

Ecphyadophorinae

Species belonging to Ecphyadophorinae are among the most remarkable of all Tylenchidae. So far little is known about this subfamily, but several morphological traits (contrasting head, amphidial aperture, vulva and bursa shape) and molecular data (*Lelenchus* and *Ecphyadophora* are separated in 18S rRNA phylogeny) suggest that Ecphyadophorinae is a heterogeneous group. Currently, no specific trait has been found for this subfamily except the extremely slender body (Siddiqi, 2000; Geraert, 2008).

Ecphyadophora

This genus is probably related to *Tremonema* and *Mitranema* based on its similar pore-like amphidial aperture and lobed bursa. It is different from other Ecphyadophorinae by a pore-like amphidial aperture and the absence of a pouch-like amphidial aperture. Molecularly, *Ecphyadophora*is grouped with *Filenchus misellus*, *F. chilensis* and *Labrys chinensis*. However, *Ecphyadophora* is the type genus for the subfamily and thus the validation of Ecphyadophorinae should be reconsidered. Yet, due to a lack of molecular data a taxonomical act reflecting this position is not appropriate at this time.

Ecphyadophoroides, *Lelenchus* and *Tenunemellus*

These genera may be closed related based on a similar long slit-like amphidial aperture and distinct amphidial fovea, and this is supported by molecular phylogeny. These genera may also closely related to *Chilenchus* and *Malenchus*, see Fig. 2.

Epicharinema

This genus has an unknown position. *Epicharinema* has dorso-ventrally fattened cephalic region (probably because of pouch-like amphidial aperture) and long slit-like amphidial aperture, which resemble to *Ecphyadophoroides*, *Lelenchus* and *Tenunemellus*. However, the pronounced median bulb with a valve in the pharynx and well-developed stylet suggest it may represent a different lineage. Currently, no molecular data are available for this genus and therefore no taxonomical act was taken in this thesis.

Ultratenella

Ultratenella This genus was assigned to Ecphyadophorinae, only because of its exceedingly thin body. However, it is probably related to with *Ecphyadophora* based on its similar vulva region and amphidial aperture (described as invisible for *Ultratenella* but probably pore-like).

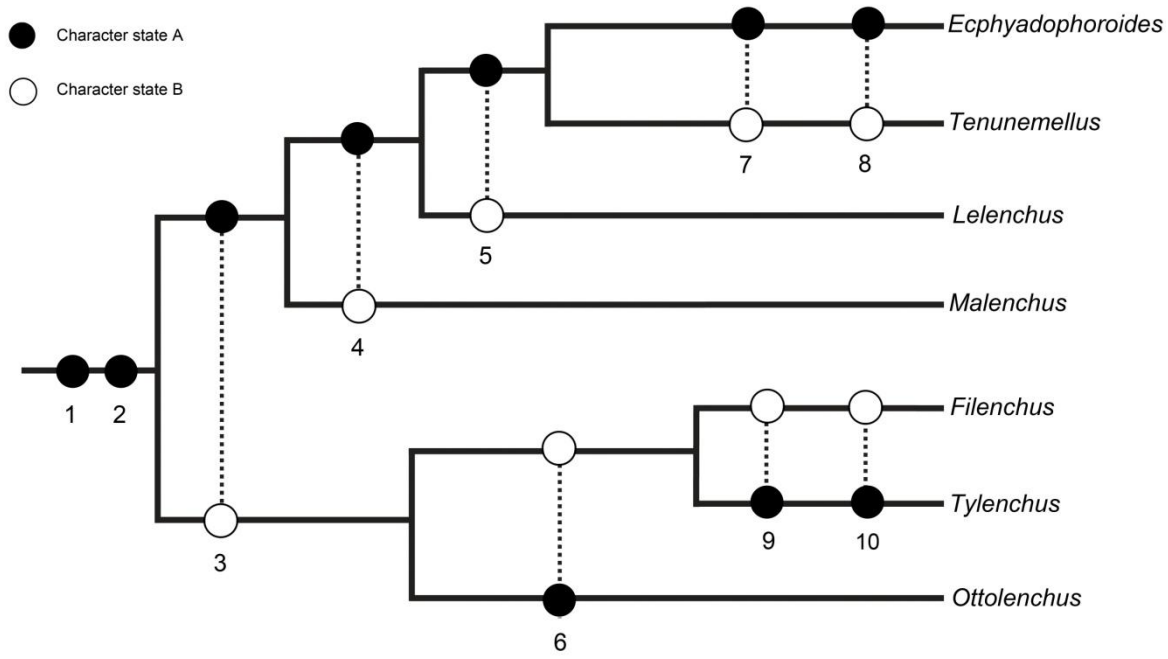

Figure 2 Hypothesis of cladogram based on informative morphology traits in combination with a molecular phylogeny of Qing *et al.* (2017). The tree shows possible phylogenetic relationship of genus *Malenchus* and other related genera based on morphology characters. *Ottolenchus* is treated as a valid genus (*vs* synonym of *Filenchus* [Raski & Geraert, 1986; Brzeski, 1997; Geraert, 2008]) as two and four incisures *Filenchus* nested in divergent lineages. Character states are arranged as A/B. Character 1. filiform tail. 2. monodelphic female. 3. Conspicuous pouch shape amphideal fovea/indistinct amphideal fovea. 4. vagina wall thin/vagina wall well developed. 5. bursa rectangular/bursa simple with convex margins. 6. lateral region one ridge forming two incisures/ lateral region with four incisures. 7. cuticle coarsely annulated/cuticle relatively smooth. 8. cuticle with longitudinal lines/cuticle without longitudinal lines. 9. Heavily sclerotized stylet with cone half of total length/weakly sclerotized stylet with cone less than half. 10. large, round amphideal aperture confined to labial plate/slit-like aperture extending 3-4 annuli beyond labial plate (Qing *et al.*, 2017)

Tylodorinae

Currently relatively few information is available for the Tylodorinae. The only available molecular data suggest that *Cephalenchus* and *Eutylenchus*are related, but in a divergent phylogenetic position in respect to other Tylenchidae (Pereira *et al.*, 2017). The genus *Campbellenchus* is probably also related to *Cephalenchus* and *Eutylenchus* because of the similar stylet, but molecular data are needed to confirm this view. *Tylodorus* and *Arborityncus*have extreme long stylets and a different pharynx (procorpus bulbous *vs* elongated in most Tylenchidae) and these two genera may also represent divergent clades.

Problems and perspective in the molecular phylogeny of Tylenchidae

Problems in molecular phylogeny

DNA Extraction and PCR

Tylenchidae are small and morphological traits are difficult to observe. Given most of

Tylenchidae species are not culturable under laboratory conditions and soil samples often contain different similar species of the same genus, accurate sample extraction in combination with a successful PCR can be challenging. In some cases, a single soil sample contains even more than five very similar species from same genus (*e.g. Filenchus*) and this makes identification challenging. A proper identification and photo/video vouchering is important prior to DNA extraction. Moreover, during DNA extraction the single specimen needs to be cut and transfer to PCR tube, a process that needs handling with caution to avoid losing the specimen. In addition, the small quantities of DNA templates obtained from single specimen only allow limited PCR attempts and thus the risk of running without templates is high if the binding of the universal primer fails.

28S and 18S rRNA

In this study we have used 28S and 18S rRNA genes, which are the two most common regions in nematode phylogenetic studies. However, both regions have some limitations to analyse the phylogeny of Tylenchidae. The 28S rRNA has a high substitution rate that introduces multiple substitutions at the same sites and in some taxa (*e.g. Malenchus*, *Lelenchus* and *Miculenchus*) this is likely to cause long-branch attraction (Felsenstein, 1978) and thus obscure the phylogenetic relationships among sequences (Arbogast *et al.*, 2002). Hence, the reliability of 28S rRNA phylogenies for Tylenchidae is limited, even with the use of likelihood methods, which are less sensitive to long-branch attraction (Felsenstein, 1981). However, despite these severe limitations, the 28S rRNA gene has widely been used in phylogeny of Tylenchomorpha (Subbotin *et al.*, 2005; Subbotin *et al.*, 2006; Subbotin *et al.*, 2007; Subbotin *et al.*, 2008; Subbotin *et al.*, 2011), and for Tylenchidae three of the last five studies have been based on 28S rRNA alone (Atighi *et al.*, 2013; Panahandeh *et al.*, 2015a; Panahandeh *et al.*, 2015b; Qing *et al.*, 2015; Yaghoubi *et al.*, 2015). However, based on our results, the obtained tree topologies should be interpreted with caution, and it is recommended that future phylogenetic studies of Tylenchidae do not solely rely on the 28S rRNA gene.

On the other hand, 18S rRNA data have an appropriate substitution rate, but considerable length variation of the reference sequences in GenBank result in a scarcity of homologous

sites in an alignment (coverage limitations). Moreover, 18S rRNA has inadequate informative sites to resolve early diverging Tylenchomorpha (=tylenchs with supposedly ancestral characters, including Tylenchidae, Anguinidae and Sphaerularioidea), even based on full length sequences, the resolution among early diverging group is low (Bert *et al.*, 2008, 2010; Holterman *et al*., 2008; van Megen*et al.*, 2009) and these resolution problems are not likely to be resolved by adding more taxa (Chapter III).

Polymorphism is an additional problem to reconstruct the Tylenchidae phylogeny. The rRNA is supposed to evolve in a concerted manner, such that the different repeats are not independent from one another but instead are homogenized by different mechanisms (*e.g.* gene conversion, unequal crossing over) collectively termed concerted evolution (Dover, 1982). As a result, rDNA polymorphism within a species is expected to be very low or absent. For nematodes, polymorphisms of 18S, 28S rRNA and ITS have been found in *Halicephalobus gingivalis* (Yoshiga, 2014), *Rotylenchulus reniformis* (Nyaku *et al.*, 2013; Van Den Berg*et al.*, 2016,) and two genera of Tylenchidae: *Cephalenchus*(Pereira & Baldwin, 2016) and *Malenchus* (Chapter IX). However, these high intragenomic variations may exist in more taxa of Tylenchidae and their impact to phylogeny still need to be evaluated.

In addition, discordances have been found between 18S and 28S rRNA (*e.g.* monophyly/polyphyly of *Malenchus* and placement of *Lelenchus*), and also considerable variations of support values (PP/BS), even for the same gene, have been observed in this study. This increased the difficulties to interpret Tylenchidae phylogeny.

Taxa density

The sampled taxa used for phylogenetic studies are also limited. Among 44 genera in Tylenchidae, 22 genera do not have any sequence representative. Furthermore, the genera with associated sequences are either only represented by single sequence or by few very short fragments (less than 800 bp or 500 bp in 18S and 28S rRNA, respectively). The lack of taxa has subsequently hampered our understanding of Tylenchidae phylogeny, especially for those divergent genera with many unique characters (*e.g.* in subfamily Ecphyadophorinae, *Tanzanius*).

Perspectives for Tylenchidae phylogeny

Gene selection

The gene selection is important in future phylogenetic studies of the family Tylenchidae.18S and 28S do not provide adequate phylogenetic signals but are certainly still important, as they represent the majority of the Tylenchidae references sequences in GenBank. The multi-genes based phylogenic approaches have been used for many other taxa and show many advantages and several candidate genes are potentially valuable: *e.g.* Hsp90, EF1a alpha, ATPase, ATPsyn, MAT, IF, CAT, Tropo, ALD, GAPDH, PFK, Mio and H3 (Shultz & Regier, 2000; Anderson *et al.*, 2004; Yurchenko *et al.*, 2006; Kim *et al.*, 2008; Paps *et al*., 2009). Evidently, whole-genome phylogeny has a great power to resolve long standing phylogenetic problems (Jarvis *et al.*, 2014) and with the ever-increasing development of NGS techniques, this method will be more affordable and doable in the near future.

Primer design

PCR amplification failure of universal primers (*e.g.* D2A/D3B, G18S4/18P) was relatively common problem in this study and this shows that the primer binding regions are relatively divergent in Tylenchidae.. As few reference sequences are available for Tylenchidae, the design of an universal primer for this family is difficult. Currently, the best option is to use the different available primer pairs and find out the most efficient combinations. However, primers with too short target regions (800 bp in 18S or 500 bp 28S rRNA) should be avoided as they may cause substantially problems in alignment and tree reconstruction (e.g. limited sequences coverage when comparing with reference sequence in database).

Alignment and Tree reconstruction

Phylogenetic trees should be reconstructed with caution, especially for Tylenchidae. The use of the most appropriate alignment method appears to be important, especially when sequences are divergent. Also the use of secondary structures can result in different phylogenetic results which usually have better quality and are more trusted. In tree reconstruction, it’s necessary to use at least two methods (in favor of BI and ML) and interpret the results based on both

support values, as these topologies and support values may differ or even not agree with each other (Chapter VI).

New techniques in morphology and taxonomy

Current problem and limitations

Nematodes are usually vermiform and share a number of plesiomorphic similarities that mask phylogenetic relations. However, on a detailed level they are actually exceedingly diverse in morphology, but light microscopy often fails to provide the appropriate resolution (De Ley, 2000). The family Tylenchidae is a typical example of a taxon that combines small body size and the lack of clearly homologous characters. Hence, taxonomy of Tylenchidae solely based on LM is problematic, although SEM and TEM are relatively widely used for nematodes but both have signification limitations (*e.g.* time consuming, laborious sample preparation).

Data acquisition and processing

Several techniques have been developed to extract detailed morphology in zoology (Hall, 1995; Bumbarger *et al.*, 2006; Beutel *et al.*, 2008; Bumbarger *et al.*, 2009; Wipfler *et al.*, 2012; Handschuh *et al.*, 2013; Nguyen *et al.*, 2014), but not all of them are suitable of nematodes (*e.g.*µ-CT, X-ray), given their minute size and high transparency. In the field of nematology, very limited attempts have been made to introduce alternative techniques. De Ley and Bert (2002) introduced a video capture system to replace images based on only a single image focal plane. Jay Burr and Baldwin (2016) have used confocal microscopy to label the cell boundaries with fluorescent antibodies to analyses stoma structures.3D reconstruction based on serial images based on TEM and LM was used to reconstruct internal structure or head structures (Ragsdale *et al.*, 2008, 2009, 2011,Apolonio Silva De Oliveira *et al.*, 2012). However, these methods are either very time consuming or limited in resolution. Nowadays, with the developing of image acquisition equipment's and processing techniques, increasing number of methods can be used in nematode morphology analysis. For example, focus stacking (a digital image processing technique which combines multiple images taken at

different focus which provide an image with a greater depth of field) was used in this study to improve image quality at high magnifications (unpublished data) using *Helicon* and *Adobe Photoshop*; High-dynamic-range imaging (HDR) that can represent a greater range of luminance levels was used to improve LM morphology visualization, in both dark and bright parts of a LM image (unpublished data); Image J is useful for counting and morphometric measurements; NeuronJ plugin (Popko *et al.*, 2009) can facilitate the tracing and quantification of elongated image structures (*e.g.* pharynx, gonad etc.), and *Voloom* may be potentially useful to reconstruct 3D images based on histological images.

Visualisation of morphological data

The morphology variations in nematodes are small compared with larger animals such as vertebrates and hard to present in a straightforward way. However, a proper visualization of morphology is important for both research and education. Below are some examples that can be used in nematology:

Line drawing

Drawing is the most traditional and most basic technique for taxonomists. The ink line drawing has many advantages especially to present detailed fine structures in high quality. However, drawing is time consuming and difficult to edit afterwards. Computer assisted drawing programs, such as *Adobe Illustrator* are getting recently more popularly, but the standard simple lines usually fail to provide a realistic picture. In this study, a new method was developed based on a combination of *Adobe Illustrator*, *Adobe Photoshop* and ink line drawing. The ink line drawings were scanned and used as brush library in *Adobe Illustrator* to provide gradually varying dots and lines for more complex details while the digital drawing pad together with *Adobe Illustrator* was used for simple lines (*e.g.* body cuticle) or repetitive structures (*e.g.* annulations). Both parts were combined and modified in *Adobe Photoshop* to achieve the final image. The combination of these methods has been used, to the best of our knowledge, for the first time in Nematology and appeared to be very successful

3D modeling

In the field of nematology, 3D modeling has an increasing number of applications in different fields as it eases the understanding by enhancing the representation of complex 3D structures and objects. In this study we introduced in the field of nematology observation/imagination based 3D modeling known from the cartoon and animation business (Chapter IX). This technique improves the illustration of some of complex structures of Tylenchidae (*e.g.* lip region and amphidial fovea of *Malenchus* and *Cephalenchus*, Chapter II). This technique is especially valuable in comparative studies that need to represent a series of structures that can be hardly differentiated based on only 2D line drawings.

3D printing

3D printing technology has been around since the 1980s, it has only recently gained real momentum as a technique as the technology matures and awareness grows. This study explored the possibility of 3D printing in Nematology. The parameters and materials are optimized to fit the requirements of nematology research and printed plastic models have been used for lectures and practical in the framework of International MSc in Agro and Environmental Nematology (Ghent University). Although the accuracy of our models is not comparable to 3D reconstruction based on serial TEM sections or electron tomography techniques, the models are useful and time-efficient complements' to pictures and drawings of species descriptions to illustrate complex 3D structures. Moreover, this fast pipeline to build models and resulting printing is useful for education, as a broad category of structures (*e.g.* stylet, muscles in cephalic region of tylenchs, neural systems, and sensory etc.) can be modeled and printed for an acceptable cost (Chapter V).

Future taxonomical applications can also be extended to virtual reality approaches that allow observation and dissection without damaging precious specimens, which represents a promising direction for both taxonomy and education.

Conclusion

In conclusion, although the backbone of the Tylenchidae phylogeny cannot be fully resolved based on current approaches, we were able to demonstrate that some frequently-used morphological characteristics fail to delimitate the genera within the family Tylenchidae, while other morphological traits have been proven to be congruent with molecular

phylogenies.

A taxonomy study based on morphological data only from LM, and phylogenetic study that solely relies on 28S or 18S rRNA should be avoid. It has been demonstrated that the use of some other, either existing or new technologies (*e.g.* SEM, TEM for other structures, multiple genes phylogeny, phylogenomics) are needed to extract more informative molecular data and/or morphological characters. Nevertheless, even with the newest techniques, nematode taxonomists still need to test and revise the congruence of morphology-based systematics and molecular phylogenetics. For the time being, in order to obtain a comprehensive understanding of Tylenchidae, the understanding of the major phylogenetic patterns and clades must be the key priority, rather than focusing on a compilation of a never-ending catalogue of single taxonomic units.

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APPENDIX

Table S1. Morphometric data for all recovered Tylenchidae species in this study. All measurements are in μm and in the form: mean±s.d.(range).

Character	*Basiria duplexa*	*Boleodorus thylactus*	*Cephalenchus cephalodiscus*	*Cephalenchus leptus*	*Cephalenchus leptus*
	5f#	7 f#	3 f#	6 f#	4 f#
a	38.9±2.8 (35.4-41.5)	24.1±3.1 (20.9-27.1)	37.1±0.4(36.8-37.4)	39.9±2.3 (37.8-42.4)	33.5±1.5 (32.4-34.5)
c	4.9±0.17 (4.7-5.1)	6.9±0.26 (6.6-6.7)	2.7±0.06 (2.6-2.7)	3.0±0.11 (2.9-3.2)	2.9±0.08 (2.9-3.0)
c'	12.7±0.63 (12.1-13.4)	6.4±0.6 (5.9-7.1)	20.7±0.22 (20.5-20.8)	21.1±1.03 (2.9-3.2)	23.0±1.3 (22.1-23.8)
V	65.1±0.37 (64.7-65.5)	64.0±0.26 (63.7-64.2)	53.7±0.133 (53.6-53.8)	55.4±0.52 (2.9-3.2)	53.7±0.11 (22.1-23.8)
V'	82.3±1.1 (81.1-83.4)	74.9±0.67 (74.4-75.7)	86.0±1.4 (85.0-87.0)	83.0±2.18 (2.9-3.2)	81.7±0.96 (80.9-82.3)
T/VA	1.4±0.13(1.1-1.6)	0.68±0.05 (0.64-0.73)	4.3±0.60 (3.9-4.8)	3.0±0.51 (2.9-3.2)	2.8±0.27 (2.7-3.0)
MB	41±2.2(37.3-45)	50±0.4 (50.2-49.4)	44.4±3.2 (42.1-46.7)	44.2±3.0 (2.9-3.2)	41.7±0.44 (41.4-42.1)
L	667±44 (637-741)	469±7.0 (464-477)	618±6.4 (614-623)	675±9.5 (2.9-3.2)	675±7.78 (670-681)
Stylet	9.8±0.7(8.1-11.1)	11±0.4 (11-12)	21±0.49 (21-22)	18±0.76 (2.9-3.2)	18±0.42 (17-18)
Pharynx	102±9.3(89-126)	85.9±4.4 (81-90)	112±14 (102-122)	86±2.6 (2.9-3.2)	104±9.9 (97-111)
E pore	68.4±4.9(62.1-74.2)	73±4.0 (71-78)	71±2.8 (69-73)	69±4.3 (2.9-3.2)	66±6.4 (62-71)
nerve ring	58±3.2(52-66)	60±1.7 (62-59)	61±1.4 (60-62)	63±3.5 (2.9-3.2)	61±3.2 (58-63)
Body Width	17±3.1 (14.1-21.2)	20±2.5 (17-22)	17±0.35 (16-17)	17±1.0 (2.9-3.2)	20±1.1 (19-21)
Vulva to anterior end/Spicule	434±34(401-451)	300±4.9 (297-306)	332±4.2 (329-335)	374±6.6 (2.9-3.2)	362±4.9 (359-366)
PUS/Gubernaculum	12±4.3(7.2-14)	9.1±2.8 (5.9-11)	14±0.78 (14-15)	11±1.1 (2.9-3.2)	16±1.2 (15-17)
Anus/Cloacal width	10±0.5(9.1-12)	11±1.2 (9.5-12	11±0.49 (11-12)	11±0.51 (2.9-3.2)	10±0.71 (9.6-11)
Tail	130±23(111-171)	68±1.9 (67-70)	232±7.8 (227-238)	224±9.9 (2.9-3.2)	231±3.5 (229-234)

Table S1. (continued)

Character	*Coslenchus costatus*	*Coslenchus oligogyrus*	*Filenchus afghanicus*	*Filenchus balcarceanus*	*Filenchus cylindricus*	
	4 f#	6 f#	7 f#	4 f#	8 f#	2 m#
a	25.4±2.9 (22.7-29.3)	26.9±0.74(26.3-27.7)	30.6±1.3 (29.3-32.3)	32.5±1.1 (31.4-34.0)	40.4±2.4 (38.1-42)	39.6±2.3 (37.9-41.2)
c	5.1±0.30 (4.7-5.4)	5.4±0.34(5.1-5.8)	4.7±0.15 (4.5-4.9)	3.9±0.25 (3.7-4.2)	5.3±0.1 (5.2-5.4)	4.6±0.19 (4.5-4.7)
c'	8.7±0.95 (7.9-10.1)	8.2±1.3(6.7-9.1)	10.4±1.2 (9.3-12.1)	13.0±1.7 (10.9-14.7)	10.6±0.42 (10.3-11.0)	12.2±0.47 (11.9-12.5)
V	65.6±1.1 (64.4-66.7)	67.4±2.6(64.5-69.7)	62.9±2.3 (61.2-66.3)	58.7±1.6 (57.2-60.9)	62.1±0.86 (61.5-63.1)	-
V'	81.7±2.1 (80.2-84.8)	82.8±2.9(79.5-84.6)	80.0±2.2 (78.4-0.83.3)	78.7±1.8 (76.6-80.2)	76.6±1.4 (75.5-78.1)	-
T/VA	1.4±0.28 (1.2-1.8)	1.3±0.23(1.1-1.6)	1.4±0.12 (1.3-1.5)	1.6±0.2 (1.3-1.9)	1.0±0.08 (0.92-1.1)	-
MB	48.5±1.3 (47.7-50.4)	48±4.6(42.4-50.5)	44.8±1.8 (42-46)	44±1.1 (43.2-45.7)	43.1±1.44 (41.5-44.4)	43±0.08 (42.9-43.1)
L	465±21 (436-481)	492±37(462-533)	542±37 (498-588)	392±26 (358-421)	913±76 (861-1001)	869±5.7 (865-873)
Stylet	12±0.62 (11-13	11±0.32(11-12.0)	9.0±0.42 (8.6-9.6)	9.5±0.12 (9.3-9.6)	13±0.30 (12.5-13.1)	12±0 (12.3-12.3)
Pharynx	85±4.2 (79-89)	97±4.0(92-99)	91±6.8 (83-99)	79±7.2 (71-88)	138±4.6 (133-142)	139±3.5 (137-142)
E pore	63±2.3 (61-67)	78±2.0(76-80)	60±2.2 (57-62)	56±4.3 (51-61)	110±2.0 (108-112)	113±1.4 (112-114)
nerve ring	58±1.6 (56-60)	74±3.6(71-78)	54±2.9 (50-57)	50±4.2 (46-56)	93±5.0 (88-98)	93.5±6.4 (89-98)
Body Width	18±2.0 (16-21)	18±0.95(17-19)	18±1.3 (17-20)	12±0.96 (11-13.0)	23±2.4 (20-25)	22±1.4 (21-23)
Vulva to anterior end/Spicule	305±13 (289-319)	331±11(322-344)	341±16 (330-365)	230±9.9 (218-241)	567±46 (530-618)	21±0.49 (21-22)
PUS/Gubernaculum	4.3±0.70 (3.6-5.2)	0±0(0-0)	8.9±0.98 (8.1-10.3)	6.8±0.81 (5.8-7.7)	14±0.25 (13-14)	5.7±1.5 (4.6-6.8)
Anus/Cloacal width	10±0.44 (10-11)	11±0.72(11-12)	11±0.80 (10.0-12)	7.7±0.26 (7.4-7.9)	16±0.85 (15.4-17.1)	15±0.07 (15.4-15.5)
Tail	92±9.0 (81-102)	92±10(80-100)	116±10 (102-125)	100±12 (86-111)	172±15 (159-189)	188±6.4 (184-193)

Table S1. (continued)

Character	*Filenchus discrepans*	*Filenchus hamuliger*	*Filenchus magnus*	*Filenchus misellus*	*Filenchus misellus*
	7 f#	4 f#	5 f#	8 f#	8 f#
a	34.3±1.9(32.9-34.4)	35.1±2.3 (32.1-37.4)	34.8±3.0 (31.8-37.7)	38±3.4 (34.7-42.7)	34.4±1.5(33.4-36.1)
c	3.4±0.08(3.4-3.5)	4.8±0.17 (4.6-5.0)	3.3±0.15 (3.2-3.4)	7.3±1.2 (6.4-9.0)	4.4±0.39 (4.0-4.8)
c'	15.9±2.5(14.1-17.6)	16.3±1.3 (15.3-18.2)	16.9±1.3 (15.4-18.0)	7.1±0.45 (6.5-7.5)	11.8±0.91 (10.9-12.7)
V	58.5±0.23(58.3-58.7)	64.4±1.5 (62.8-66.4)	49.0±2.9 (45.9-51.7)	70.6±1.26 (68.8-71.7)	60.6±2.4 (57.9-62.2)
V'	82.6±1.1(81.8-83.3)	81.4±2.0 (78.6-83.5)	70.0±2.9 (67.05-72.8)	82.0±1.64 (79.9-83.6)	78.4±1.7 (76.9-80.3)
T/VA	2.4±0.21(2.2-2.5)	1.4±0.18 (1.2-1.5)	1.4±0.06 (1.4-1.5)	0.91±0.19 (0.62-1.0)	1.4±0.16 (1.2-1.5)
MB	46.5±1.9(45-48)	41.4±1.4 (39.4-42.6)	44.6±1.2 (43.9-46.0	66.2±5.4 (59.1-72.3)	46.8±0.89 (46.0-47.8)
L	396±6.4(392-401)	574±14 (556-589)	369±11 (356-377)	384±29 (346-408)	381±17 (368-400)
Stylet	6.8±0.05(6.7-6.8)	8.5±0.45 (8.1-9.1)	6.9±0.25 (6.7-7.2)	6.5±0.33 (6.2-7.0)	7.0±0.21 (6.8-7.2)
Pharynx	79±1.02(78-80)	103±6.4 (96-110)	83±3.7 (79.7-87)	63±2.6 (60-66)	62.0±3.6 (58-65)
E pore	55±4.0(52-58)	84±8.1 (76-93)	59±3.8 (56-63)	60±4.0 (55-65)	47±1.4 (45-48)
nerve ring	48±2.3(46-50)	74±6.6 (67-81)	54±2.8 (51-56)	50±2.6 (48-53)	39±3.3 (37-43)
Body Width	11±0.46(11-12.0)	16.4±1.14 (15.3-18)	11±0.67 (9.9-11)	10±0.68 (9.5-11)	11±0.75 (10-12.0)
Vulva to anterior end/Spicule	232±2.8(230-234)	370±15 (349-384)	181±11 (173-193)	271±25 (238-291)	231±15 (217-247)
PUS/Gubernaculum	9.5±0.03(9.4-9.5)	6.0±0.65 (5.1-6.6)	6±1.7 (4.3-7.8)	4.6±1.2 (3.5-6.3)	6.5±0.55 (6.0-7.1)
Anus/Cloacal width	7.4±1.2(6.5-8.2)	7.4±0.25 (7.1-7.7)	6.6±0.25 (6.3-6.8)	7.5±1.5 (5.6-8.9)	7.3±0.28 (7.0-7.6)
Tail	115±0.71(115-116)	120±7.0 (112-129)	111±7.4 (105-119)	53±8.2 (42-60)	86.3±5.8 (83-93)

Table S1. (continued)

Character	*Filenchus sheri*		*Filenchus tenuis*	*Filenchus vulgaris*	*Filenchus vulgaris*	
	10 f#	2 f#	5 f#	5 f#	1 m#	4 f#
a	34.1±2.7 (31.0-37.2)	38.7±2.1 (37.2-40.1)	27.6±0.88 (26.5-27.9)	30.6±5.5(25.5-36.4)	30.1	29.8±0.92(28.8-30.6)
c	3.9±0.43 (3.3-4.2)	4.7±0.19 (4.5-4.8)	5.7±0.18 (5.5-5.9)	4.6±0.23(4.4-4.8)	4.2	4.4±0.08 (4.3-4.5)
c'	14.4±0.93 (13.6-15.7)	12.1±1.8 (10.8-13.4)	7.4±0.40 (7.0-7.8)	11.8±0.09 (11.7-11.9)	9.9	12.4±0.32 (12.1-12.8)
V	60.0±2.4 (58.4-63.2)	-	65.8±1.69 (63.1-67.3)	60.8±0.84 (59.9-61.6)	-	60.4±0.68 (59.7-61.1)
V'	79.4±0.86 (78.6-80.6)	-	79.9±1.6 (77.2-81.5)	77.6±1.4 (76.1-78.9)	-	78.2±0.65 (77.5-78.6)
T/VA	1.70±0.30 (1.4-2.1)	-	1.1±0.06 (0.98-1.1)	1.2±0.15 (1.1-1.4)	0.31	1.3±0.04 (1.3-1.4)
MB	47±3.1 (43.9-51.1)	48.3±1.3 (47.4-49.2	46.1±1.7 (44-48)	46.5±2.0 (45.1-48.7)	48.9	50±1.5 (49.4-52.3)
L	447±34 (397-470)	440±7.8 (435-446)	359±12 (349-378)	640±18 (630-661)	512	559±21 (535-577)
Stylet	8.3±1.7 (7.3-10.9)	7.7±0.3 (7.5-7.9)	7.5±0.11 (7.4-7.7)	10±0.51 (9.7-10.6)	10	11±0.38 (10-11.0)
Pharynx	87±11 (72-98)	94±1.5 (93-95)	75±3.8 (70-77)	98±6.2 (91-103)	94	80±4.9 (76-86)
E pore	64±7.0 (53-69)	71±1.1 (70-72)	53±2.2 (50-55)	75±3.0 (72-78)	66	68±2.8 (65-71)
nerve ring	56±2.6 (52-59)	63±2.7 (61-65)	46±1.7 (44-49)	62±3.2 (59-65)	57	61±2.5 (58-63)
Body Width	13±0.62 (12-13.0)	11±0.42 (11.1-11.7)	13±0.50 (12.7-13.8)	21±3.7 (17-24)	17	19±1.1 (18-20)
Vulva to anterior end/Spicule	264±30 (220-283)	14±0.14 (13.9-14.1)	236±6.3 (231-247)	389±6.6 (383-396)	16	338±11 (327-349)
PUS/Gubernaculum	8.3±0.64 (7.6-9.1)	4.0±0.23 (3.8-4.1)	8.77±0.70 (7.6-9.5)	11±1.4 (9.6-12)	4.4	8.8±0.95 (7.9-9.8)
Anus/Cloacal width	8.0±0.54 (7.5-8.7)	7.9±1.0 (7.2-8.6)	8.6±0.19 (8.3-8.7)	12±0.61 (11-12)	12	10±0.5 (9.8-11)
Tail	114±5.8 (109-120)	94±2.1 (93-96)	63±4.1 (59-68)	139±6.8 (131-144)	122	127±7.2 (119-133)

Table S1. (continued)

Character	*Lelenchus leptosoma*	*Malenchus acarayensis*	*Miculenchus salvus*	*Neopislenchus longicaudatus*	*Neopsilenchus magnidens*
	4 f#	4 f#	5 f#	5 f#	5 f#
a	30.4±1.0 (29.2-31.1)	21.4±0.68 (20.8-22.2)	23.8±0.18 (23.6-24.0)	30.6±1.7 (28.7-31.8)	31.1±1.7 (29.2-32.4)
c	3.7±0.05 (3.6-3.7)	4.5±0.24 (4.2-4.7)	5.7±0.40 (5.2-5.9)	4.9±0.19 (4.7-5.0)	6.2±0.58 (5.8-6.8)
c'	15.9±0.45 (15.4-16.3)	9.3±0.70 (8.5-9.9)	8.0±0.41 (7.5-8.3)	12.0±1.3 (10.5-12.9)	8.6±0.59 (7.9-9.1)
V	55.9±1.1 (54.9-57.0)	61.7±0.31 (61.4-62.0)	63.2±1.3 (62.1-64.6)	59.7±4.8 (54.2-63.0)	66±1.46 (64.5-67.4)
V'	76.9±1.9 (75.2-78.9)	79.4±1.6 (78.2-81.3)	76.7±1.1 (75.6-77.8)	75.1±6.5 (67.7-80.1)	78.9±2.3 (77.4-81.5)
T/VA	1.6±0.16 (1.5-1.8)	1.4±0.22 (1.2-1.7)	0.92±0.09 (0.83-1.0)	1.08±0.29 (0.77-1.4)	0.93±0.19 (0.76-1.1)
MB	43.5±2.4 (42.0-46.3)	47.7±0.64 (47.0-48.2)	46.5±2.8 (43.8-49.4)	54.9±3.4 (51.2-57.8)	49.7±1.4 (48.6-51.2)
L	617±29 (585-642)	377±8.2 (368-384)	351±19 (331-369)	614±17 (596-631)	635±8.7 (625-642)
Stylet	8.5±0.23 (8.4-8.8)	8.8±0.35 (8.4-9.1)	8.7±0.40 (8.3-9.1)	11±0.51 (10-11.0)	13±0.67 (12-13)
Pharynx	118±3.7 (114-121)	86±3.1 (83-89)	89±6.0 (83-95)	84±3.2 (82-88)	76±5.1 (70-80)
E pore	87±4.2 (83-91)	67±2.8 (64-70)	73±5.0 (68-78)	75±1.7 (74-77)	59±3.2 (57-63)
nerve ring	78±4.5 (74-83)	56±2.7 (53-58)	59±2.1 (57-61)	60±3.0 (57-63)	55±2.3 (54-58)
Body Width	20±1.5 (19-22)	18±0.88 (17-18)	14.8±0.91 (14-16)	20±1.6 (19-22)	20±1.4 (19-22)
Vulva to anterior end/Spicule	345±20 (326-366)	233±6.2 (226-238)	222±7.5 (214-229)	366±23 (342-387)	419±12 (411-433)
PUS/Gubernaculum	12±0.83 (11.4-13)	11±1.2 (9.3-12)	7.3±0.50 (6.8-7.8)	10±1.0 (9.2-11)	12±1.0 (11-13)
Anus/Cloacal width	11±0.31 (10-11)	9.0±0.66 (8.3-9.6)	7.7±0.65 (7.3-8.5)	10±1.4 (9.2-12)	12±0.56 (11-13)
Tail	168±9.5 (159-178)	84±6.2 (79-91)	61.9±7.6 (56-70)	125±6.0 (119-131)	103±11 (91-111)