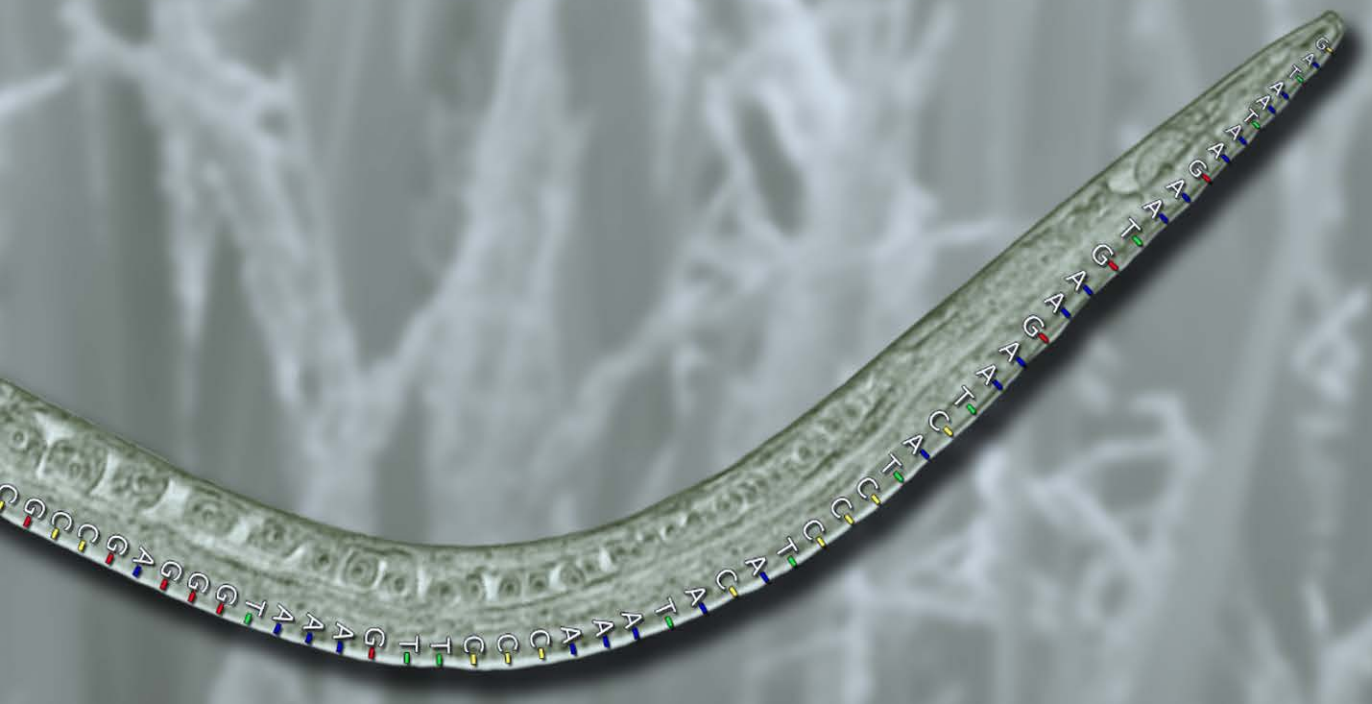
FACULTY OF SCIENCES

Nematology

Diversity, diagnosis and phylogeny of the genus *Aphelenchoides* (Aphelenchoidea: Nematoda), with focus on the plant-parasitic species

Gerardo Alcides Sánchez-Monge

Diversidad,diagnóstico y filogenia del género *Aphelenchoides* (Aphelenchoidea: Nematoda) con énfasis en especies fitoparásitas



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Promotor:
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Examination Committee:

Prof. Wim Bert
Promotor, Ghent University, Belgium

Prof. Tom Moens
Chairman, Ghent University, Belgium

Prof. Wilfrida Decraemer
Secretary, Ghent University, Belgium

Dr. Sue Hockland
Emeritus Fellow Food and Environment Research Agency,
Independent Plant Nematology Consultant, UK

Dr. Katarzyna Rybarczyk-Mydłowska
Museum and Institute of Zoology, Polish Academy of Sciences, Warsaw, Poland

Prof. Sergei Subbotin
Plant Pest Diagnostic Center, California Department of Food and Agriculture, USA

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Summary

Problem and objective

Foliar nematodes are part of one of the four evolutionary lineages of plant-parasitic nematodes. Four main species, namely *Aphelenchoides besseyi*, *A. fragariae*, *A. ritzemabosi* and *A. subtenuis*, and 10 other less known species, *i.e. A. arachidis, A. bicaudatus, A. blastophthorus, A. dalianensis, A. ensete, A. nechaleos, A. panaxofolia, A. paranechaleos, A. saprophilus*, and *A. sphaerocephalus,* have been described as plant parasites in a broad range of hosts, and especially damaging to certain crops. Yet, these species reside in a mostly mycophagous genus and their phylogenetic relationships with closely related genera remain unsolved. Moreover, morphology-based diagnosis is extremely difficult due to poor descriptions and a generally conserved morphology, while molecular data are only available for few species.

The main objective of this study was to examine and update the knowledge of the genus *Aphelenchoides* by focusing on four main aspects: 1) appraise the host range of *Aphelenchoides* species by reviewing the plant species associated with plant-parasitic *Aphelenchoides*; 2) evaluate the potential of commonly used molecular markers for the identification of *Aphelenchoides*, with focus on the Cytochrome Oxidase I (COI); 3) construct the best possible phylogenetic framework to provide an insight into the evolution of plant-parasitism within the genus; and 4) asses the correlation of morphological features with phylogenetic clades within the genus.

Approach and methodology

1) The number of plant-parasitic *Aphelenchoides* and associated plants was defined after a careful review of available literature, species descriptions and reports on databases. A dataset was constructed by compiling published reports on associated plants after discarding -when possible- doubtful parasitic relationships. This compilation was plotted on a hosts supertree made to botanical order (or family). All available data were used to appraise the potential/overlapping ranges that a single or a specific combination of species could have.

2) Molecular data of three molecular markers, one mitochondrial (mtCOI) and two rDNA (18S and 28S) regions, were generated from several free-living and plant-parasitic species to evaluate the diagnostic usefulness and phylogenetic resolution for those markers. Single gene trees and concatenated topologies were reconstructed, and the intra- vs inter- specific differences were analyzed.

3) Digital morphological vouchers of the molecularly-analyzed specimens were used to explore morphological features against the single- and multi-gene phylogenetic topologies following the reverse taxonomic approach. Each morphometrical feature was analyzed *per* phylogenetic clade to define statistically-significant traits among clades using ANOVA, followed by a post-hoc Tukey HSD test. The K2P distances and differences in base pairs between sister taxa were calculated to estimate the number of putative species in the dataset.

Results and concluding remarks

Based on our compilation, the number of plant-parasitic *Aphelenchoides* is 14: the foliar nematodes, *i.e. Aphelenchoides besseyi*, *A. fragariae* and *A. ritzemabosi*, and eleven other plant-parasitic species, namely *A. arachidis*, *A. bicaudatus*, *A. blastophthorus*, *A. dalianensis*, *A. ensete*, *A. nechaleos*, *A. panaxofolia*, *A. paranechaleos*, *A. saprophilus*, *A. sphaerocephalus* and *A. subtenuis*, which have been reported from a limited number of plant species. We compiled a new data-base (freely available at http://nematodes.myspecies.info/) of the associated plants for these fourteen species, this comprehensive list includes 1105 reports from 126 botanical families. *Aphelenchoides besseyi*, *A. fragariae* and *A. ritzemabosi* represent 94% of the reports, most of which correspond to flowering plants and ferns, and with only three records on conifers. Most plant-parasitic *Aphelenchoides* show a remarkably broad diversity of associated plants and do not appear to have specific plant hosts (*i.e.* are generalists). At the same time, biology and dispersal of *Aphelenchoides* species is largely unknown, so are the potential interactions with microorganisms in the infection process. The obtained broad host ranges, which are likely to represent only a fraction of the actual ranges, combined with the absence of more intimate interactions with the associated plants

highlights the primitive mode of parasitism in *Aphelenchoides* species, which is potentially interesting for further studies on the evolution of plant-parasitism.

The Cytochrome Oxidase I gene (COI), albeit being the standard barcode for almost all animal groups, is explored for the first time as a diagnostic tool for *Aphelenchoides*. We generated 69 mtCOI and 123 rDNA sequences of diverse *Aphelenchoides* taxa; including the first mtCOI sequences of *A. fragariae* and the first mtCOI and 28S sequences of *A. subtenuis*. We were also able to locate several misidentified sequences of plant-parasitic *Aphelenchoides* in existing databases. Phylogenetic trees based on the three studied markers, *i.e*. 18S, D2D3 (28S) and mtCOI, are partially in agreement with each other, which together with the large inter-specific differences obtained from the K2P analyses, validate their use for *Aphelenchoides'* diagnosis. The mtCOI and rDNA markers had a similar success rate for PCR amplification. The generated sequences not only benefit the diagnosis of *Aphelenchoides* taxa but also contribute to a more comprehensive framework for phylogenetic and biodiversity studies of *Aphelenchoides* and related groups. The concatenated analysis from the three markers resulted in a more robust insight into the phylogeny and evolution of *Aphelenchoides*, revealing that plant-parasitism has independently evolved at least three times within this genus, presumably from fungal-feeding ancestors. The presence of four genera, *i.e*. *Ficophagus*, *Laimaphelenchus, Martininema* and *Schistonchus* imbedded within *Aphelenchoides,* confirms the paraphyly of the genus *Aphelenchoides*.

Plotting and statistically analyzing morphological traits on a molecular framework revealed that, despite 46 features and ratios were evaluated, only the tail terminus shape (*i.e*. mucro shape) and the measurements related to the position of the secretory-excretory pore relative to the median bulb unequivocally correspond with molecular-defined clades. Two features related to the excretory pore position were both, statistically significantly different and with no overlapping values between clade II-6 and the other clades. For the morphological traits, only the tail terminus shape appears to correspond with natural groups, thus we propose a grouping system to delineate supra-specific groups based on this feature. Following a very conservative estimation to assess species diversity in *Aphelenchoides,* we obtained a total of 29 putative species based on only 47 newly generated and eight GenBank sequences, with

other 15 described species represented in the same topology. Remarkably, only two of the free-living retrieved *Aphelenchoides* specimens could be assigned to an identified species, *i.e. A. fujianensis*. The fact that, based on our obtained sequences, nearly all analyzed samples result in putative new species underlines the enormous diversity in *Aphelenchoides*. Furthermore, our data suggests that *A. besseyi* is probably a complex of cryptic species.

Thus, despite the scope of this research was not a quantitative analysis of diversity, the number of retrieved potential species, in respect to the number and type of samples analyzed, obviously suggest that neglected substrates hide an important number of aphelenchoidids, and that the diversity foreshadowed in the phylogenetic trees is just a glimpse of the real amount of species waiting to be discovered, particularly on bark and wood samples.

Keywords: barcoding, concatenated analysis, COI, rDNA, morphological identification

Samenvatting

Probleem en doelstellingen

Het onderzochte genus *Aphelenchoides* behoort tot één van de vier evolutionaire lijnen van plantparasitaire nematoden. Binnen dit genus zijn *Aphelenchoides besseyi*, *A. fragariae*, *A. ritzemabosi* en *A. subtenuis* beschreven als plantparasieten in een breed spectrum aan gastheren, en bijzonder schadelijk voor bepaalde gewassen. Andere plantparasitaire soorten binnen dit genus zijn minder bekend, namelijk *A. arachidis, A. bicaudatus, A. blastophthorus, A. dalianensis, A. ensete, A. nechaleos, A. panaxofolia, A. paranechaleos, A. saprophilus* en *A. sphaerocephalus*. De andere soorten *Aphelenchoides* zijn meestal fungivoor en hun fylogenetische relaties met elkaar en met nauw verwante genera zijn niet goed gekend. Bovendien is een morfologie-gebaseerde diagnose zeer moeilijk als gevolg van slechte beschrijvingen en morfologisch minimalisme. Moleculaire gegevens zijn alleen beschikbaar voor een beperkt aantal soorten.

De belangrijkste doelstellingen van dit onderzoek waren: 1) Het gastheer-bereik van *Aphelenchoides* inschatten door een literatuurstudie van de plantensoorten die geassocieerd zijn met de plantparasitaire *Aphelenchoides* soorten; 2) de mogelijkheden exploreren van moleculaire merkers voor de identificatie van *Aphelenchoides*, in het bijzonder het cytochroom oxidase I (COI); 3) het construeren van de best mogelijke fylogenetische verwantschapsboom om inzicht te krijgen in de evolutie van plantparasitisme binnen het genus; en 4) morfologische kenmerken correleren met fylogenetische clades binnen het genus.

Aanpak en methodologie

1) Het aantal plantparasitaire *Aphelenchoides* soorten en geassocieerde planten werd gedefinieerd na een zorgvuldige evaluatie van de beschikbare literatuur en databases. Een dataset werd geconstrueerd op basis van gekende plantassociaties maar twijfelachtige waarnemingen werden niet opgenomen. Deze compilatie werd geplot op een gastheer-supertree op basis van plantordes (of familie). Alle beschikbare gegevens werden gebruikt om de potentiële gastheerradius in te schatten.

2) Moleculaire sequenties van drie moleculaire merkers, één mitochondriaal (mtCOI) en twee ribosomaal DNA (18S en 28S) genetische regio's, werden gegenereerd uit meerdere vrijlevende en plantparasitaire soorten om het diagnostische potentieel en fylogenetische resolutie voor die markers te evalueren. Zowel fylogenetische bomen op basis van individuele genen als op basis van samengevoegde genen werden gereconstrueerd, en de intra- versus interspecifieke verschillen werden geanalyseerd.

3) Digitale vouchers van de moleculair-geanalyseerde specimen werden gebruikt om morfologische kenmerken te analyseren in combinatie met de individueel en multi-gen fylogenetische bomen. Er werd gezocht naar significant verschillende morfometrische kenmerktoestanden, gebruik makende van ANOVA, gevolgd door een post-hoc Tukey HSD-test. De sequentie-verschillen tussen zustertaxa werden berekend om het totaal aantal mogelijke soorten in de dataset in te schatten.

<u>Resultaten en conclusies</u>

Op basis van onze compilatie zijn er 14 plantparasitaire *Aphelenchoides* soorten: de bekende bladaaltjes, namelijk *Aphelenchoides besseyi*, *A. fragariae* en *A. ritzemabosi*, en elf andere plantparasitaire soorten die zijn gemeld uit een beperkt aantal plantensoorten, namelijk *A. arachidis, A. bicaudatus, A. blastophthorus, A. dalianensis, A. ensete, A. nechaleos, A. panaxofolia, A. paranechaleos, A. saprophilus, A. sphaerocephalus* en *A. subtenuis*. We stellen een nieuwe database van de planten, waarmee deze 14 soorten geassocieerd zijn ter beschikking. Deze uitgebreide lijst bevat 1.105 rapporten van 126 botanische families (http://nematodes.myspecies.info/). *Aphelenchoides besseyi, A. fragariae* en *A. ritzemabosi* vertegenwoordigen 94% van de meldingen, waarvan de meeste van bloeiende planten en varens, en met slechts drie meldingen van coniferen. De meeste plantparasitaire *Aphelenchoides* vertonen een opmerkelijk grote gastheer-diversiteit, blijken dus niet gastheer-specifiek te zijn en zijn dus generalisten. De biologie en verspreiding van *Aphelenchoides* soorten zijn weliswaar nog grotendeels onbekend, dus ook de mogelijke interacties met micro-organismen in het infectieproces. Het breed gastheerbereik en het ontbreken van duidelijke interacties met de gastheer wijst op een eerder primitieve wijze van

parasitisme in het genus, wat interessant is om de evolutie van plantparasitisme in nematoden beter te begrijpen.

Het cytochroom oxidase I gen (COI), dat de standaard moleculaire barcode is voor bijna alle diergroepen, wordt hier voor het eerst grondig onderzocht als een diagnostische tool voor *Aphelenchoides*. We genereerden 69 mtCOI en 123 ADNR sequenties van diverse *Aphelenchoides* taxa; waaronder de eerste mtCOI sequenties van *A. fragariae* en de eerste mtCOI en 28S sequenties van *A. subtenuis*. Op basis van onze gegevens werden verschillende foutieve *Aphelenchoides* sequenties in bestaande databases ontmaskerd. Fylogenetische bomen op basis van de drie onderzochte markers, 18S, D2D3 (28S) en mtCOI, zijn grotendeels in overeenstemming met elkaar. Door duidelijke inter-specifieke verschillen zijn alle drie de markers bruikbaar voor de diagnose van *Aphelenchoides* soorten. De gegenereerde sequenties zijn niet enkel van belang voor identificatie van *Aphelenchoides* soorten maar kan ook bijdragen tot een beter inzicht in verwantschap en biodiversiteitsstudies van *Aphelenchoides* en verwante groepen. De gecombineerde analyse van de drie markers resulteerde in een meer robuust inzicht in de fylogenie en de evolutie van *Aphelenchoides*, waaruit blijkt dat plantparasitisme drie keer onafhankelijk van elkaar is geëvolueerd, vermoedelijk van fungivore voorouders. De aanwezigheid van vier genera ingebed binnen *Aphelenchoides*, namelijk *Ficophagus*, *Laimaphelenchus*, *Martininema* en *Schistonchus,* bevestigt de parafilie van het geslacht *Aphelenchoides*.

Het plotten van en statistisch analyseren van 46 morfologische kenmerken en metingen op de fylogenetische boom, leerde dat enkel de morfologie van de staartterminus en de afmetingen met betrekking tot de positie van de excretieporus overeen komen met moleculair gedefinieerde clades. Op basis van de morfologie van de staartterminus, die dus natuurlijke groepen vertegenwoordigt, werden supra-specifieke groepen binnen *Aphelenchoides* afgebakend. Op basis van onze nieuwe moleculaire dataset bleek de soortenrijkdom bijzonder hoog en nog zeer slecht gekend. Slechts 47 nieuw gegenereerde sequenties en acht GenBank sequenties bleken vermoedelijk 29 verschillende soorten te weerspiegelen, en dit op basis van een zeer conservatieve schatting. Opmerkelijk, slechts twee van de sequenties komende van vrijlevende *Aphelenchoides* soorten kunnen worden

toegewezen aan een gekende soort, namelijk. *A. fujianensis*. Dus, het feit dat, op basis van onze verkregen sequenties, bijna alle geanalyseerde stalen resulteren in mogelijke nieuwe soorten, onderstreept de enorme diversiteit in *Aphelenchoides*. Bovendien blijkt uit onze gegevens dat *A. besseyi* waarschijnlijk een complex is van cryptische soorten.

Ok al was dit onderzoek niet gefocust op een kwantitatieve analyse van de diversiteit, het aantal gevonden soorten ten opzichte van het gelimiteerde aantal stalen maakt duidelijk dat slecht onderzochte substraten een gigantische onbekende diversiteit herbergt. Wat we nu kennen is slechts een fractie van het totaal aantal bestaande *Aphelenchoides* soorten, vooral in schors en houtachtige substraten leven een groot aantal soorten te wachten om ontdekt te worden.

Resumen

Problema y objetivo

Los nemátodos foliares son parte de uno de los cuatro linajes evolutivos de nematodos fitoparásitos. Cuatro especies principales: *Aphelenchoides besseyi*, *A. fragariae*, *A. ritzemabosi* y *A. subtenuis*, y otras 10 especies menos conocidas; *A. arachidis*, *A. bicaudatus*, *A. blastophthorus*, *A. dalianensis*, *A. ensete*, *A. nechaleos*, *A. panaxofolia*, *A. paranechaleos*, *A. saprophilus* y *A. sphaerocephalus*, han sido descritas como fitoparásitas en una amplia gama de huéspedes y son especialmente dañinas para ciertos cultivos. Sin embargo, estas especies pertenecen a un género principalmente micófago y sus relaciones filogenéticas con géneros estrechamente relacionados no son del todo claras. Además, el diagnóstico basado en morfología es extremadamente difícil debido a las malas descripciones y a una morfología general conservada, mientras que los datos moleculares están disponibles solamente para pocas especies.

El objetivo principal de este estudio fue examinar y actualizar los conocimientos del género *Aphelenchoides*, centrándose en cuatro aspectos principales: 1) evaluar la variedad de hospederos de especies de *Aphelenchoides* mediante la revisión de las especies de plantas asociadas con *Aphelenchoides* fitoparásitos; 2) evaluar el potencial de los marcadores moleculares de uso común para la identificación de *Aphelenchoides*, con énfasis en el Citocromo Oxidasa I (COI); 3) construir el mejor marco filogenético posible para proporcionar una visión de la evolución del fitoparasitismo dentro del género; y 4) evaluar la correlación de características morfológicas con clados filogenéticos dentro del género.

Enfoque y metodología

1) El número de *Aphelenchoides* fitoparásitos y plantas asociadas se definió después de una cuidadosa revisión de la literatura disponible, descripciones de especies e informes y bases de datos. Se construyó un base de datos recopilando informes publicados sobre plantas asociadas después de descartar -si era posible- relaciones parasitarias dudosas. Esta recopilación fue sobrepuesta en un superárbol de hospederos hecho al nivel de orden

botánico (o familia). Todos los datos disponibles se utilizaron para evaluar los rangos, potenciales o sobrepuestos, que una o una combinación específica de especies podría tener.

2) Se generaron secuencias con tres marcadores moleculares, uno mitocondrial (mtCOI) y dos regiones de ADNr (18S y 28S), de varias especies libres y fitoparásitas para evaluar tanto la utilidad diagnóstica como la resolución filogenética de dichos marcadores. Se reconstruyeron árboles de genes únicos y topologías concatenadas, y se analizaron las diferencias intra- e inter-específicas.

3) Se generaron respaldos digitales de los especímenes analizados molecularmente para explorar características morfológicas contra las topologías filogenéticas basadas ya fuera en un solo gen o en la combinación de varios, siguiendo el enfoque taxonómico inverso. Cada característica morfométrica se analizó por clado filogenético para definir rasgos estadísticamente significativos utilizando ANOVA, seguido de una prueba post-hoc (Tukey HSD). Las distancias K2P y las diferencias en los pares de bases entre taxa hermanos se calcularon para estimar el número de potenciales especies en el conjunto de datos.

Resultados y conclusiones

Basado en nuestra recopilación, el número de especies de *Aphelenchoides* fitoparásitos es 14: los nemátodos foliares, es decir, *Aphelenchoides besseyi*, *A. fragariae* y *A. ritzemabosi*, y otras once especies parasíticas de plantas, a saber, *A. arachidis*, *A. bicaudatus*, *A. blastophthorus*, *A. dalianensis*, *A.* sinte, *A. nechaleos*, *A. panaxofolia*, *A. paranechaleos*, *A. saprophilus*, *A. sphaerocephalus* y *A. subtenuis*, que han sido reportados de un número limitado de especies de plantas. Se generó una nueva base de datos de las plantas asociadas con estas catorce especies (disponible gratuitamente en http://nematodes.myspecies.info/), esta lista incluye 1105 informes de 126 familias botánicas. *A. besseyi*, *A. fragariae* y *A. ritzemabosi* representan el 94% de los informes, la mayoría de los cuales corresponden a plantas con flores y helechos, y con sólo tres registros en coníferas. La mayoría de *Aphelenchoides* fitoparásitos muestran una diversidad notablemente amplia de plantas asociadas y no parecen tener hospederos específicos (es decir, son generalistas). Al mismo tiempo, la biología y la dispersión de especies de *Aphelenchoides* son en gran parte desconocidas, así como las

posibles interacciones con microorganismos en el proceso de infección. Los amplios rangos de hospederos obtenidos, que probablemente representan sólo una fracción de la amplitud real, combinadas con la ausencia de interacciones más íntimas con las plantas asociadas, destacan el modo primitivo de parasitismo en las especies de *Aphelenchoides*, que resulta potencialmente interesante para futuros estudios sobre la evolución del fitoparasitismo.

Pese a ser el código de barras estándar para casi todos los grupos de animales, el gen Citocromo Oxidasa I (COI), es explorado por primera vez como una herramienta de diagnóstico para *Aphelenchoides*. Se generaron 69 secuencias de mtCOI y 123 de ADNr de diversos taxones de *Aphelenchoides*; incluyendo la primera secuencia mtCOI de *A. fragariae* y las primeras secuencias mtCOI y 28S de *A. subtenuis*. También pudimos señalar varias secuencias de *Aphelenchoides* fitoparásitos erróneamente identificadas en bases de datos existentes. Los árboles filogenéticos basados en los tres marcadores estudiados, es decir, 18S, 28S y mtCOI, concuerdan en forma parcial, lo que junto a las diferencias inter-específicas obtenidas de los análisis K2P validan su uso para el diagnóstico de *Aphelenchoides*. Las tasas de éxito para la amplificación por PCR fueron similarres para los tres marcadores. Las secuencias generadas no sólo benefician el diagnóstico de *Aphelenchoides* sino también contribuyen a construir un marco más amplio para los estudios filogenéticos y de biodiversidad de *Aphelenchoides* y grupos relacionados. El análisis concatenado de los tres marcadores dio lugar a una visión más sólida de la filogenia y la evolución de *Aphelenchoides*, revelando que el fitoparasitismo ha evolucionado de forma independiente al menos tres veces en este género, probablemente a partir de ancestros micófagos. La presencia de cuatro géneros, es decir *Ficophagus*, *Laimaphelenchus*, *Martininema* y *Schistonchus* embebidos dentro de *Aphelenchoides*, confirma la parafilia del género *Aphelenchoides*.

A pesar de que 46 características y proporciones fueron contempladas, la sobreposición y el análisis estadístico de los rasgos morfológicos dentro del marco molecular revelaron que sólo la forma terminal de la cola y las medidas relacionadas con la posición del poro secretor-excretor respecto al bulbo medio coinciden inequívocamente con clados definidos molecularmente. Dos características relacionadas con la posición del poro secretor-excretor fueron significativamente diferentes y sin traslape de medidas entre el clado II-6 y los otros.

De los rasgos morfológicos, sólo la forma terminal de la cola parece corresponder con grupos naturales, y basado en esto, se propone un sistema de agrupación para delimitar grupos supra-específicos. A fin de evaluar la diversidad de especies en *Aphelenchoides*, se hizo una estimación muy conservadora que obtuvo un total de 29 potenciales especies nuevas de únicamente 47 nuevas secuencias y ocho no identificadas de GenBank, con otras 15 especies reconocidas representadas en la misma topología. Sólo dos de los ejemplares de *Aphelenchoides* de vida libre se podrían asignar a una especie identificada, es decir, a *A. fujianensis*. El hecho de que casi todas las muestras analizadas resulten en nuevas potenciales especies subraya la enorme diversidad de *Aphelenchoides*. Además, nuestros datos sugieren que *A. besseyi* es probablemente un complejo de especies crípticas.

Por lo tanto, a pesar de que el objetivo de esta investigación no fue un análisis cuantitativo de la diversidad, el número de potentiales especies recuperadas respecto al número y tipo de muestras analizadas claramente sugiere que sustratos menos estudiados ocultan un número importante de aphelenchoideos, y que la diversidad vislumbrada en los árboles filogenéticos es solamente un vistazo a la cantidad real de especies que esperan ser descubiertas, particularmente en muestras de corteza y madera.

CHAPTER I
General introduction and thesis outline

Nematodes are one of the most diverse and abundant group of metazoans (Heip *et al.* 1985; Ye *et al.* 2007); they have colonized almost all terrestrial habitats as well as marine environments (De Ley 2000). Plant-parasitism has arisen independently several times in Nematoda: in Trichodoridae Thorne, Longidoridae Thorne (Meyl), Tylenchina Chitwood and Aphelenchina Geraert; with an estimated cost to world agriculture of US$125 billion annually (Bakhetia *et al.* 2005).

Within "Aphelenchs", the superfamily Aphelenchoidoidea (*sensu* Hodda 2011) comprises 7 families and includes fungal-feeding species, insect parasites, predators but also some damaging plant pathogens (Hunt 2008). *Aphelenchoides,* the type genus of the family Aphelenchoididae (Nickle, 1970), was proposed 122 years ago by Fischer in 1894 and harbors a very diverse group of nematodes exhibiting all mentioned feeding behaviors except for predators. The number of described *Aphelenchoides* species increased throughout the years, by the early 60s less than 40 species were recognized (Fig. I.1) but for 1981, Maggenti (1981) mentioned the existence of 197 species. Between 140-150 spp. were recognized in the 90s (Ebsari 1991; Hunt 1993; Shahina 1996) (Fig. I.1), but in 2001, Hockland published a revised list and recognized only 86 species, regarding several taxa as *species indeterminatae* due to, among other reasons, the low number of specimens in original descriptions. The checklist by Hunt (2008) contemplates 154 species plus 19 *species inquerendae;* however, several new species have been published since. Considering Hockland's (2001) revision and the new species described after Hunt (2008), the number of valid/described species is 118 (Table I.1), other 79 species are regarded as *species inquirendae vel insertae sedis* or *indeterminatae* (Table I.2) (Fig. I.1). However, we did not check the taxonomic validity of the newly described species after Hockland's (2001) while updating the lists (Tables I.1 and I.2).

Several taxonomical changes have occurred in the study of aphelenchs since the first description in 1864 (Fig. I.2). *Aphelenchus*, the first aphelenchid genera *sensu* Siddiqi (1980), was proposed in 1865 by Bastian, followed by *Aphelenchoides* Fischer almost 30 years later, in 1894 (Fig. 2). The similar morphology of these groups, especially the strongly developed median bulb (shared by all aphelenchs), led to confusion about their boundaries, and several species were transferred mainly from *Aphelenchus* to *Aphelenchoides* (Hunt 2008). Whereas

Aphelenchus has a pharynx with a distinctive isthmus and the nerve ring surrounding the pharynx, *Aphelenchoides* usually lacks an isthmus and the nerve ring surrounds both, the pharynx and intestine. Furthermore, the tail shape is usually short and with a broadly rounded terminus in *Aphelenchus*, while conoid with a variable terminus in *Aphelenchoides* (Hunt 1993). The number of incisures in the lateral field can also be informative for these genera, *i.e.* usually six or more in *Aphelenchus* and less than six in *Aphelenchoides*, but exceptions have been described (see Chapter IV). 37 years after *Aphelenchoides*, Fuchs (1931) proposed *Seinura* to accommodate species that, although generally similar to *Aphelenchoides*, had elongate filiform tails instead of short and conical; and six years after *Seinura*, Fuchs (1937) created two more genera to separate those species in which males had a terminal bursa (*i.e. Bursaphelenchus)* and those with females showing a cuticular projection over the vulva (vulval flap) (*i.e. Laimaphelenchus*) (Fig. I.2). However, these and subsequent taxonomical changes (see detailed compilation in Hunt 1993) have not been able to solve all taxonomic conundrums in this family. Furthermore, and even with new information, *i.e.* molecular data, we have not been able to reconstruct their natural history and the understanding of their relationships.

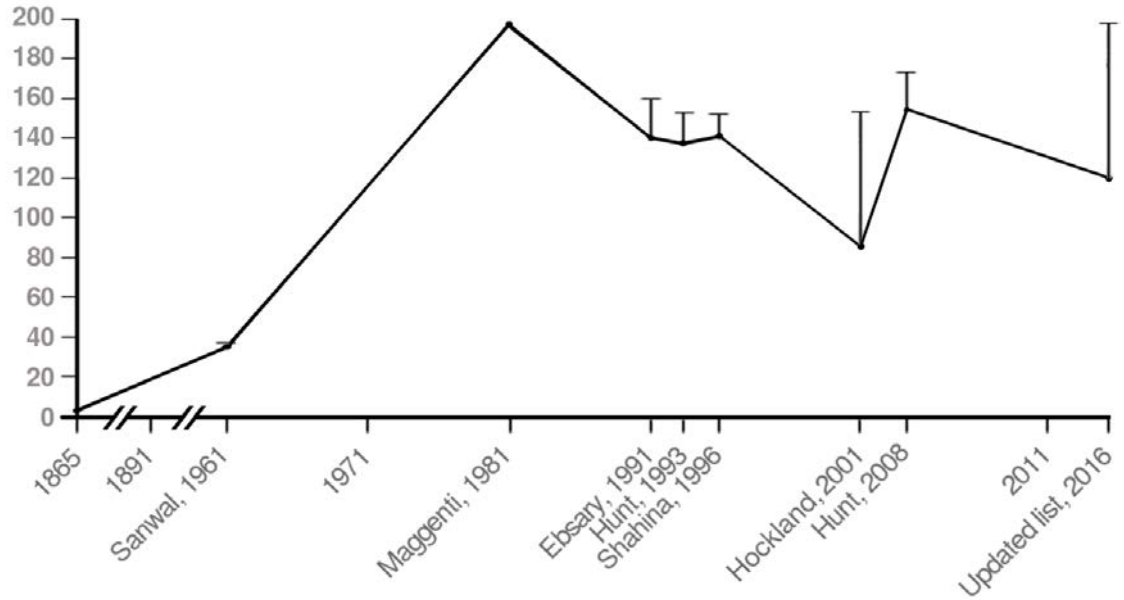

Figure I.1. Number of *Aphelenchoides* species reported in literature *per* reference; error bars represent the number of *species inquerendae, insertae sedis* and *indeterminatae* reported in each case.

The potential of molecular data for taxonomy became evident in the late 90s by the evolutionary framework of Nematoda published by Blaxter *et al.* (1998), after which a

growing number of techniques, protocols and molecular markers have been proposed and implemented for nematode diagnosis and studies in several groups (Abebe *et al.* 2013), but specially for economically important species, *i.e.* plant-parasitic nematodes (PPN) (Abrantes

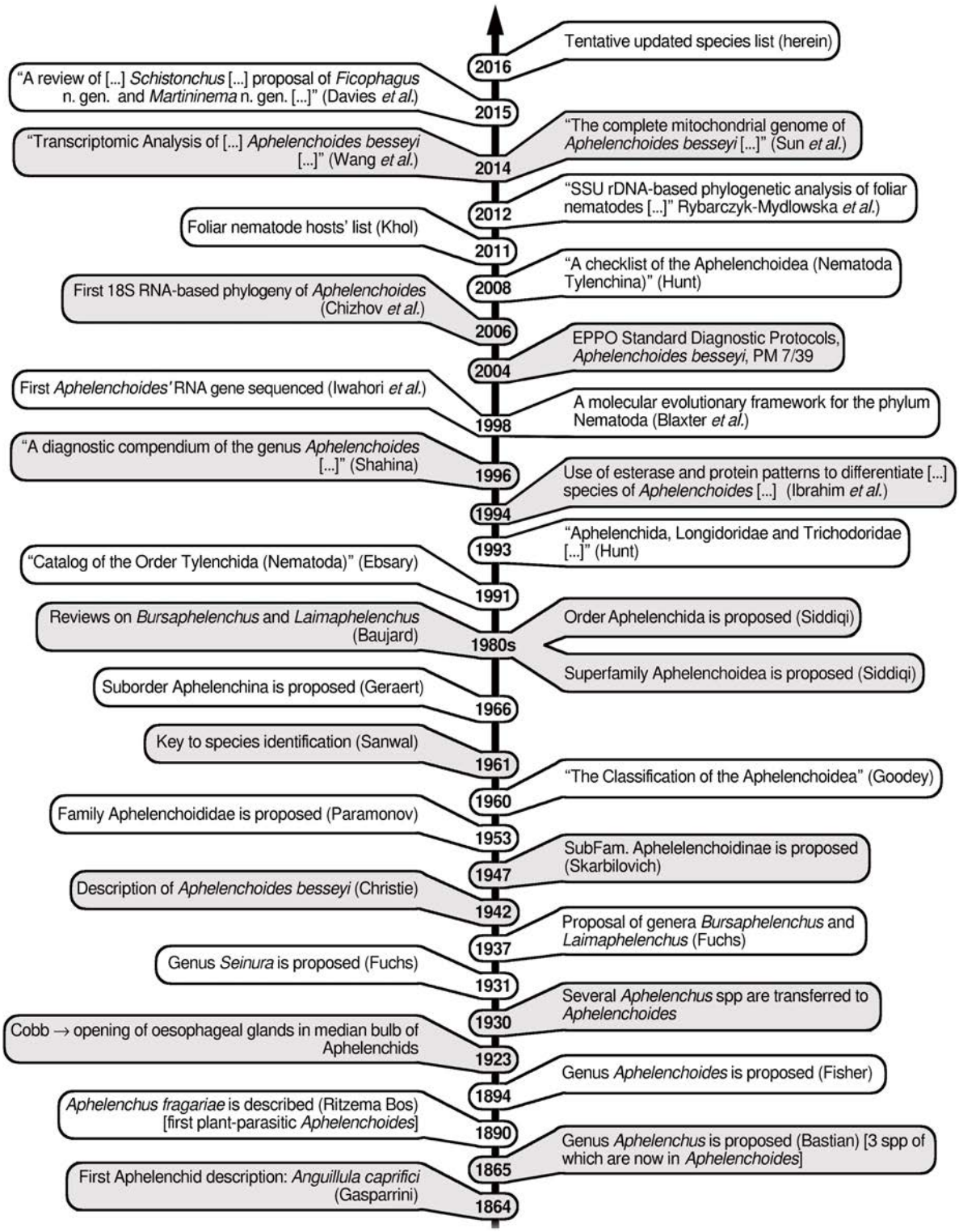

Figure I.2. Some key taxonomical and molecular publications of *Aphelenchoides*-related studies. For references see Hunt (1993; 2008) and Chapter I

et al. 2004). More recently, great advances have been made in the characterization of several taxa, including selected *Aphelenchoides* species such as *A. besseyi* and *A. fragariae* (Rybarczyk-Mydłowska *et al.* 2012; Sun *et al.* 2014; Wang *et al.* 2014) (Fig. I.2).

However, most *Aphelenchoides* species are not yet associated with discriminating molecular data and the original descriptions are usually not detailed enough to enable morphology-based identification. Even the widely-known plant-parasitic species require both, expertise and appropriate equipment, to be properly diagnosed by specialists given the large intra-specific variation and minimal inter-specific relationships (Hockland 2001). Molecular data are, therefore, particularly relevant for the identification of *Aphelenchoides* species and have been included in several new species descriptions, also allowing the reconstruction of a better framework to understand *Aphelenchoides* diversity and the evolution of plant parasitism within this genus.

- Plant parasitic *Aphelenchoides*: few but important species

Currently, around 4000 species of nematodes have been described as plant-parasitic (Decraemer & Hunt 2013), classified according to three main types of feeding groups: migratory ectoparasites, semi-endoparasites and endoparasites (migratory or sedentary) (Decraemer & Hunt 2013; Jones *et al.* 2013). The most important plant-parasitic genera belong to the superfamilies Tylenchoidea and Criconematoidea with circa 19 and 7 genera, respectively, whereas Sphaerularioidea, Dorylaimoidea and Diphterophoroidea each contain three genera comprising important plant parasites (Decraemer & Hunt 2013). Two other genera with important plant-parasitic species are found in the superfamily Aphelenchoidea *sensu* de Ley & Blaxter (2002), namely *Bursaphelenchus* and *Aphelenchoides* in the families Parasitaphelenchidae and Aphelenchoididae, respectively.

Bursaphelenchus species have been extensively studied, particularly the pinewood nematode *i.e. B. xylophilus* (Jones *et al.* 2013; Kanzaki 2008; Ryss *et al.* 2005; Ye *et al.* 2007), conversely, and except for the few facultative plant parasites, *Aphelenchoides* remains largely unexplored. Three of the main plant-parasitic *Aphelenchoides* species show similar life cycles and affect the above ground plant parts; they are collectively known as the "foliar and bulb

nematodes": *Aphelenchoides besseyi* Christie, 1942, *A. fragariae* (Ritzema Bos, 1890) Christie, 1932 and *A. ritzemabosi* (Schwartz, 1911) Steiner & Buhrer, 1932. *A. besseyi* is mainly a seed-borne ectoparasite in rice (Duncan & Moens 2013) and is widely spread throughout rice fields of the world where it causes the "white tip disease", *i.e.* a chlorotic pattern in the young leaves, representing up to 60% of the yield losses in some cases (Duncan & Moens 2013; Kanzaki & Giblin-Davis 2012; Nicol *et al.* 2011). This species can also affect the fecundity, size and germination of rice seeds (Kanzaki & Giblin-Davis 2012) and has been reported from over 90 plant species; moreover, it was identified as the causal agent of the "black spot disease" on beans (Chaves *et al.* 2013). Because of its economic impact, *A. besseyi* was listed within the top ten PPN (Jones *et al.* 2013) and more recently, its transcriptomic analysis and mitochondrial genome characterization were published (Sun *et al.* 2014; Wang *et al.* 2014) (Fig. I.2).

A. fragariae and *A. ritzemabosi*, respectively the strawberry and the chrysantemum nematodes, have been reported in a wide variety of plants, moreover, they are the most common parasitic nematodes on aerial parts of ornamental plants (McCuiston *et al.* 2007). The two species have similar life cycles, they enter (and exit) the leaves through upper and underside stomata (Duncan & Moens 2013; Kohl 2008), initial symptoms include leaf blotches (caused by the damage in the leaf mesophyll) that turn into malformations or leaf distortions. Furthermore, these nematodes can kill the growing point and prevent flowering when infecting buds (Duncan & Moens 2013; Kanzaki & Giblin-Davis 2012).

Notably, these plant-parasitic *Aphelenchoides*, together with parasitic members of the families Anguinidae and Parasitaphelenchidae, are unique among PPN in parasitizing the above-ground parts of plants (Moens & Perry 2009). However, unlike plant-parasitic *Bursaphelenchus* species (Parasitaphelenchidae) which exhibit a complex life-cycle involving a vector insect (Duncan & Moens 2013; Jones *et al.* 2013), the infection of *Aphelenchoides* and Anguinidae occurs through stomata, wounds or by direct penetration (Duncan & Moens 2013; Kanzaki & Giblin-Davis 2012; Kohl 2008), after which these nematodes are able to move, thrive and reproduce inside the plant, feeding while migrating through the hosts' cells (Duncan & Moens 2013). In addition to the above *Aphelenchoides* species, *A. subtenuis,* which

atypically penetrates and feeds on root tissue, is regarded as a fourth important plant-parasitic species affecting a more reduced range of hosts (Deimi *et al.* 2006; Maggenti 1981).

The number of plants associated with the main plant-parasitic *Aphelenchoides* (MPPA) (see Chapter II) *i.e.* the three foliar species and *A. subtenuis*, has increased in recent years, revealing a broad host range compared to other PPN; with over 700 species from 85 botanical families (Kohl 2008, 2011).

- Molecular markers and diagnosis of *Aphelenchoides* species

To assist in the taxonomical complexity of nematodes, molecular tools have gradually became an almost-mandatory input in present-day taxonomy; particularly in groups where morphology is insufficient or too difficult for accurate diagnosis. Several genetic markers and techniques have been developed for nematodes' DNA barcoding, e.g. marine species (Bhadury *et al.* 2006; Derycke *et al.* 2010b), soil taxa (Floyd *et al.* 2002) and animal and plant-parasitic species (McKeand 1999; Powers 2004). In the latter case, molecular diagnosis is especially relevant as false or incorrect identifications can lead to economic repercussions (Kiewnick *et al.* 2014). Among others, the ribosomal RNA array (particularly the 18S and 28S regions) and to a lesser extend the mitochondrial genome, have been routinely used as molecular markers for plant-parasitic nematodes (PPN) e.g. *Meloidogyne* spp., *Pratylenchus* spp. and *Scutellonema* spp. (Holterman *et al.* 2009; Janssen *et al.* 2016; Lesufi *et al.* 2015; Powers 2004).

The first biochemical approaches to characterize *Aphelenchoides* species were done in the late 60s for two main plant-parasitic species, *i.e. A. fragariae* and *A. ritzemabosi* (Abrantes *et al.* 2004); in the 90s, Ibrahim *et al.* (1994) successfully differentiated *Aphelenchoides* species using esterase and protein patterns, and only four years latter, Iwahori *et al.* (1998) published the first *Aphelenchoides'* rDNA gene sequence. Yet, the first 18S-based phylogenetic tree of the genus has been only reconstructed recently by Chizhov *et al.* in 2006 (Fig. I.2). Besides the small and large RNA subunits (*i.e.* 18S and 28S regions), the ITS regions and the 5.8S genes have also been implemented to diagnose *Aphelenchoides* (Ibrahim *et al.* 1994; Kanzaki

& Giblin-Davis 2012; McCuiston *et al.* 2007; Rybarczyk-Mydłowska *et al.* 2012), however, the mitochondrial Cytochrome Oxidase I gene (COI) has been explored only in a limited number of nematode species (Palomares-Rius *et al.* 2014) including marine taxa (Derycke *et al.* 2010b) and several plant-parasitic species (van den Berg *et al.* 2013; Kanzaki & Giblin-Davis 2012; Kiewnick *et al.* 2014; Troccoli *et al.* 2016; Ye *et al.* 2007). Circa fifty mtCOI sequences of *Aphelenchoides* are available in GenBank, but those comprise only two plant-parasitic species, *i.e. A. besseyi* and *A. ritzemabosi*; hence, the potential of mtCOI for barcoding and species diagnosis in this genus remained unexplored.

- On the taxonomy and diversity of the genus via reverse taxonomy

Given the limitations explained above, *i.e.* poorly described species, lack of molecular data plus intra and inter-specific variability alongside a high number of species, the taxonomy of *Aphelenchoides* is still under construction. From a molecular perspective, *Aphelenchoides* is said to be polyphyletic or paraphyletic (Azizi *et al.* 2015; Cardoza *et al.* 2008; Esmaeili *et al.* 2016; Kanzaki & Giblin-Davis 2012; Kanzaki *et al.* 2014; Zhao *et al.* 2008) with sequences of other genera, *i.e. Ficophagus* Davies & Bartholomaeus, 2015, *Laimaphelenchus, Martininema* Davies & Bartholomaeus, 2015 and *Schistonchus* Cobb, 1927, imbedded within *Aphelenchoides s*equences. However, sequences of these genera, except for *Ficophagus,* are scarce and their phylogenetic positions are not fully resolved.

Among the genetic tools and approaches applied to taxonomical research; reverse taxonomy was intended to help the study of those cases where traditional approaches were not sufficient to elucidate diversity (Markmann & Tautz 2005). Rather than based on morphological similarities, this approach relies on sequences from usually anonymous taxa to reconstruct their phylogenetic relationships and subsequently assign them to taxonomical groups (Markmann & Tautz 2005; Randrianiaina *et al.* 2010). This methodology, although not always explicitly, has been applied in other nematological studies dealing with species complexes, cryptic species and new taxa descriptions (Apolônio Silva de Oliveira *et al.* 2012; Derycke *et al.* 2010a; Kanzaki *et al.* 2012). In cryptic speciation' studies, molecular data are analyzed first to subsequently validate the new species with morphological data

(Palomares-Rius *et al.* 2014). In this thesis, the reverse taxonomic approach was not intended to identify species but rather to define supra-specific phylogenetic groups in *Aphelenchoides*, after which we explored morphological features to support such clades.

According to Hockland (2001), distinguishing characters in *Aphelenchoides*' original descriptions include 1) body length, 2) body length/maximum body width, 3) tail terminus, 4) length of the post-uterine sac, 5) stylet length, 6) lateral lines, 7) position of the secretory-secretory-excretory pore (relative to the nerve ring), 8) stylet shape, 9) tail shape, 10) tail length, 11) vulva position, 12) head and lip region, 13) ovary length, 14) body length/tail length, 15) tail length/anal body width, 16) position of the nerve ring, 17) total length/pharyngeal length, 18) body length/length from anterior end to end of pharyngeal glands, and 19) body shape when relaxed. We evaluated these characters except for numbers 2, 8, 12, 13, 17, 18 and 19; in addition, we explored the following features and ratios, with special attention to the median bulb as the most characteristic feature of aphelenchoids: 1) body length/anal body width, 2) body length / body width at middle of median bulb, 3) body length / distance from anterior end to the middle of the median bulb, 4) body length / body width at vulva, 5) knobs width, 6) knobs height, 7) knobs ratio, 8) lips width at base, 9) lips height, 10) lips maximum width, 11) lips ratio, 12) median bulb valves length, 13) median bulb valves width, 14) ratio of the median bulb valves, 15) median bulb length, 16) median bulb width, 17) median bulb ratio, 18) length from the middle of the valves to the anterior end of the median bulb, 19) length from the middle of the valves to the posterior end of the median bulb, 20) valves position in the median bulb, 21) length from the middle of the median bulb to the anterior end, 22) body width at the median bulb, 23) body width at the median bulb/lips width at base, 24) length from the tip of the pharyngeal gland lobe to the anterior end, 25) distance of the secretory-excretory pore to the anterior end, 26) distance of the secretory-excretory pore to the middle of the median bulb, 27) position of the secretory-excretory pore as % of the body length, 28) length from the anterior end to the middle of the median bulb/distance of the secretory-excretory pore to the anterior end, 29) body width at vulva and 30) length from vulva to anus and 31) anal body width.

By constructing a molecularly-based phylogenetic tree and plotting morphological and biological data on the topology, we aimed to enlight the understanding of both, phylogenetic relationships and morphological characters of *Aphelenchoides*, and, to a certain extend, closely-related genera.

However, it should be mentioned that the morphological part of this thesis focuses on the morphological support of molecularly-defined supra-specific *Aphelenchoides*' groups and not on the characteristics that are informative for genus or species diagnosis. The evaluation of potentially species-informative traits requires specimens from different populations and geographic locations, which was not possible given the remarkably high diversity, *i.e*. virtually each sample resulted in a different species (Chapter V). Furthermore, taxonomical units are here considered putative species (Chapter V) not based on morphological features, but if intra-specific sequences' differences exceeded those of established species. Thus, by measuring and comparing the intra- and inter-specific differences to discriminate possible independent lineages, *i.e*. barcode-gap (Hebert *et al.* 2004).

The barcode-gap method implies the use of subjective thresholds that may not be accurate for delineating closely related species, especially in groups that require more taxonomical studies (Meyer & Paulay 2005). Hence, given the experienced constraints, especially the lack of representation of populations in both datasets (morphological and molecular), taxa delimitation in this thesis does not follow a particular species' concept. Despite sequence differences can reveal clear differences among taxa (gaps), they do not fulfill other criteria that are needed for species delimitation, such as additional differences in morphological and biological characters and/or evidence of common ancestry (Luc *et al*. 2010).

- Objectives and thesis outline

The aim of this thesis is to contribute and update the knowledge on several aspects of the genus *Aphelenchoides*, including the range of plants associated with plant-parasitic species, a comparison between two commonly used molecular markers (18S and 28S rDNA) and the

unexplored mtCOI for diagnosis, and the phylogenetic and taxonomic relationships within the genus with comments on its diversity.

Chapter II presents an updated list of plant associated with the plant-parasitic *Aphelenchoides* species. In addition to the four MPPA (see above), ten other plant-parasitic species are included: *A. arachidis*, *A. bicaudatus*, *A. blastophthorus*, *A. dalianensis*, *A. ensete*, *A. nechaleos*, *A. panaxofolia*, *A. paranechaleos*, *A. saprophilus* and *A. sphaerocephalus*. This compilation allows to define generalist and specialist plant-parasites within this genus, as well as shared associated-plants among the MPPA species and other PPA species. Remarkably, such shared associations are relatively few, despite most species appear to have no specific plant hosts (*i.e.* are generalists). The broad host ranges of these species and absence of more intimate interactions with their associated plants highlights the primitive mode of parasitism in *Aphelenchoides* species, and even though the compiled list of associated plants is long, it probably only represents a fraction of the actual range.

In **Chapter III,** the phylogenetic relationships of the MPPA, the evolution of plant parasitism in *Aphelenchoides* and the use of molecular barcodes to diagnose *Aphelenchoides* species is studied. As rDNA markers are widely used but mtCOI remains relatively unexplored, mtCOI is evaluated as a diagnostic marker for plant-parasitic species and to improve the phylogenetic resolution of *Aphelenchoides*. To accomplish this, we generated 69 mtCOI and 123 rDNA sequences of *Aphelenchoides* taxa; including the first mtCOI and 28S sequences of *A. subtenuis* and the first mtCOI sequence of *A. fragariae*. Besides the numerous advantages of using mtCOI as a barcode, mtCOI had a similar rate for PCR amplification, moreover, phylogenetic trees based on the studied markers are generally in agreement with each other, validating their use for *Aphelenchoides* diagnosis. These analyses also allowed us to spot several misidentified sequences of plant-parasitic *Aphelenchoides* in databases. Finally, the concatenated analysis, the first for this genus, included data from one mitochondrial and two nuclear ribosomal genes and resulted in a more robust insight in the phylogeny and evolution of *Aphelenchoides*. The obtained topology revealed that plant-parasitism has independently evolved at least three times within this genus, presumably from fungal-feeding ancestors.

In **Chapter IV**, all rDNA sequences obtained in this thesis (mainly from Chapter III) are combined and analyzed in order to obtain the best possible phylogenetic hypothesis to illuminate *Aphelenchoides'* complexity. The morphology of the sequenced specimens is retained via digital vouchers, and the agreement between the obtained phylogeny and morphological traits is analyzed *via* the reverse taxonomic approach, by constructing a molecular framework, based on two different molecular markers (18S rDNA and 28S rDNA), and plotting morphological and biological features on the topology. Morphological traits that support phylogenetic clades are presented and discussed, additionally we propose an amendment of the genus diagnosis together with a tentative species classification system based on tail-terminus features. A general discussion (**Chapter V**) highlights the main results and conclusions of each chapter, and addresses several comments on the diversity and future study of *Aphelenchoides*. Finally, a (short) general conclusion lists the major findings, remarks and achievements of this thesis towards a better understanding of this complex genus.

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Table I.1 List of *Aphelenchoides* species, after Hunt's checklist (2008) and Hockland's taxonomic revision (2001)

1	Type species *A. kuehnii* Fischer, 1894 = *A.* (*Aphelenchoides*) *kuehnii* Fischer, 1894 (Filipjev, 1934)
2	*A. absari* Husain & Khan, 1967
3	*A. aerialis* Bina Chanu, Mohilal & Shah, 2013*
4	*A. africanus* Dassonville & Heyns, 1984
5	*A. aligarhiensis* Siddiqi, Husain & Khan, 1967
6	*A. allius* Feng, 2012*
7	*A. andrassyi* Husain & Khan, 1967
8	*A. angusticaudatus* Eroshenko, 1968
9	*A. appendurus* Singh, 1967
10	*A. arachidis* Bos, 1977
11	*A. arcticus* Sanwal, 1965
12	*A. asterocaudatus* Das, 1960
13	*A. asteromucronatus* Eroshenko, 1967
14	*A. besseyi* Christie, 1942 = *Aphelenchoides oryzae* Yokoo, 1948 = *Asteroaphelenchoides besseyi* (Christie, 1942) Drozdovski, 1967
15	*A. bicaudatus* (Imamura, 1931) Filipjev & Stekhoven, 1941 = *Aphelenchus bicaudatus* Imamura, 1931
16	*A. blastophthorus* Franklin, 1952
17	*A. brassicae* Edward & Misra, 1969
18	*A. brevistylus* Jain & Singh, 1984
19	*A. brushimucronatus* Bajaj & Walia, 1999*
20	*A. chalonus* Chawla & Khan, 1979 = *Aphelenchoides teres* Chawla, Bhamburkar, Khan & Prasad, 1968
21	*A. chamelocephalus* (Steiner, 1926) Filipjev, 1934 = Aphelenchus chamelocephalus Steiner, 1926
22	*A. chauhani* Tandon & Singh, 1974
23	*A. chinensis* Husain & Khan, 1967
24	*A. cibolensis* Riffle, 1970
25	*A. composticola* Franklin, 1957
26	*A. confusus* Thorne & Malek, 1968
27	*A. conimucronatus* Bessarabova, 1966
28	*A. dactylocercus* Hooper, 1958
29	*A. dalianensis* Cheng, Hou & Lin, 2009*

Table I.1 *Continued*

30	*A. delhiensis* Chawla, Bhamburkar, Khan & Prasad, 1968
31	*A. depressospicularis* Negi, Kalia, Walia, Bajaj, 2009*
32	*A. dhanachandi* Bina Chanu, Mohilal & Shah, 2012*
33	*A. disccaudatus* Feng & Liu 2008*
34	*A. dubitus* Ebsary, 1991 = *Aphelenchoides dubius* Wasilewska, 1969 *nec* Fuchs, 1930
35	*A. editocaputis* Shavrov, 1967
36	*A. eltayebi* Zeidan & Geraert, 1992
37	*A. emiliae* Romaniko, 1966
38	*A. ensete* Swart, Bogale & Tiedt, 2000
39	*A. eximius* Khusainov, 2013*
40	*A. fragariae* (Ritzema-Bos, 1890) Christie 1932 = *Aphelenchoides* (*Aphelenchoides*) *fragariae* (Ritzema Bos, 1890) Christie, 1932 = *Aphelenchoides* (*Chitinoaphelenchus*) *fragariae* (Ritzema-Bos, 1890) Christie, 1932 = *Aphelenchoides* (*Aphelenchoides*) *longicollis* (Schwartz, 1911) Goodey, 1933 = *Aphelenchoides olesistus* (Ritzema-Bos, 1892) Steiner, 1932 = *Aphelenchoides* (*Aphelenchoides*) *olesistus* (Ritzema-Bos, 1892) Steiner, 1932 = *Aphelenchoides* (*Chitinoaphelenchus*) *olesistus* (Ritzema-Bos, 1892) Steiner, 1932 = *Aphelenchus olesistus longicollis* Schwartz, 1911 = *Aphelenchoides olesistus* var. *longicollis* (Schwartz,1911) T. Goodey, 1933 = *Aphelenchoides pseudolesistus* (Goodey, 1928) Goodey, 1933 = *Aphelenchoides* (*Aphelenchoides*) *pseudolesistus* (Goodey, 1928) Goodey, 1933 = *Aphelenchus fragariae* Ritzema-Bos, 1890 = *Aphelenchus longicollis* Schwartz, 1911 = *Aphelenchus olesistus* Ritzema-Bos, 1892 = *Aphelenchus ormerodis* of Jegen, 1920 nec Ritzema-Bos, 1891 = *Aphelenchus pseudolesistus* Goodey, 1928
41	*A. fuchsi* Esmaeili, Heydari, Ziaie & Gu, 2016*
42	*A. fujianensis* Zhuo, Cui, Ye, Luo, Wang, Hu & Liao, 2010*
43	*A. goldeni* Suryawanshi, 1971
44	*A. goodeyi* Siddiqi & Franklin, 1967
45	*A. gynotylurus* Timm & Franklin, 1969
46	*A. haguei* Maslen, 1979
47	*A. hamatus* Thorne & Malek, 1968
48	*A. heidelbergi* (Zhao, Davies, Riley & Nobbs, 2007) Carta, Li, Skantar & Newcombe, 2016* = *Laimaphelenchus heidelbergi* Zhao, Davies, Riley & Nobbs, 2007*
49	*A. helicosoma* Maslen, 1979
50	*A. helicus* Heyns, 1964
51	*A. huntensis* Esmaeili, Fang, Li & Heydari, 2016*
52	*A. hylurgi* Massey, 1974

Table I.1 *Continued*

53	*A. hypotris* Shah, Siddiqi & Handoo, 2015*
54	*A. indicus* Chawla, Bhamburkar, Khan & Prasad, 1968
55	*A. involutus* Minagawa, 1992
56	*A. iranicus* Golhasan, Heydari, Alvarez-Ortega, Esmaeili, Castillo & Palomares-Rius, 2016*
57	*A. jacobi* Husain & Khan, 1967
58	*A. jonesi* Singh, 1977
59	*A. lichenicola* Siddiqi & Hawksworth, 1982
60	*A. lilium* Yokoo, 1964
61	*A. limberi* Steiner, 1936 = *Paraphelenchoides limberi* (Steiner, 1936) Haque, 1967
62	*A. longiurus* Das, 1960
63	*A. longistylus* Bina Chanu & Mohilal, 2014*
64	*A. marinus* Timm & Franklin, 1969
65	*A. microspermi* Negi, Kalia, Walia, Bajaj 2009*
66	*A. microstylus* Kaisa, 2000
67	*A. montanus* Singh, 1967
68	*A. nechaleos* Hooper & Ibrahim, 1994
69	*A. neoechinocaudatus* Bina Chanu, Mohilal, Shah 2012*
70	*A. neominoris* Bina Chanu & Mohilal, 2014*
71	*A. obtusicaudatus* Eroshenko, 1967
72	*A. obtusus* Thorne & Malek, 1968
73	*A. pannocaudus* (Massey, 1966) Sánchez-Monge, Janssen, Couvreur, Hockland & Bert, *in prep*. = *Aphelenchoides* (*Laimaphelenchus*) *pannocaudus* (Massey, 1966) Hirling 1986 = *Laimaphelenchus pannocaudus* Massey, 1966
74	*A. parabicaudatus* Shavroz, 1967
75	*A. parabrushmucronatus* Feng, 2009*
76	*A. paradalianensis* Cui, Zhuo, Wang & Liao, 2011*
77	*A. paranechaleos* Hooper & Ibrahim, 1994
78	*A. parasaprophilus* Sanwal, 1965
79	*A. parascalacaudatus* Chawla, Bhamburkar, Khan & Prasad, 1968
80	*A. parasubtenuis* Shavrov, 1967
81	*A. parietinus* (Bastian, 1865) Steiner, 1932 = *Aphelelenchoides* (*Aphelenchoides*) *parietinus* (Bastian, 1865) Steiner, 1932 = *Aphelenchus aquaticus* Micoletzky, 1913 = *Aphelenchus littoralis* Hofmaner, 1915 = *Aphelenchoides modestus* (de Man, 1876) Filipjev, 1934 = *Aphelenchus parietinus* Bastian, 1865

Table I.1 *Continued*

	= *Aphelenchus rivalis* Blitschli, 1873 = *Aphelenchus striatus aquaticus* Micoletzky, 1913 = *Pathoaphelenchus parietinus* (Bastian, 1865) Steiner, 1931
82	*A. petersi* Tandon & Singh, 1970
83	*A. pinusi* Bajaj & Walia, 1999*
84	*A. pityokteini* Massey, 1974
85	*A. rarus* Eroshenko, 1968
86	*A. resinosi* Kaisa, Harman & Harman, 1995
87	*A. richardsoni* Grewal, Siddiqi & Atkey, 1991
88	*A. ritzemabosi* (Schwartz, 1911)Steiner & Buhrer, 1932 = *Aphelenchoides* (*Aphelenchoides*) *ribes* (Taylor 1917) Goodey, 1933 = *Aphelenchoides* (*Chitinoaphelenchus*) *ribes* (Taylor, 1917) Goodey, 1933 = *Aphelenchoides* (*Aphelenchoides*) *ritzemabosi* (Schwartz, 1911) Steiner & Buhrer, 1932 = *Aphelenchoides* (*Chitinoaphelenchus*) *ritzemabosi* (Schwartz, 1911) Fuchs, 1937 = *Aphelenchus omerodis apud*, Ritzema-Bos, 1892 *(partim)* = *Aphelenchus phyllophagus* Stewart, 1921 = *Aphelenchus ribes* (Taylor, 1917) Goodey, 1923 = *Aphelenchus ritzemabosi* Schwartz, 1911 = *Pathoaphelenchus ritzemabosi* (Schwartz, 1911) Steiner, 1932 = *Pseudaphelenchoides ritzemabosi* (Schwartz, 1911) Drozdovski, 1967 = *Tylenchus ribes* Taylor, 1917
89	*A. robustus* Gagarin, 1997
90	*A. rotundicaudatus* Fang, Wang, Gu & Li, 2014*
91	*A. rutgersi* Hooper & Myers, 1971
92	*A. sacchari* Hooper, 1958
93	*A. sanwali* Chaturvedi & Khera, 1979
94	*A. scalacaudatus* Sudakova, 1958
95	*A. sexlineatus* Eroshenko, 1967
96	*A. siddiqi* Fortuner, 1970
97	*A. silvester* Andrássy, 1968
98	*A. sinodendroni* Rühm, 1957 = *Aphelenchoides sinodendroni* Rühm, 1957
99	*A. spasskii* Eroshenko, 1968
100	*A. sphaerocephalus* Goodey, 1953
101	*A. spicomucronatus* Truskova, 1973
102	*A. spinohamautus* Bajaj & Walia, 1999*
103	*A. srinagrensis* (Kaul, 1985) Hassan, Chishti, Rasheed & Lone, 2009*
104	*A. stellatus* Fang, Gu, Xang & Li, 2014*
105	*A. subparietinus* Sanwal 1962

Table I.1 *Continued*

106	*A. subtenius* (Cobb, 1926) Steiner & Buhrer 1932 = *Aphelenchoides hodsoni Goodey, 1935* = *Aphelenchoides* (*Aphelenchoides*) *subtenuis* (Cobb, 1926) Steiner & Buhrer, 1932 = *Aphelenchoides* (*Chitinoaphelenchus*) *subtenuis* (Cobb, 1926) Steiner & Buhrer, 1932 = *Aphelenchus subtenuis Cobb, 1926*
107	*A. suipingensis* Feng & Li, 1986
108	*A. taraii* Edward & Misra, 1969
109	*A. trivialis* Franklin & Siddiqi, 1963
110	*A. tsalolikhini* Ryss, 1993
111	*A. tumulicaudatus* Truskova, 1973
112	*A. tuzeti* B'Chir, 1979
113	*A. unisexus* Jain & Singh, 1984
114	*A. varicaudatus* Ibrahim & Hooper, 1994
115	*A. vaughani* Maslen, 1979
116	*A. wallacei* Singh, 1977
117	*A. xui* Wang, Wang, Gu, Wang & Li 2013*
118	*A. xylocopae* Kanzaki, 2006*

* species not listed by Hunt (2008), their taxonomic validity was not checked

Table I.2 List of *Aphelenchoides species inquirendae vel insertae sedis* and *indeterminatae*, after Hunt's checklist (2008) and Hockland's taxonomic revision (2001)

1	*A. abyssinicus* (Filipjev, 1931) Filipjev, 1934 = *Aphelenchus abyssinicus* Filipjev, 1931
2	*A. agarici* Seth & Sharma, 1986
3	*A. bengalensis* Singh & Khera, 1978
4	*A. bimucronatus* Nesterov, 1985
5	*A. brevicaudatus* Das, 1960
6	*A. brevionchus* Das, 1960 Murali Mohan, 1982
7	*A. breviuteralis* Eroshenko, 1968
8	*A. buckleyi* Tandon & Singh, 1974
9	*A. capsuloplanus* (Haque, 1967) Andrassy, 1976 = *Paraphelenchoides capsuloplanus* Haque, 1967
10	*A. centralis* Thorne & Malek, 1968
11	*A. clarolineatus* Baranovskaya, 1958
12	*A. clarus* Thorne & Malek, 1968
13	*A. coffeae* (Zimmeman, 1898) Filipjev, 1934 = *Aphelenchus coffeae* Zimmeman, 1898
14	*A. colocasiai* Tandon & Singh, 1974
15	*A. conophthori* Massey, 1974
16	*A. curiolis* Gritsenko, 1971
17	*A. cyrtus* Paesler, 1957
18	*A. daubichaensis* Eroshenko, 1968
19	*A. dubius* (Fuchs, 1930) Filipjev, 1934 = *Parasitaphelenchus dubius* Fuchs, 1930
20	*A. echinocaudatus* Haque, 1968
21	*A. elongatus* Schuurmans Stekhoven, 1951
22	*A. eradicitus* Eroshenko, 1968
23	*A. ferrandini* Meyl, 1954
24	*A. fluviatilis* Andrássy, 1960
25	*A. franklinae* Singh, 1969
26	*A. goeldii* (Steiner, 1914) Filipjev, 1934 = *Aphelenchus goeldii* Steiner, 1914 = *Aphelenchoides* (*Aphelenchoides*) *goeldii* (Steiner, 1914) Filipjev, 1934
27	*A. graminis* Baranovskaya & Haque, 1968
28	*A. graminophilus* Verma, Bisen, Verma & Kumar-Singh, 1981
29	*A. hainanensis* (Rahm, 1938) Goodey 1951

Table I.2 *Continued*

	= *Aphelenchus hainanensis* Rahm, 1938
30	*A. helophilus* (de Man, 1880) Goodey, 1933 = *Aphelenchoides* (Aphelenchoides) *helophilus* (de Man, 1880) Goodey, 1933 = *Aphelenchus elegans* Micoletzky, 1913 = *Aphelenchus helophilus* de Man, 1880 = *Aphelenchus hessei* Rahm, 1925 = *Aphelenchus parietinus helophilus* de Man, 1880
31	*A. hessei* (Rahm, 1925) Filipjev, 1934
32	*A. hyderabadensis* Das, 1960
33	*A. jodhpurensis* Tikyani, Khan & Bhatnagar, 1970
34	*A. kungradensis* Karimova, 1957
35	*A. lagenoferrus* Baranovskaya, 1963
36	*A. lanceolatus* Tandon & Singh, 1974
37	*A. littoralis* Hofmänner, 1915 = *Aphelenchus littoralis* (Hofmänner, 1915) Filipjev, 1934
38	*A. loofi* Kumar, 1982
39	*A. lucknowensis* Tandon & Singh, 1973
40	*A. macromucrons* Slankis, 1967
41	*A. macronucleatus* Baranovskaya, 1963
42	*A. menthae* Lisetzkaya, 1971
43	*A. minimus* Meyl, 1953
44	*A. minor* (Cobb, 1893) Steiner & Buhrer, 1933 = *Aphelenchus minor* Cobb, 1893
45	*A. minoris* Ebsary, 1991 = *Aphelenchoides minor* Seth & Sharma, 1986 *nec* Cobb, 1893
46	*A. mucronatus* Paesler, 1946
47	*A. myceliophagus* Seth & Sharma, 1986
48	*A. naticochensis* (Steiner, 1920) Filipjev, 1934 = *Aphelenchus naticochensis* Steiner, 1920
49	*A. neocomposticola* Seth & Sharma, 1986
50	*A. nonveilleri* Andrássy, 1959
51	*A. ormerodis* (Ritzema Bos, 1891) Steiner, 1932 = *Aphelenchus ormerodis* Ritzema Bos, 1891
52	*A. orientalis* Eroshenko, 1968
53	*A. panaxi* Skarbiolovich & Potekhina, 1959
54	*A. panaxofolia* Liu, Wu, Duan & Liu, 1999
55	*A. paramonovi* Eroshenko & Kruglik, 2004
56	*A. parasexlineatus* Kalinich, 1984

Table I.2 *Continued*

57	*A. platycephalus* Eroshenko, 1968
58	*A. polygraphi* Massey, 1974
59	*A. pusillus* (Thorne, 1929) Filipjev 1934 = *Aphelenchus pusillus* Thorne, 1929 = *Aphelenchoides* (*Aphelenchoides*) *pusillus* (Thorne, 1929) Filipjev, 1934
60	*A. retusus* (Cobb, 1927) Goodey, 1951 = *Aphelenchus retusus* Cobb, 1927
61	*A. rhytium* Massey, 1971
62	*A. richtersi* (Steiner, 1914) Filipjev, 1934 = *Aphelenchus richtersi* Steiner, 1914
63	*A. rosei* Dmitrenko, 1966
64	*A. saprophilus* Franklin, 1957
65	*A. seiachicus* Nesterov, 1973
66	*A. shamimi* Khera, 1970
67	*A. sinensis* (Wu & Hoeppli, 1929) Andrássy, 1960 = *Aphelenchus parietinus* var. *sinensis* Wu & Hoeppli, 1929
68	*A. singhi* Das, 1960
69	*A. speciosus* Andrassy, 1958
70	*A. spinocaudatus* Skarbilovich, 1957
71	*A. spinosus* Paesler, 1957
72	*A. stammeri* Körner, 1954
73	*A. steineri* Rühm, 1956
74	*A. submersus* Truskova, 1973
75	*A. swarupi* Seth & Sharma, 1986
76	*A. tagetae* Steiner, 1941
77	*A. teres* (Schneider, 1927) Filipjev, 1934 = *Aphelenchus teres* Schneider, 1927
78	*A. vigor* Thorne & Malek, 1968
79	*A. zeravschanicus* Tulaganov, 1949

Nomina nuda

A. coffeae (Noack, 1898) Filipjev, 1934
= *Aphelenchus coffeae* Noack, 1898
A. henansis Li, Feng & Xu, 1985 (Misspelled as "*henanensis"* in Hunt, 2008)
A. longiuteralis Eroshenko, 1967

Last taxonomical changes in the genus:

- *Aphelenchoides ipidicola*, listed by Hunt (2008), is now regarded as *Ruehmaphelenchus ipidicola* (Kanzaki *et al*. 2014)

- *Laimaphelenchus heidelbergi* was transferred to the genus *Aphelenchoides* as *A. heidelbergi* by Carta *et al*. (2016)

- *Laimaphelenchus pannocaudus* was transferred to the genus *Aphelenchoides* by Hirling (1986) together with other *Laimaphelenchus* without a vulval flap, however, this was not accepted by Hunt (2008). Given the molecular and morphological data of this species (see discussion in Chapter IV), its transfer to *Aphelenchoides* can be accepted, *i.e.* as *A. pannocaudus*. This publication is currently in preparation.

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CHAPTER II
Plant-parasitic *Aphelenchoides* and associated plants

Modified from

Sánchez-Monge, A.[1,2], Flores, L.[3] , Salazar, L.[3] , Hockland, S.[4] & Bert, W.[1] (2015) An updated list of the plants associated with plant-parasitic *Aphelenchoides* (Nematoda: Aphelenchoididae) and its implications for plant-parasitism within this genus. *Zootaxa* 4013, 207–224.

[1] *Ghent University, Department of Biology, Nematology Research Unit, Ledeganckstraat 35, B-9000 Ghent, Belgium.*
[2] *Universidad de Costa Rica, Escuela de Estudios Generales, 2060, Costa Rica*
[3] *Universidad de Costa Rica, Laboratorio de Nematología, Centro de Investigación en Protección de Cultivos, 2060, Costa Rica*
[4] *Independent Plant Nematology Consultant, Harrogate, North Yorkshire, England, UK.*
www.plantparasiticnematodes.com

Abstract:

Few *Aphelenchoides* spp. are facultative plant-parasites (foliar and bulb nematodes); three of them are well known in agricultural systems, namely *Aphelenchoides besseyi, A. fragariae* and *A. ritzemabosi*. Eleven other plant-parasitic species: *A. arachidis, A. bicaudatus, A. blastophthorus, A. dalianensis, A. ensete, A. nechaleos, A. panaxofolia, A. paranechaleos, A. saprophilus, A. sphaerocephalus* and *A. subtenuis*, have been reported from a limited number of plant species. We compiled a new database of the associated plants for these fourteen species, a comprehensive list that includes 1105 reports from 126 botanical families. *A. besseyi, A. fragariae* and *A. ritzemabosi* represent 94% of the reports, circa 83% and 16% of the total reports correspond to flowering plants and ferns, respectively, with three records on conifers and two from other botanical groups also listed. 50% of the plant-parasitic *Aphelenchoides* spp. show a remarkably broad diversity of associated plants and appear to have no specific plant hosts (*i.e.* are generalists). The broad host ranges of these species and the absence of more intimate interactions with their associated plants highlights the primitive mode of parasitism in *Aphelenchoides* species, making them potentially interesting in the study of the evolution of plant parasitism. Even though the compiled list of associated plants is long, it probably only represents a fraction of the actual range. The complete compilation has been uploaded to http://nematodes.myspecies.info/.

Keywords: crops, evolution, ferns, flowering plants, foliar nematodes, phylogeny

Resumen:

Pocas especies de *Aphelenchoides* son parásitos facultativos de plantas (nematodos foliares y del bulbo), tres de ellas: *Aphelenchoides besseyi, A. fragariae* y *A. ritzemabosi* son muy importantes en sistemas agrícolas. Otras once especies; *A. arachidis, A. bicaudatus, A. blastophthorus, A. dalianensis, A. ensete, A. nechaleos, A. panaxofolia, A. paranechaleos, A. saprophilus, A. sphaerocephalus y A. subtenuis*, han sido informadas en un número reducido de especies de plantas. Se recopiló una nueva base de datos de plantas asociadas a las catorce especies, e incluye 1105 registros de 126 familias botánicas. *A. besseyi, A. fragariae* and *A. ritzemabosi* representan el 94% de los registros, cerca del 83% y 16% del total de los mismos corresponden a plantas con flores y helechos, respectivamente, tres registros en coníferas y dos en otros grupos botánicos fueron también enlistados. La mitad de los *Aphelenchoides* fitoparásitos muestran una amplia diversidad de plantas asociadas y no tienen hospederos específicos (generalistas). Los amplia variedad de estas especies y la ausencia de relaciones más íntimas con sus plantas asociadas destacan el modo primitivo de parasitismo de *Aphelenchoides*, haciéndolos potencialmente interesantes en el estudio de la evolución del fitoparasitismo. A pesar de la amplitud de la lista recopilada, es probable que solo represente una parte de la diversidad de asociaciones. Este listado está disponible en http://nematodes.myspecies.info/.

Palabras clave: cultivos, evolución, filogenia, helechos, nematodos foliares, plantas con flores

Introduction

Around 4000 species of nematodes have been described as plant-parasitic (Decraemer & Hunt 2013), *i.e.* those that can feed on plant tissue, and some of them have a serious economic impact on crops. Plant-parasitism has arisen independently several times in Nematoda: in Trichodoridae Thorne, 1935, Longidoridae Thorne, 1935 (Meyl, 1961) and in the order Panagrolaimida Hodda, 2007 specifically in the suborders Tylenchina Chitwood, 1950 and Aphelenchina Geraert, 1966 (*sensu* Hodda 2011). However, the position of "tylenchs" (=Tylenchina Chitwood, 1950 *sensu* Hodda 2011 or Tylenchida Thorne, 1949 *sensu* Siddiqi 1980) versus "aphelenchs" (=Aphelenchina Geraert, 1966 *sensu* Hodda 2011 or Aphelenchida Siddiqi, 1980) is controversial, and thus the point at which plant-parasitism arose remains hypothetical. Phylogenetic hypotheses based on nuclear SSU rDNA (Bert *et al.* 2008, van Megen *et al.* 2009) suggested that Aphelenchoidea Fuchs, 1937 (Thorne, 1949) is a sister to tylenchs while Aphelenchoidoidea Skarbilovich, 1947 (Siddiqi, 1980) have an independent origin. However, a recent phylogenetic analysis based on mitochondrial genomes (Kim *et al.* 2015) indicate a monophyletic status for aphelenchs, independent from the tylenchs.

The superfamily Aphelenchoidoidea (*sensu* Hodda 2011) comprises 7 families and includes fungal-feeding species, insect parasites, predators but also some damaging plant pathogens in the genera *Bursaphelenchus* Fuchs, 1937 and *Aphelenchoides* Fischer, 1894 (Nickle, 1970). Although most species of *Aphelenchoides* are fungivores (Kanzaki & Giblin-Davis 2012), fourteen species have been reported as plant-parasitic in a wide variety of plants. Special attention has been paid to three predominantly plant-parasitic species within the "foliar and bulb nematodes" (Aphelenchoididae Skarbilovich, 1947 (Paramonov, 1953)) namely *Aphelenchoides besseyi* Christie, 1942, *A. fragariae* (Ritzema Bos, 1890) Christie, 1932 and *A. ritzemabosi* (Schwartz, 1911) Steiner & Buhrer, 1932, that have been extensively studied due to their economic impact and yield losses. Notably, *A. besseyi* was listed within the top ten plant-parasitic nematodes (PPN) according to its scientific and economic importance (Jones *et al.* 2013), while *A. fragariae* and *A. ritzemabosi* are the most common parasitic nematodes on aerial parts of ornamental plants (McCuiston *et al.* 2007). In addition to the plant-parasitic *Aphelenchoides,* a few mycophagous species have gained a quarantine status, *i.e. A.*

agarici Seth & Sharma, 1986, *A. composticola* Franklin, 1957, *A. sacchari* Hooper, 1958 and *A. swarupi* Seth & Sharma, 1986 (Singh *et al.* 2013).

The number of plants associated with *Aphelenchoides* has increased in recent years showing a broad host range compared to other PPN, with over 700 species from 85 botanical families being reported (Kohl 2008, 2011). However, Koch's Postulates, *i.e.* 1) the pathogen is present in all cases of the disease, 2) the pathogen is able to grow in pure culture when isolated, 3) the pathogen from the pure culture is able to cause the disease when inoculated on a healthy and susceptible host and 4) once re-isolated the pathogen must be identified as the originally inoculated organism, have not been fulfilled in most cases and the term "associated host" is preferred to denote a possible parasitic relationship (Kohl 2011). The high number of nominal species of *Aphelenchoides* (circa 180), of which the majority have not been described sufficiently to enable reliable identification, has led to notorious determination problems. Moreover, in addition to a large intra-specific variation and minimal inter-specific relationships, most taxa are not yet associated with discriminating molecular data, muddling the taxonomic work on this genus (Zhao 2006). By the beginning of 2015, the databases of the International Nucleotide Sequence Database Collaboration (INSDC) had more than 600 nucleotide sequences (mostly mitochondrial DNA and RNA subunits) that belonged to *Aphelenchoides* samples, but for only 17 named species while the number of taxa tagged only as "*Aphelenchoides* sp." was 34. Some of these taxa are represented only by a single sequence.

Based on the number of their hosts, parasites are either classified as specialists or generalists (Koprivnikar & Randhawa 2013). Both feeding strategies are probably present in the genus *Aphelenchoides* as some species have been reported only on one or two related plant species while others have been reported on plant groups not closely related. In this paper, we present a compiled list of the plant species associated with plant-parasitic *Aphelenchoides* to appraise the potential/overlapping ranges that a single or a specific combination of species could have. Based on the compiled data of *Aphelenchoides* records and relationships, respectively plotted on a plant and *Aphelenchoides* spp phylogenetic framework, we also provide some insights on plant-parasitism of this genus.

Materials and methods

Data on *Aphelenchoides* species and their associated plants were compiled from the available literature (papers, bulletins, theses, data sheets), on-line publications (Kohl 2011) and the University of California Davis on-line database (http://plpnemweb.ucdavis.edu). Reports considered by the source as "Doubtful" or "Mistake" were excluded from the list as well as those that originated from soil samples. When the nematodes were found in roots, only those plants explicitly described as hosts or associated hosts were listed to avoid the inclusion of non-parasitic species that may live in the surroundings of the sampled plant. For the same reason, *Aphelenchoides* species described as fungivores and found on plant samples or their vicinities were also excluded from the main list when a parasitic relationship was not clear or stated. Excluded cases were compiled in a secondary list.

Plant species reported only with the common name, except for crops, were excluded to avoid confusion on their identity. Those reports on varieties, hybrids or subspecies were treated as independent entries to facilitate the use of this new compilation. Taxonomic information for the reported plants (family, class, order, genus and synonymy) was updated to the most recent classification available (The Angiosperm Phylogeny Group 2009).

Reports of *Aphelenchoides* species were plotted on an associated plants' supertree based on consensus trees of angiosperms (The Angiosperm Phylogeny Group 2009) and ferns (Lehtonen 2011). This reconstruction was made to order level, or to family level if relevant, such as for the Pteridophyta (ferns). Ten families of this group with reports for only *A. fragariae* were excluded from the supertree to simplify its layout. A schematic overview of the phylogenetic relations of *Aphelenchoides* was made as a combination of the SSU rDNA-based topologies published by Kanzaki *et al.* (2014a, 2014b), Rybarczyk-Mydłowska *et al.* (2012) and Ryss *et al.* (2013), mainly based on plant-parasitic species of this genus. Both trees were made on Mesquite Version 3.01 (Mesquite Project Team 2014), and the subsequent editing as done on the GNU Image Manipulation Program (GIMP) 2.8.10 (Kimball *et al.* 2014).

Results and discussion

118 species of *Aphelenchoides* (plus 79 of uncertain status) have been described (Tables I.1 and I.2) but only 14 have been reported as plant-parasitic species (Table II.1). The most commonly reported species were *A. besseyi, A. ritzemabosi* and *A. fragariae*, with 91, 321 and 620 associated plant species, respectively. Six of the plant-parasitic *Aphelenchoides* spp. are only known from single hosts and *A. dalianensis* Cheng, Hou & Lin, 2009 is found exclusively on two species within Pinophyta (Table II.1). In the latter group some reports were made from wood or decaying samples and thus, a parasitic relationship cannot be confirmed.

Table II.1: Number of associated plants reported for 14 plant-parasitic *Aphelenchoides* species on Pteridophyta, Pinophyta and Magnoliophyta.

Aphelenchoides species	Pteridophyta	Pinophyta	Magnoliophyta	Total
A. arachidis			6	6
A. besseyi *	4	2	85	91
A. bicaudatus		2	16	18
A. blastophthorus			16	16
A. dalianensis		2		2
A. ensete			1	1
A. fragariae *	162	2	456	620
A. nechaleos			1	1
A. panaxofolia			1	1
A. paranechaleos	7		1	1
A. ritzemabosi			314	321
A. saprophilus			1	1
A. sphaerocephalus			1	1
A. subtenuis			23	23
Total	173	8	922	1103

* *A. besseyi* and *A. fragariae* were also found associated with Lycopodiophyta and Marchantiophyta, respectively.

According to our database, a total of 25 families of ferns (Pteridophyta), and 99 Spermatophyta families (36 orders of Angiosperms and one of Gymnosperms) have at least one plant species associated with *Aphelenchoides*. 83.4% of the total reports belong to flowering plants (Magnoliophyta), 15.7% to ferns (Pteridophyta) and only one family, 0.7%, in the conifers (Pinophyta) (Table II.1); the distribution of the reports in Magnoliophyta is given in Table II.2. The complete data of these reports as well as a complementary list including reported fungivorous *Aphelenchoides,* specimens identified only to genus level and findings on pine trees or diverse samples are available at http://nematodes.myspecies.info/.

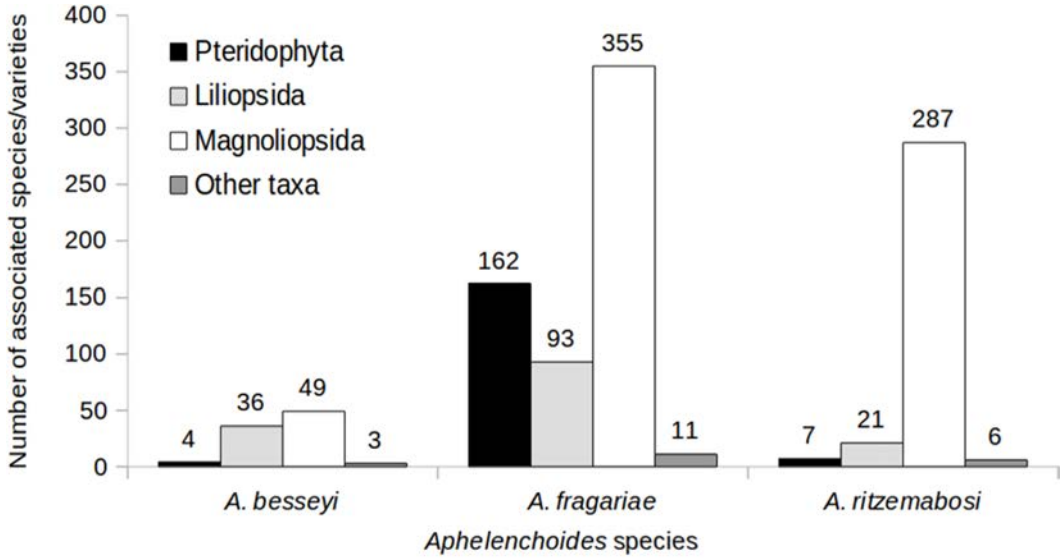

Figure II.1. Number of species/varieties of plants associated with foliar nematodes in ferns (Pteridophyta), monocots (Liliopsida), dicots (Magnoliopsida) and other botanical groups

Plant-parasitic *Aphelenchoides* (PPA) and associated plants

Aphelenchoides arachidis Bos, 1977

A. arachidis was found in *Arachis hypogagea* L. (Fabaceae Lindl.) (Minton & Baujard 1990) as an endoparasite of groundnut testa in Nigeria (Bos 1977a) and more recently in Egypt and South Africa (Montasser *et al.* 2008, Lesufi *et al.* 2015). Although the symptoms have only been described on *Arachis*, roots of some Poaceae Barnhart species (*Oryza sativa* L., *Saccharum officinarum* L., *Sorghum* sp., *Pennisetum glaucum* (L.) R. Br. and *Zea mays* L.) had high quantities of this species and were reported as hosts (Bos 1977b, CABI 2010, Escuer & Bello 2000). This nematode was also found on roots of non-specified wild grasses (Bos 1977b, CABI 2010). According to Bos (1977b) two biotypes, one on cereals and the other on cereals and groundnuts, may be occurring in fields.

Aphelenchoides besseyi Christie, 1942

Known as the causal agent of the "white tip disease" in rice (Hockland 2004) and recently identified as the causal agent of the "black spot disease" on beans (*Phaseolus vulgaris* L., Fabaceae) (Chaves *et al.* 2013), *A. besseyi* has been reported on 90 other plants, ranging from lycopodiums (Lycopodiophyta) and ferns (Pteridophyta) (Kohl 2008, UCDavis Nemabase 2010) to flowering plants (Magnoliophyta) (Table II.1, Fig. II.1). Zhuo *et al.* (2010) mentioned the presence of *A. besseyi* on pine wood from China (*Pinus massoniana* Lamb. and *P. taeda* L.,

Table II.2. Number of reported associations (N) of flowering plants (Magnoliophyta) with plant-parasitic *Aphelenchoides* spp. (complete database available at http://nematodes.myspecies.info/)

Order	Family	N	Order	Family	N
Alismatales	Alismataceae	1	Lamiales	Acanthaceae	9
	Araceae	12		Bignoniaceae	1
	Hydrocharitaceae	3		Calceolariaceae	3
	Potamogetonaceae	1		Gesneriaceae	12
Apiales	Apiaceae	9		Lamiaceae	56
	Araliaceae	4		Lentibulariaceae	1
Arecales	Arecaceae	1		Linderniaceae	1
Asparagales	Amaryllidaceae	21		Oleaceae	5
	Asparagaceae	23		Phrymaceae	2
	Iridaceae	12		Plantaginacea	1
	Orchidaceae	20		Plantaginaceae	27
	Xanthorrhoeaceae	3		Scrophulariaceae	10
Asterales	Asteraceae	146		Verbenaceae	10
	Campanulaceae	5	Liliales	Alstroemeriaceae	2
Boraginaceae	Boraginaceae	14		Colchicaceae	5
Brassicales	Brassicaceae	12		Liliaceae	32
Caryophyllales	Amaranthaceae	4		Melanthiaceae	3
	Cactaceae	3		Philesiaceae	1
	Caryophyllaceae	7	Malpighiales	Hypericaceae	1
	Phytolaccaceae	1		Passifloraceae	1
	Plumbaginaceae	9		Salicaceae	1
	Polygonaceae	8		Violaceae	4
	Portulacaceae	1	Malvales	Malvaceae	4
Ceratophyllales	Ceratophyllaceae	1	Myrtales	Lythraceae	1
Chloranthales	Chloranthaceae	1		Myrtaceae	1
Commelinales	Commelinaceae	4		Onagraceae	3
	Haemodoraceae	1	Nymphaeales	Cabombaceae	1
Cornales	Cornaceae	2	Oxalidales	Oxalidaceae	2
	Hydrangeaceae	7	Piperales	Aristolochiaceae	3
Cucurbitales	Begoniaceae	15		Piperaceae	8
	Cucurbitaceae	1	Poales	Cyperaceae	4
Dioscoreales	Dioscoreaceae	1		Poaceae	36
Dipsacales	Adoxaceae	2	Ranunculales	Berberidaceae	4
	Caprifoliaceae	9		Papaveraceae	3
	Dipsacaceae	12		Ranunculaceae	64
	Morinaceae	1	Rosales	Moraceae	16
Ericales	Balsaminaceae	4		Rosaceae	31
	Diapensiaceae	1		Ulmaceae	1
	Ericaceae	3		Urticaceae	4
	Myrsinaceae	3	Sapindales	Rutaceae	1
	Polemoniaceae	8	Saxifragales	Crassulaceae	8
	Primulaceae	18		Grossulariaceae	7
Fabales	Fabaceae	16		Haloragaceae	1
Gentianales	Apocynaceae	1		Paeoniaceae	8
	Rubiaceae	9		Saxifragaceae	41
Geraniales	Geraniaceae	10	Solanales	Convolvulaceae	7
Gunnerales	Gunneraceae	1		Solanaceae	16
			Vitales	Vitaceae	2
			Zingiberales	Marantaceae	2
				Musaceae	3
				Strelitziaceae	1

Pinaceae Lindley) but the parasitic relationship remains uncertain since, like other *Aphelenchoides* species, *A. besseyi* has the ability to feed on fungi (Jones *et al.* 2013) and therefore the nematodes are more likely to be thriving on mycelia rather than the tree tissue itself. Further research should address the association between pine trees and this nematode species. Also noteworthy is the presence of *A. besseyi* on seeds of *Brachiaria brizantha* (Hochst. ex A.Rich.) R.Webster (Poaceae) (Tenente *et al.* 2006).

Aphelenchoides bicaudatus (Imamura, 1931) Filipjev & Schuurmans Stekhoven, 1941

Asparagus aethiopicus L. (Asparagaceae Juss.), *Fragaria glandiglora* Ehrn. (Rosaceae Juss.), *Lupinus angustifolius* L. (Fabaceae) and *Setaria palmifolia* Stapf. (Poaceae) are listed in the UCDavis Nemabase (2010) as hosts for this species. Escuer & Bello (2000) listed a dozen plant species as associated with *A. bicaudatus*, mostly monocots (see online database) and was also found in soil from banana (Liao & Feng 1999) and coffee plantations (Souza 2000). Recently, Zhao (2006). Zhuo *et al.* (2010) also listed this species as associated with *Pinus radiata* D. Don (Pinaceae) and *P. thunbergii* Parl. *,* respectively. A parasitic relationship was not confirmed on such plant species. According to Escuer & Bello (2000), *A. bicaudatus* is known as a mycophagous species and can feed on algae as well as plant tissue; it is relatively common to find in ornamental nurseries (Jen *et al.* 2012). It is also able to grow and survive on roots of rice (*Oryza sativa*, Poaceae) and *Phalaenopsis* sp. (Orchidaceae Juss.) despite the absence of symptoms of infestation (Jen *et al.* 2012).

Aphelenchoides blastophthorus Franklin, 1952

This species is commonly found on ornamental plants of the genera *Anchusa* L. (Boraginaceae Juss.), *Begonia* L. (Begoniaceae C. Agardh)*, Caltha* L. and *Trollius* L. (Ranunculaceae Juss.)*, Cephalaria* Schrad. ex Roem. & Schult. (Dipsacaceae Juss.)*, Convallaria* L. (Asparagaceae)*, Dipsacus* L. (Dipsacaceae)*, Geum* L. (Rosaceae), *Narcissus* L. (Amaryllidaceae J.St.-Hil.) *and Viola* L. (Violaceae Batsch) (Escuer & Bello 2000, Ortuño & Oros 2002), *Anemone* L. (Ranunculaceae) (McCuiston *et al.* 2007), *Iris* L. (Iridaceae Juss.) and *Hepatica* Mill. (Ranunculaceae) (UCDavis Nemabase 2010) but it is particularly important in *Scabiosa caucasica* M. Bieb. (Caprifoliaceae Juss.) on which it destroys the inflorescence and causes laminae distortion (Singh *et al.* 2013). Haukeland & Brekke (2000) also showed the

damaging potential of this species on strawberry (*Fragaria* x *ananasa* Duchesne, Rosaceae) in Norway.

Aphelenchoides dalianensis Cheng, Hou & Lin, 2009

A. dalianensis was extracted from wood slices of *Pinus thunbergii* (Pinaceae) and subsequent experiments showed its ability as a parasite on *Pinus massoniana* (Pinaceae) (Cheng *et al.* 2009). No other hosts have been reported for this species and its origin is still unknown since the affected samples were found close to a trade port (Cheng *et al.* 2009).

Aphelenchoides ensete Swart, Bogale & Tiedt, 2000

A. ensete was found on *Ensete ventricosum* (Welw.) Cheesman (Musaceae Juss.) leaves showing the "black leaf streak" disease in Ethiopia (Swart *et al.* 2000) but it has also been extracted from fresh root samples of the same host (Bogale *et al.* 2004).

Aphelenchoides fragariae (Ritzema Bos, 1890) Christie, 1932

The most striking augmentation in the number of reported plant associations of *Aphelenchoides* spp. belongs to *A. fragariae*. Escuer & Bello (2000) and Fu (2012) quote Siddiqi (1975) and mention 250 plant species belonging to 47 families, but as shown in Table II.1 and Fig. II.1, at least 621 plant species and varieties from 287 genera are associated with *A. fragariae*, most of them (84%) within flowering plants. *A. fragariae* is also the species that is reported on the highest number of ferns, followed by *A. ritzemabosi* and *A. besseyi* with 162, 7 and 4 species respectively (Fig. II.1). To date, it is also the only *Aphelenchoides* species reported on Marchantiophyta (Kohl 2008). Several hosts are shared with other *Aphelenchoides* species (Fig. II.2).

Aphelenchoides nechaleos Hooper & Ibrahim, 1994

A. nechaleos was extracted from stems of rice in Sierra Leone. Since this species occurred with *A. besseyi* it was originally thought as a variant of the latter, but it was later described as a new species (Hooper & Ibrahim 1994).

Aphelenchoides panaxofolia Liu, Wu, Duan & Liu, 1999

A. panaxofolia was described as a parasite on the leaves of *Panax quinquefolius* L. (American ginseng) in China, no other host is known for this species.

Aphelenchoides paranechaleos Hooper & Ibrahim, 1994

A. paranechaleos was extracted from stems of rice in Vietnam and, like *A. nechaleos*, mistakenly thought to be *A. besseyi* (Hooper & Ibrahim 1994). It is also similar to *A. nechaleos* but their populations are unable to interbreed (Hooper & Ibrahim 1994). According to our data *A. nechaleos, A. paranechaleos, A. arachidis* and *A. besseyi* are the only four species in this genus considered as plant-parasites of rice; further surveys are needed to confirm *A. bicaudatus*' parasitism on this crop (Escuer & Bello 2000; Jen *et al.* 2012) (Fig. II.3).

Aphelenchoides ritzemabosi (Schwartz, 1911) Steiner & Buhrer, 1932

After *A. fragariae, A. ritzemabosi* has the highest number of reported hosts in both flowering plants and ferns (Table II.1, Fig. II.1). It is particularly problematic on *Chrysanthemum* L. and has previously been reported from circa 200 plant species (Escuer & Bello 2000, McCuiston 2007). That number increases to 321 in this paper with 314 flowering plants and seven ferns (Table II.1). Escuer & Bello (2000) mentioned that several weed species or non-cultivated plants are also suitable hosts for this species and further studies should be addressed to confirm this possibility. Reports in Asteraceae Bercht. & J. Pres are especially numerous (see online database) and around 85 species of this family have been mentioned as associated plants with *A. ritzemabosi*.

Aphelenchoides saprophilus Franklin, 1957

A. saprophilus was described as being commonly found in rotting plant tissues (Franklin 1957) and has been found parasitizing garlic (*Allium sativum* L.*,* Amaryllidaceae) on which it could be an important pest (Singh *et al.* 2013). This nematode was intercepted from *Aralia* sp. (Araliaceae Juss.) at ports in Canada (Sewell 1977) and from *Festuca vaginata* Waldst. & Kit. ex Willd. clumps (Poaceae) (Krnjaic & Krnjaic 1976) but a parasitic relationship with these plant species needs to be confirmed.

Aphelenchoides sphaerocephalus Goodey, 1953

A. sphaerocephalus was described from *Evodia roxburghiana* Benth. (Rutaceae Juss.) on dry leaves with yellow specks (Goodey 1953). This is the only known host for this nematode (UCDavis' Nemabase 2010), and to the best of our knowledge, that report is also the only one on the family Rutaceae for any plant-parasitic *Aphelenchoides*. It should be noted that *Ditylenchus drepanocercus* Goodey, 1953 (Tylenchida Thorne, 1949: Anguinidae Nicoll, 1935) was found occurring with *A. sphaerocephalus* in the same symptomatic samples (Goodey 1953).

Aphelenchoides subtenuis (Cobb, 1926) Steiner & Buhrer, 1932

A. subtenuis was first described on *Narcissus* bulbs (Amaryllidaceae) (Goodey 1933) and it is mostly found on monocots hosts of the genera *Allium* L. (Amaryllidaceae) and *Narcissus*, *Crocus* L. and *Iris* (Iridaceae), *Scilla* L. and *Tulipa* L. (Liliaceae Juss.) and *Colchicum* L. (Colchicaceae DC.) (UCDavis' Nemabase 2010). The only reports from dicotyledonous plants are on *Phlox* sp. (Polemoniaceae Juss.) (UCDavis Nemabase 2010) and *Trifolium pratense* L. (Fabaceae) (Deimi *et al.* 2006).

Main Plant-Parasitic *Aphelenchoides* spp (MPPA): *A. besseyi, A. fragariae* and *A. ritzemabosi*

A. besseyi, A. fragariae and *A. ritzemabosi* are the most important species of foliar and bulb nematodes in terms of both, host ranges and economic yields. In Spermatophyta, these three species are present in 98 out of 99 families within 36 of 37 orders of plants associated with PPA. Only the order Sapindales Dumortier has no associations with the MPPA but only with *A. sphaerocephalus* on *Evodia roxburghiana* (Rutaceae) (Goodey 1953). Flowering plants are the most important group in terms of associations (83% of the reports; see Tables II.1 and II.2) and within them, dicots hosts are almost five times the number of monocots (Fig. II.1). Ferns (Pteridophyta) also represent an important group with 25 families having at least one plant species associated, especially for *A. fragariae* as 25% of its reports belong to the latter group (Table II.1, Fig. II.1).

Remarkably, despite the high number of reported associations and their unspecialized feeding behavior, it is not common for a plant species to have more than one MPPA. From a

total of 925 plant species/varieties only six have reports of the three species, *i.e. Dahlia pinnata* Cav., *Zinnia elegans* Jacq., and *Z. violacea* Cav. (Asteraceae), *Fragaria* x *ananasa* (Rosaceae), *Saintpaulia ionantha* H. Wendl. (Gesneriaceae Rich. & Juss.) and the fern *Asplenium nidus* L. (Aspleniaceae Newm.) on which their inter-specific and host-related interactions have not been documented. In addition to those species, the MPPA have been reported on unidentified species of the genera *Begonia*, *Chrysanthemum* and *Fragaria* L. (see online database). Plant and nematode distribution aside from environmental factors could be affecting potential associations, unfortunately the number of studies on such topic and *Aphelenchoides* spp. is limited and focused on selected species on specific hosts *e.g. A. fragariae* in Lantana (Kohl *et al.* 2010), *A. ritzemabosi* in Alfalfa (Williams-Woodward & Gray 1999).

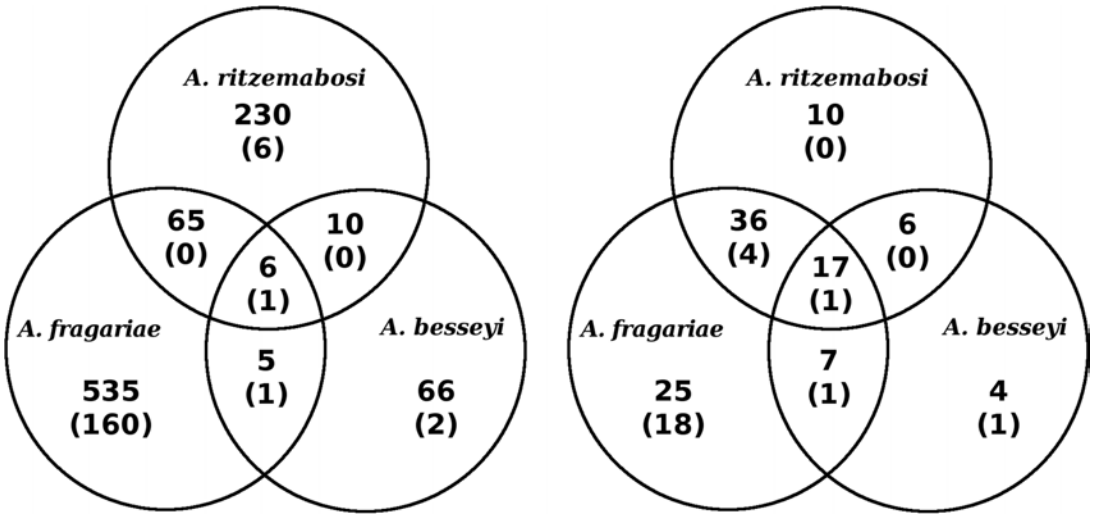

Figure II.2. Number of single and shared associated plant species (left) and families (right) of foliar nematodes (*Aphelenchoides*). Between brackets is the corresponding number of ferns for each case

According to the compiled data, *A. fragariae* and *A. ritzemabosi* are more likely to be found on the same plant species than any other combination of MPPA (65 species, Fig. II.2). *A. fragariae* and *A. besseyi* have only 6 plant species in common; *Impatiens balsamina* L. (Balsaminaceae Rich.), *Ficus elastica* Roxb. (Moraceae Gaudich.), *Allium cepa* L. (Amaryllidaceae), *Pinus massoniana* (Pinaceae) and the ferns *Asplenium jamaicense* Jenman (Aspleniaceae) and *Lygodium circinatum* (Burm.f.) Sw. (Lygodiaceae C.Presl.). *A. fragariae* and *A. besseyi* have also been found on *Lemna* sp. (Asteraceae). Finally, *A. besseyi* and *A. ritzemabosi* record 10 hosts from 6 families in common; *Calendula officinalis* L.,

Chrysanthemum morifolium Ramat., *Chrysanthemum maximum* L., *Lactuca sativa* L. and *Leucanthemum maximum* (Ramond) DC. (Asteraceae), *Phaseolus vulgaris* (Fabaceae), *Solenostemon scutellarioides* (L.) R.Br. (Lamiaceae Martinov), *Fragaria vesca* L. (Rosaceae), *Nicotiana tabacum* L. (Solanaceae Juss.) and *Polianthes tuberosa* L. (Asparagaceae). The two species were also detected on *Tagetes* sp. (Asteraceae). However, new associations of *Aphelenchoides* on non-reported plant species as well as new combinations on already reported associations are plausible.

The following plant families have reported associations with the MPPA on different plant species: the dicots Asteraceae, Balsaminaceae, Begoniaceae, Brassicaceae Burnett, Caryophyllaceae Juss., Convolvulaceae Juss., Fabaceae, Gesneriaceae, Hydrangeaceae Dumort, Lamiaceae, Oleaceae Hoffmanns. & Link, Plantaginaceae Juss., Rosaceae and Solanaceae and the monocots Amaryllidaceae, Asparagaceae and Poaceae (see online database).

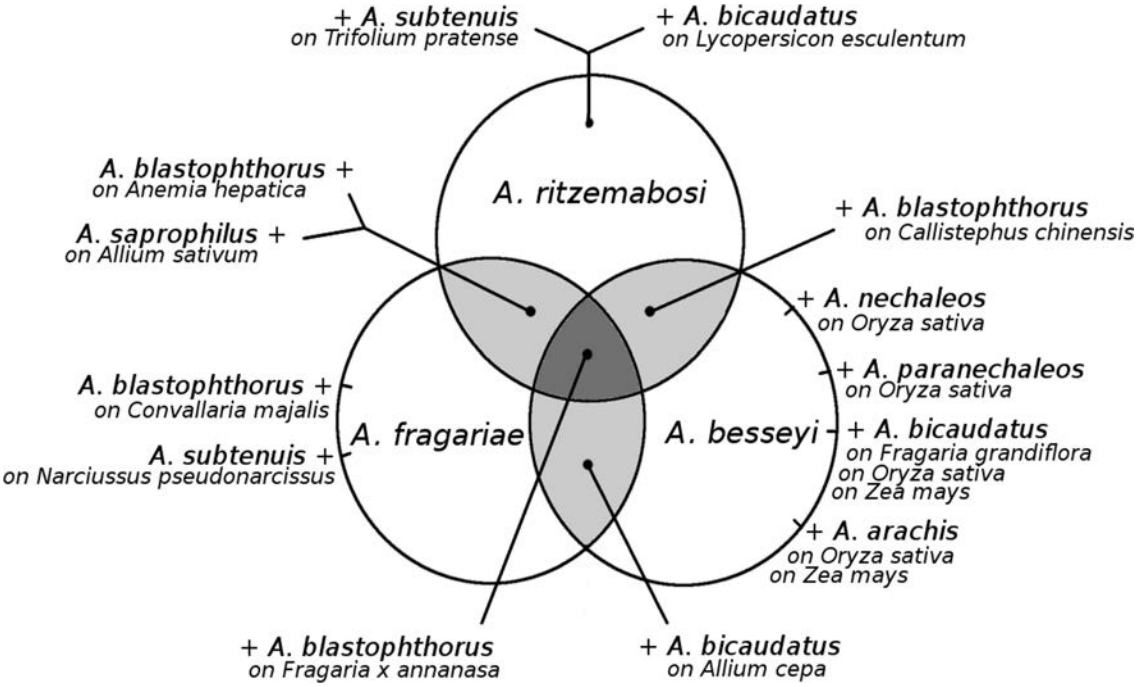

Figure II.3. Possible combinations of other plant-parasitic *Aphelenchoides* and main foliar nematodes on associated plants. Gray areas indicate a combination of the overlapping foliar nematodes

Other plant-parasitic *Aphelenchoides*

While the MPPA represent 95% of the total reports, the other 10 species have been reported only from a limited number of plants (Table II.1). Noteworthy, some of them were reported

on hosts with records of the MPPA species, and special attention should be paid in such instances for an accurate identification (Fig. II.3). Some plant genera could shelter a combination of the following species: *A. blastopthorus* + *A. subtenuis* on *Narcissus* sp.,

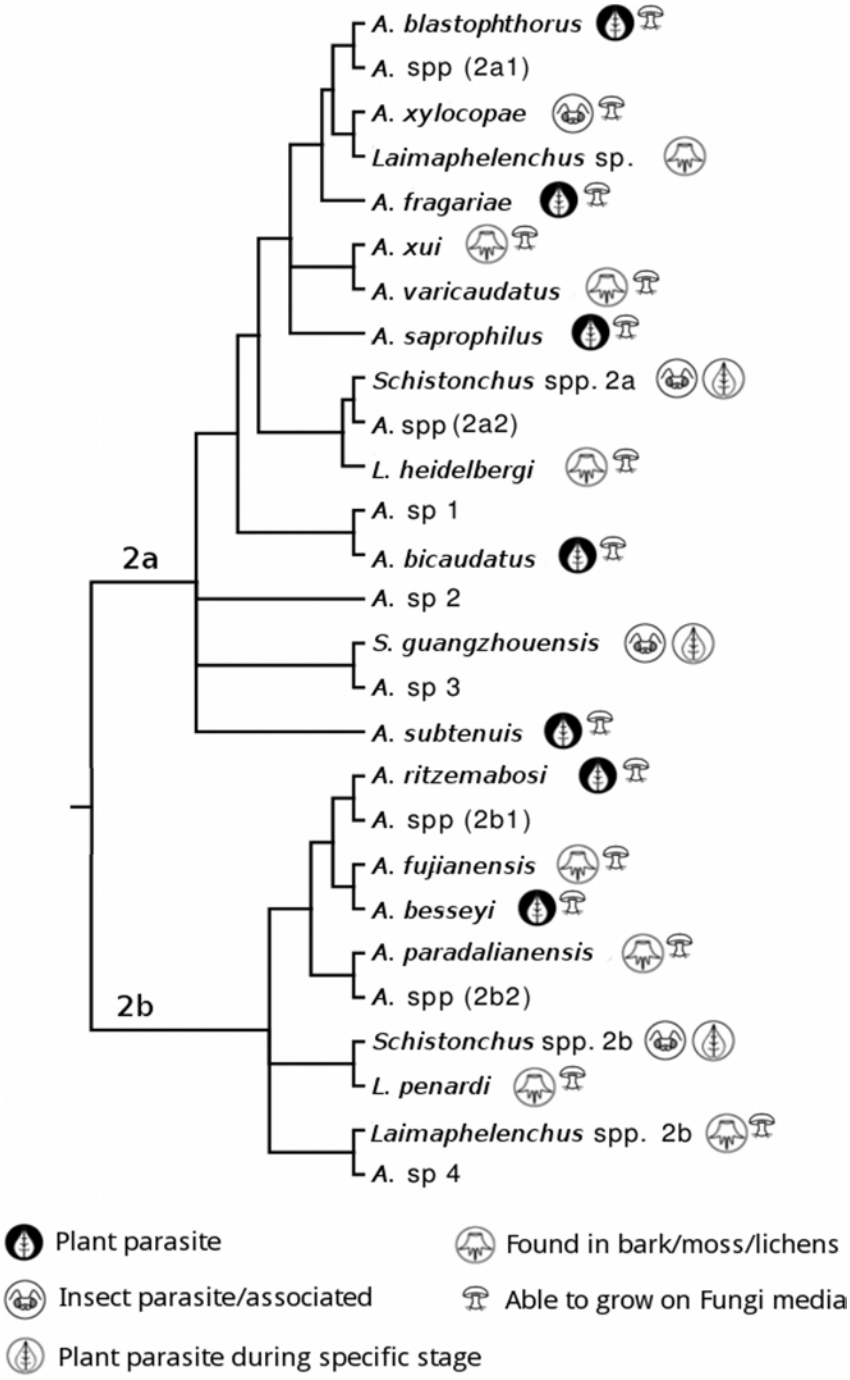

Figure II.4. Schematic overview of the phylogeny of *Aphelenchoides* and related taxa (*Laimaphelenchus* and *Schistonchus* s.l.) after the topologies provided by Kanzaki *et al.* 2014a, 2014b, Rybarczyk-Mydłowska *et al.* 2012 and Ryss *et al.* 2013 based on SSU sequence analyses; clusters 2a and 2b are consistent in the 4 topologies. Feeding behavior is plotted on the tree. (Tree reconstructed using Mesquite 3.01)

A. blastopthorus + *A. fragariae* + *A. ritzemabosi* + *A. subtenuis* on *Iris* sp. (Iridaceae)*, A. fragariae* + *A. ritzemabosi* + *A. subtenuis* on *Colchicum* sp. (Colchicaceae) and *A. ritzemabosi* + *A. subtenuis* on *Tulipa* sp. (Liliaceae). Species reported from a limited number of plant species in particular locations, *i.e. A. ensete, A. nechaleos* and *A. paranechaleos*, require more sampling and laboratory tests to confirm their specificity.

Pine trees and *Aphelenchoides* spp.

Although several samples of *Pinus* L. species (Pinaceae) have been particularly rich for *Aphelenchoides,* most of the species found do not appear to be parasites; the only confirmed plant parasite is *A. dalianensis* (Cheng *et al.* 2009). Several species including *A. besseyi* and *A. fragariae* were found on samples of *Pinus massoniana*, and *A. fujianensis* Zhuo, Cui, Ye, Luo, Wang, Hu & Liao, 2010 was originally described from this species (Zhuo *et al.* 2010). The same authors mentioned *A. bicaudatus* and *A. macronucleatus* Baranovskaya, 1963 from *P. thunbergii* and *A. composticola* from *P. elliottii* Engelm., while Negi *et al.* (2009) described *A. depressospicularis* Negi, Kalia, Walia & Bajaj, 2009 and *A. microspermi* Negi, Kalia, Walia & Bajaj, 2009 from *Pinus roxburghii* Sarg.*,* and Kaisa (2000) described *A. microstylus* Kaisa, 2000 as an associated species to a bark beetle from *Pinus sylvestris* L. The number of *Aphelenchoides* species isolated from pine trees and wood samples has recently increased as a result of the protocols implemented for detection and certification of pinewood nematodes *i.e. Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 (Zhuo *et al.* 2010). It would be likely to find more *Aphelenchoides* species if other substrates were examined with such attention.

Most of these reports were made from dead material and as discussed before, the ability to thrive on fungi would let them survive on mycelia rather than feeding directly on the plant tissue, as other species in Aphelenchoidoidea. This could be also the case for *A. paradalianensis* Cui, Zhuo, Wang & Liao, 2011 (Cui *et al.* 2011) and *A. rotundicaudatus* Fang, Wang, Gu & Li, 2014 (Fang *et al.* 2014a)*,* both extracted from packaging wood from South Korea and *A. aerialis* Bina Chanu, Mohilal, Victoria & Manjur Shah, 2013 (Bina Chanu *et al.* 2013)*, A. xui* Wang, Wang, Gu, Wang & Li, 2013 (Wang *et al.* 2013) and *A. stellatus* Fang, Gu, Wang & Li, 2014 (Fang *et al.* 2014b) in India, South Africa and Japan, respectively.

Few of the reported mycophagous species pose phytosanitary risks (Singh *et al.* 2013) and can be found on plant material such as bark, wood or roots, but in such cases a parasitic relationship has not been confirmed. Nevertheless, the presence of living nematodes could indicate non-sterile media or packaging, and so these taxa are potentially useful a bioindicators.

Dispersal and potential interactions of PPA

Little is known about the biology and dispersal of the *Aphelenchoides* species. Clues could be found in the relationship with the reported pine species and possible insect vectors, as they play a crucial role in the dispersal of other Aphelenchoidoidea taxa, e.g. longhorn beetles and *B. xylophilus* (Kikuchi *et al.* 2011, Vicente *et al.* 2012). Aside from *A. microstylus* and a bark beetle (Kaisa 2000), only two species of *Aphelenchoides* have been found associated with insects: *A. xylocopae* Kanzaki 2006 with the Japanese large carpenter bee (Kanzaki 2006) and *Aphelenchoides* sp. (Cardoza *et al.* 2008) with the spruce beetle (Cardoza *et al.* 2008). In the first case, *A. xylocopae* was isolated from the bee's oviduct, in the latter, specimens were found under the beetle's elytra. Both species of nematodes were successfully cultured on fungi media after isolation, and a phoretic phase within a fungivorous life-cycle was hypothesized. Insect phoresy could be far more important than currently known for *Aphelenchoides*. However, the high diversity of botanical families associated with PPA (Fig. II.5) indicates that the relation nematode-insect is not specific as one would expect with more specific insect-plant relationship.

Next to insects, interaction(s) or association(s) of aphelenchs with bacteria or fungi may play a role in the plant infection process. According to Li (2008) certain bacteria species have a positive effect on the reproduction rates and egg production of *B. xylophilus*. For PPA a direct positive effect on the life cycle has not been described. However, the interaction with pathogens can seriously aggravate plant damage. *A. fragariae* and *A. ritzemabosi* with the bacteria *Rhodococcus fascians* (Tilford, 1936) Goodfellow, 1984 cause the "cauliflower disease" on strawberry (Moens & Perry 2009, Duncan & Moens 2013) and *A. ritzemabosi* associated with *Phytophthora cryptogea* Pethybr. & Laff. 1919 induce a disease in gloxinia (Duncan & Moens 2013). The association of *A. fragariae* with two other bacteria species,

Pseudomonas cichorii (Swingle, 1925) Stapp, 1928 and *Xanthomonas axonopodis* Starr & Garces, 1950, showed a combined effect on *Barleria cristata* L. and rieger begonia, respectively (Duncan & Moens 2013), but each pathogen was also able to develop symptoms by its own (Lehman & Miller 1988, Riedel & Larsen 1974). More recently, Tiedt & Bogale (1999) suggested that *A. ensete* could act as a vector of *Xanthomonas campestris* (Pammel, 1895) Dowson, 1939 in a disease complex on *Ensete ventricosum,* but to our knowledge this has not been confirmed. Furthermore, fungivorous nematodes can spread fungal propagules to new locations (Griffin *et al.* 2009), consequently, further studies need to be undertaken to elucidate the extent to which microbes can be spread by PPA parasitism and/or mediate their parasitic behavior.

Plant-parasitism and host range in the genus *Aphelenchoides*

As stated above, plant parasitism arose several times in the evolution of nematodes (Sultana *et al.* 2013), with Aphelenchoidoidea being one of the resulting taxa. However, according to recent molecular phylogenetic analyses (Kanzaki & Giblin-Davis 2012, Rybarczyk-Mydłowska *et al.* 2012, Ryss *et al.* 2013, Kanzaki *et al.* 2014a, 2014b) plant parasitism arose more than once within *Aphelenchoides*. Current evidence shows two strongly supported clades; 2a and 2b (Rybarczyk-Mydłowska *et al.* 2012, see Fig. II.4) comprising both plant-parasitic and mycophagous *Aphelenchoides,* as well as *Laimaphelenchus* Fuchs, 1937 and *Schistonchus s.l.* Cobb, 1927 (Fuchs, 1937) species. *Laimaphelenchus* is associated with bark beetles, moss, lichens and algae mainly in conifers (Asghari & Eskandari, 2014) and *Schistonchus s.l.* species show highly specific tritrophic relationships with fig wasps and fig trees, in which plant parasitism has been described (DeCrappeo & Giblin-Davis 2001, Kanzaki & Giblin-Davis 2012).

In the case of the evolution of tylenchs it is hypothesized that fungal feeding ancestors evolved towards plant-parasitism via accessible plant tissues such as algae, mosses or root hairs (Bert *et al.* 2008, Holterman *et al.* 2009, Paramonov 1970, Siddiqi 1980; 1986); this could be also the case for plant-parasitic aphelenchs as they have the ability to feed on fungi, but unlike those tylenchid taxa that exclusively parasitize higher plants and form a monophyletic group (Bert *et al.* 2008, Holterman 2007, Holterman *et al.* 2009), neither

ecological patterns nor taxonomic groups are congruent with molecular phylogenies in *Aphelenchoides* (Fig. II.4). Thus, it is unclear if plant-parasitism in this genus is the ancestral state of Aphelenchoididae or whether it has emerged independently several times (Bird *et al.* 2014). Notwithstanding plant-parasitism may have emerged from fungivorous ancestors (Bird *et al.* 2014) in both, tylenchs and aphelenchs, parasitism in tylenchs and Aphelenchoidoidea apparently followed two remarkably different paths according to horizontal gene transfer (HGT) evidence. Cellulase genes, particularly glycoside hydrolases, have a bacterial origin in many tylenchs and belong to family 5 (GH5), while they have a fungal origin and belong to family 45 (GH45) in *B. xylophilus* (Gheysen & Jones 2013) and *A. besseyi* (Wang *et al.* 2014, Kikuchi *et al.* 2014). However, a GH5 hydrolase have also been reported in *A. fragariae* (Fu *et al.* 2012) and, more recently, a GH5-candidate was discovered in *A. besseyi* (Wu *et al.* 2016).

Several taxa and groups of plant-parasitic organisms tend to be specific, *e.g.* most herbivorous arthropods are highly host-specific and only 10% have a host range of more than 3 families (Skoracka 2006). For several nematodes, a broad range of hosts seems to be a successful strategy, *e.g.* the endoparasitic nematode *Meloidogyne trifoliophila* Bernard & Eisenback, 1997 was reported in 6 monocotyledonous and 23 dicotyledonous families (Bernard & Jennings 1997), and *M. arenaria* (Neal, 1889) Chitwood, 1949 was reported on several crop and weed species as well as fruit and pine trees (López-Pérez *et al.* 2011) and *Rotylenchulus reniformis* Linford & Oliveira, 1940 was listed by Khan (2005) as having 150 plant hosts in 50 families. However, an extreme case of a broad diversity of associated plants can be found for half plant-parasitic *Aphelenchoides* spp. (Table II.1) as their ranges surpass the level of orders (Table II.1, Fig. II.5). These taxa are therefore regarded as generalist species; some of them with particularly intriguing ranges such as *A. besseyi* and *A. fragariae* that expand to almost the whole plant radiation including Lycopodiophyta and Marchantiophyta, respectively (Fig. II.5). Interestingly, *A. besseyi* and *A. ritzemabosi* not only belong to the same sub-clade (Fig. II.4) but also share 13 orders of associated plants (Fig. II.5). Nonetheless, new discoveries on *A. besseyi* suggest that this species could be actually a complex of species (see Chapter V), thus, not a generalist parasite. Further research should be conducted on the MPPA to evaluate this possibility.

Final discussion and conclusion

Notably, the current list of reported associations is long but is likely to represent only a fraction of the potential ranges. Additionally, there is uncertainty not only about the parasitic relationship in several cases (Koch's postulates are not fulfilled) but also on the identification of nematodes or hosts; problems such as the use of common names for host's reports (Knight *et al.* 1997) or parasitism assumed only by the presence of nematodes in soil samples are misleading and regrettably common in PPN (Knight *et al.* 1997, Knight 2001, Robinson *et al.* 1997). Very little is known about parasitic relationships, presence in wild vegetation or non-crop hosts and possible insects/pathogens interactions, therefore, experimental work as well as surveys extending the sampling to possible hosts with accurate identifications of both, plants and nematodes, are needed for a better understanding of their real hosts, corresponding ranges and ecology. It would be also valuable to explore the implications of the potential co-existence of PPA in the same plant species (Fig. II.2 and II.3), *e.g.* symptoms, infection processes and populations dynamics; such information could provide more evidence in the study of the origin of *Aphelenchoides* parasitism.

Altogether, according to the molecular phylogenies of *Aphelenchoides* spp. which show no clear pattern (Fig. II.4) and the broad range of associated plants exhibited by most of the plant-parasitic species within this genus (Fig. II.5), flexibility of this group to switch towards plant-parasitism is underlined. Other plant-parasitic nematodes (even sedentary endoparasites *e.g. Meloidogyne* spp. and *R. reniformis*) can also be not host-specific. However, unlike these tylenchid taxa, re-differentiation of plant cells, intimate interactions such as alterations to the host physiology (Jones *et al.* 2013) and highly correlated patterns in terms of life cycles (Perry & Moens 2011) are not documented for PPA. Thus, the parasitic behavior in *Aphelenchoides* is far less complex and supposedly more primitive, which is potentially interesting in the study of the evolution of plant parasitism (Jones *et al.* 2013).

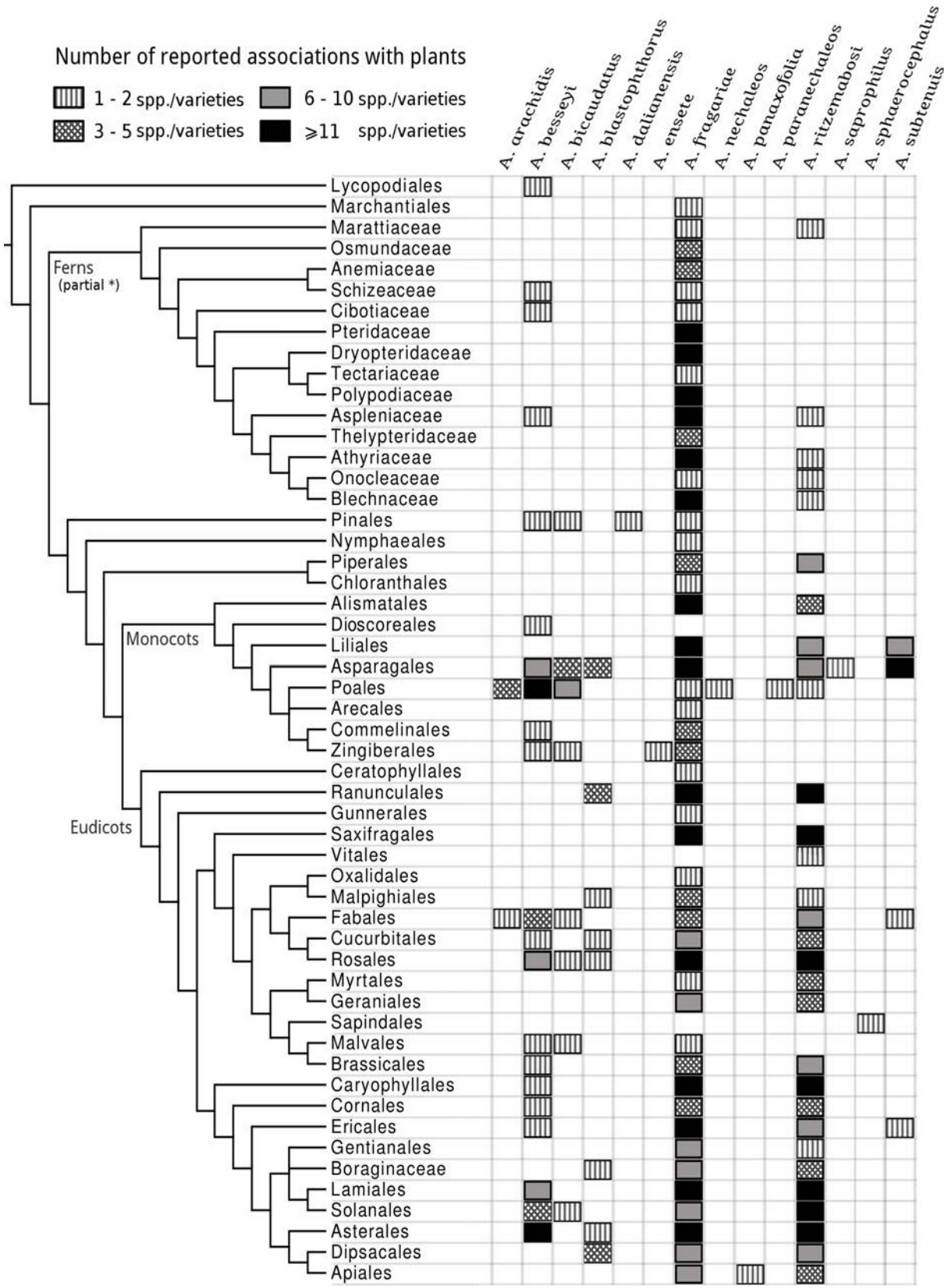

Figure II.5. Number of records of plant-parasitic *Aphelenchoides* species *per* plant host taxon. Data plotted on a supertree made using Mesquite 3.01 based on those by The Angiosperm Phylogeny Group (2009) and Lehtonen (2011). *10 families of Pteridophyta with reports for only *A. fragariae* were excluded

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CHAPTER III
Mitochondrial COI successfully identifies *Aphelenchoides* species and supports a multiple origin of plant-parasitism

Modified from

Sánchez-Monge, A.[1,2], Jansen, T.[1,] Fang, Y.[1,3], Couvreur M.[1,] Karssen G.[1,4] & Bert, W.[1] (2016) mtCOI successfully diagnoses the four main plant-parasitic *Aphelenchoides* species (Nematoda: Aphelenchoididae) and supports a multiple origin of plant-parasitism in this paraphyletic genus. *Accepted for publication in the European Journal of Plant Pathology*

[1] *Ghent University, Department of Biology, Nematology Research Unit, Ledeganckstraat 35, B-9000 Ghent, Belgium.*
[2] *Universidad de Costa Rica, Escuela de Estudios Generales, 2060, Costa Rica*
[3] *Department of Plant Pathology, Nanjing Agricultural University, Nanjing 210095, P.R. China.*
[4] *National Plant Protection Organization, Netherlands Food and Consumer Product Safety Authority, Geertjesweg 15, 6700HC Wageningen, The Netherlands.*

Abstract:

Composed mostly of fungivorous species, the genus *Aphelenchoides* also comprises 14 plant-parasitic species. The most common and devastating, *A. besseyi, A. fragariae, A. ritzemabosi* and *A. subtenuis* have been reported on more than 900 plant species. The combination of low inter-specific and high intra-specific morphological variability makes morphology-based identification extremely difficult within this genus, and has led to molecular tools being employed to ensure accurate diagnoses. rDNA markers are widely used for the identification of nematodes while the Cytochrome Oxidase I gene (COI) remains relatively unexplored despite its role as the standard barcode for almost all animal groups. To explore its suitability as a diagnostic tool, we studied a fragment of the mtCOI region of the four main plant-parasitic *Aphelenchoides* within a phylogenetic framework. We generated 69 mtCOI and 123 rDNA sequences of diverse *Aphelenchoides* taxa; 67 belong to the main plant-parasitic species including the first mtCOI sequence of *A. fragariae* and the first mtCOI and 28S sequences of *A. subtenuis*. mtCOI had a similar success rate for PCR amplification. Phylogenetic trees based on the three studied markers are generally in agreement with one another, validating their use for *Aphelenchoides* diagnosis; additionally, we were able to locate several misidentified sequences of plant-parasitic *Aphelenchoides* in existing databases. The concatenated analysis from the three markers resulted in a more robust insight into the phylogeny and evolution of *Aphelenchoides*, revealing that plant-parasitism has evolved independently at least three times within this genus, presumably from fungal-feeding ancestors.

Key words: foliar nematodes, molecular barcoding, phylogeny, rDNA

Resumen:

Compuesto en su mayoría por micófagos, el género *Aphelenchoides* también contiene 14 especies fitoparásitas, las más importantes, *i.e. A. besseyi, A. fragariae, A. ritzemabosi* y *A. subtenuis* han sido informadas en más de 1000 asociaciones con plantas, con un impacto significativo en cultivos. La combinación de baja variabilidad morfológica a nivel inter-específico y alta a nivel intra-específico, hace que la identificación morfológica sea extremadamente difícil en este género, por lo que la implementación de herramientas moleculares es necesaria para un diagnóstico certero. Los marcadores de ADN ribosomal (ADNr) son ampliamente utilizados para la identificación de nematodos, mientras que el gen Citocromo Oxidasa subunidad I (COI) se mantiene relativamente inexplorado a pesar de ser el código de barras estándar para casi todos los grupos de animales. Para explorar su utilidad como herramienta de diagnóstico, la region del COI mitocondrial de las cuatro especies principales de *Aphelenchoides* fitoparásitos fue estudiada dentro de un marco filogenético. Se generaron 69 y 123 secuencias de COI y ADNr, respectivamente. 67 pertenecen a las principales especies e incluyen las primeras secuencias de COI y de ADNr de *A. subtenuis*, y la primera COI de *A. fragariae*. Sumado a las numerosas ventajas del uso del ADN mitocondrial como códido de barras (e.g. altas diferencias intra-especificas), este gen tuvo una tasa de éxito similar a las de ADNr durante la amplificación con PCR. Los árboles filogenéticos basados en los tres marcadores moleculares concuerdan entre sí, validando su uso en el diagnóstico de *Aphelenchoides*. Adicionalmente, se hallaron varias secuencias de *Aphelenchoides* fitoparásitos erróneamente identificadas en las bases de datos. El análisis combinado de un gen mitocondrial con dos ribosomales proveyó un enfoque más robusto de la filogenia y evolución de *Aphelenchoides* y reveló que el fitoparasitismo ha aparecido de manera independiente al menos en 3 ocasiones dentro de este género, probablemente a partir de ancestros micófagos.

Palabras clave: nematodos foliares, código de barras genético, filogenia, ADNr

Introduction

Around 4000 species of nematodes are recognized as plant parasites (Decraemer and Hunt 2013) causing an estimated economic impact of US$125 billion annually (Bakhetia *et al.* 2005). Among them, the genus *Aphelenchoides* contains 14 plant-parasitic species that have been reported in a broad range of plants and crops (Rybarczyk-Mydłowska *et al.* 2012; Sánchez-Monge *et al.* 2015). Unfortunately, diagnostic taxonomic traits, for example characteristic tail-terminus shapes, are limited in this genus and the majority of species have not been described adequately enough to enable reliable identifications (de Jesus *et al.* 2016; Hockland 2001; Sun *et al.* 2014). Problematically and despite the main plant-parasitic *Aphelenchoides* (MPPA) are morphologically distinguishable from one another (Chałańska et al. 2011; Hockland 2004), species within this genus show a low inter-specific and high intra-specific morphological variability and most of them are not yet associated with discriminating molecular data. This has already led to several misidentifications, taxonomic conundrums and the presence of cryptic species (Hockland 2001; Kanzaki and Giblin-Davis 2012) including plant-parasites and alike species (de Jesus *et al*. 2016, Hockland 2001).

The relatively-easy implementation and availability of molecular tools in species diagnosis and characterization has consolidated them as one of the most important tools in present-day taxonomy; particularly in those cases where morphology is insufficient or too complicated to allow the accurate identification of key organisms. Several genetic markers and techniques have been developed for DNA barcoding of nematodes in various groups, e.g. marine species (Bhadury *et al.* 2006; Derycke *et al.* 2010), soil taxa (Floyd *et al.* 2002) and animal and plant-parasitic species (McKeand 1999; Powers 2004). In the latter case, molecular diagnosis becomes especially relevant as false or incorrect identifications can have serious economic repercussions (Kiewnick *et al.* 2014). With this end, the ribosomal RNA array (particularly the 18S and 28S regions) and to a lesser extent the mitochondrial genome, have been routinely used as molecular markers for plant-parasitic nematodes (PPN) (Holterman *et al.* 2009; Janssen *et al.* 2016; Lesufi *et al.* 2015; Powers 2004).

In addition to the RNA array, the ITS regions and the 5.8S gene have also been implemented for *Aphelenchoides* diagnosis (Kanzaki and Giblin-Davis 2012; Rybarczyk-Mydłowska *et al.*

2012), however, the region that is being used as the standard barcode for almost all animal groups, *i.e* the mitochondrial Cytochrome Oxidase I gene (COI) (Hebert *et al.* 2003), has only been explored for a limited number of nematode species (Palomares-Rius *et al.* 2014a). These species include marine taxa (Derycke *et al.* 2005; Derycke *et al.* 2010) and several plant-parasitic species e.g. *Bursaphelenchus* spp. (Kanzaki and Giblin-Davis 2012; Ye *et al.* 2007), *Meloidogyne* spp. (Kiewnick *et al.* 2014), *Pratylenchus* spp. (Troccoli *et al.* 2016) and *Scutellonema* spp. (van den Berg *et al.* 2013). For *Aphelenchoides*, only 54 mtCOI sequences are currently available in GenBank, albeit comprising only six identified species, out of which only *A. besseyi* and *A. ritzemabosi* are plant parasites. This precludes the evaluation of COI for barcoding and species diagnosis in this genus. Furthermore, although essential to infer phylogenetic relationships, only few mtCOI sequences of related genera such as *Ficophagus*, *Laimaphelenchus*, *Martininema* and *Schistonchus* are available.

In this chapter we studied a fragment of the mitochondrial COI (mtCOI), in combination with two rDNA markers, of several aphelenchs including the four MPPA species: *Aphelenchoides besseyi* (Christie, 1942), *A. fragariae* (Ritzema Bos, 1890) Christie, 1932, *A. ritzemabosi* (Schwartz, 1911) Steiner and Buhrer, 1932 and *A. subtenuis* (Cobb, 1926) Steiner and Buhrer, 1932. Our objectives were to 1) evaluate the potential of mtCOI sequences for the identification of plant-parasitic *Aphelenchoides* species and related taxa; 2) compare the resolution, sequences variability and tree topologies obtained from one mtCOI and two rDNA markers (*i.e*. 18S rDNA and the D2D3 expansion region of the 28S rDNA) and 3) use the newly available phylogenetic frameworks, also based on a concatenated analysis, to discuss its implications for the evolution of plant-parasitism in this genus.

Materials & Methods

Sampling, DNA isolation and PCR amplification

Newly generated sequences were obtained from samples collected from different habitats in geographical widespread localities (Table III.1) and pure cultures provided by the National Plant Protection Organization (Wageningen, The Netherlands). The diagnosis and description of unknown taxa was out of the scope of this chapter, therefore only descriptive code names were used as reference for newly generated sequences (see Table III.1). Each

nematode was individually mounted in temporary slides for morphological vouchering by photo documentation and video capturing (De Ley & Bert 2002); subsequently, DNA extractions were made from vouchered specimens following the protocol of Janssen *et al.* (2016) using NaOH and tween; final extraction volumes were 40µL.

The DNA fragments of the SSU rDNA (18S) gene, the D2D3 expansion region of the LSU rDNA (28S) and the cytochrome oxidase subunit I of the mitochondrial DNA (mtCOI) were amplified. Primers for the 18S fragment were, 1813F (CTGCGTGAGAGGTGAAAT) and 2646R (GCTACCTTGTTACGACTTTT) (Holterman *et al.* 2006). Primers for the 28S rDNA were D2A (ACAAGTACCGTGAGGGAAAGTTG) and D3B (TCCTCGGAAGGAACCAGCTACTA) (Nunn 1992). The mtCOI fragment was amplified using COI-F1 (CCTACTATGATTGGTGGTTTTGGTAATTG) and COI-R2 (GTAGCAGCAGTAAAATAAGCACG) (Kanzaki and Futai 2002). All PCR reactions consisted of master mix (Qiagen Taq DNA Polymerase Kit: 2.17mM $MgCl_2$, 1.08 10XBuffer, 0.22mM dNTP, 0.43µM of each primer and 0.01u/µL TopTaqPolymerase) plus 3 to 5µL of DNA extraction. The thermal cycling program for the 18S was as follows: 95°C for 5min, 5x (94°C for 30s; 45°C for 30s, 72°C for 70s), 45x (94°C for 30s; 54°C for 30s; 72°C for 70s), 72°C for 5min. The thermal cycling program for the D2D3 region was as follows: 94°C for 4min, 5x (94°C for 30s; 55°C for 30s, -1°C/cycle, Ramp 3°C/s, 72°C for 2min), 40x (94°C for 30s; 50°C for 30s; 72°C for 2min), 72°C for 10min. The thermal cycling program for the mtCOI was as follows: 94°C for 5min, 42x (94°C for 30s; 51°C for 30s, 72°C for 2min), 72°C for 10min.

Sequencing and phylogenetic analyses

PCR products were sequenced in forward and reverse direction, contigs were assembled from both reads using Geneious 9.1.3; each read was quality checked manually and subsequently subjected to a BLAST search (Altschul *et al.* 1990) to confirm their nematode origin and discard possibly contaminated samples. Multiple sequence alignments of new mtCOI, 18S and 28S data were made and supplemented with available GenBank sequences of *Aphelenchoides, Aphelenchus, Bursaphelenchus, Ficophagus, Laimaphelenchus, Martininema, Robustodorus* and *Schistonchus* species (see Supplementary Table III.1). *Aphelenchoides* sequences which were not identified to species level were omitted in order to limit the

amount of missing data in the multigene analysis. Newly generated sequences of *Neodiplogaster* sp. were used as out-group.

An alignment considering the secondary structure of the RNA was done for both rDNA alignments at http://mafft.cbrc.jp/alignment/server/ (Katoh and Standley 2013) according to the Q-INS-i algorithm. Post-alignment curation was performed with Gblocks (discarded after evaluation) and Aliscore (Mlsof and Katharina 2009) (http://aliscore.zfmk.de); poorly aligned positions and conflicting regions were excluded with Alicut (http://utilities.zfmk.de). mtCOI sequences were aligned on-line on the TranslatorX web server (Abascal *et al.* 2010) based on their amino acid encoded sequences as translated by the invertebrate mitochondrial genetic code. Conversions between bioinformatic file formats were done on-line at http://sing.ei.uvigo.es/ALTER/ (Glez-Peña *et al.* 2010). A separate alignment for each gene was ran in Geneious 9.1.3 using the Kimura-two-parameter (K2P) model to measure the intra- and inter-specific divergences of the four MPPA species.

The best-fitting base-substitution models were calculated using the Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 (Tamura *et al.* 2013) using the Bayesian information criterion; the models GTR+I+G, K2+I+G and K2+G were selected for the mtCOI, 18S and 28S phylogenetic analyses, respectively. A concatenated dataset was built with most sequences from the three data sets (See Supplementary Table 1); missing nucleotide positions were treated as "?". Bayesian phylogenetic analyses were executed on the CIPRES Science Gateway (Miller *et al.* 2010) using MrBayes 3.2.6 ran for $2x10^7$ generations; Markov Chains were sampled at intervals of 1000 generations; burnin was chosen to be 25%. The model for the amino acids sequences was defined as mixed; in the multi-gene phylogenetic analysis each gene was considered to be a separate partition. A Bayesian Inference consensus tree was created for each analysis by collapsing all clades with a posterior probability below 95 using FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) and TreeGraph2 2.7.1-563 beta (Stöver and Müller 2010), subsequent edition was done with the GNU Image Manipulation Program GIMP 2.8.14 (Kimball *et al.* 2014).

Results

Amplification and sequencing success of the mtCOI and rDNA regions

In total, 69, 56 and 91 sequences of mtCOI, 18S and 28S markers, respectively, were generated, out of which 21 (mtCOI), 16 (18S) and 30 (28S), belong to the MPPA species. These sequences include the first mtCOI sequences of *A. fragariae* and *A. subtenuis* and the first 28S sequences of the latter. Sequences were deposited in GenBank under the accession numbers indicated in Table III.1. Populations from pure cultures were morphologically identified and confirmed by the already known markers.

The amplification success of the mtCOI region (65%) was similar to rDNAs' (59% SSU and 72% LSU) based on the evaluated *Aphelenchoides* samples *per* marker, yet, sequence quality was high for most samples and markers. The mtCOI region was especially T-rich (circa A:25%, C:12%, G:19%, T:43%). After quality control and manual edition, sequences' lengths ranged between 318-693bp, 440-758bp and 396-668bp, for the mtCOI, 18S and 28S respectively. The resulting single-gene alignments comprised 128, 170, and 225 sequences, respectively and the encoded protein sequences ranged from 106 to 237 amino acids in length. The concatenated alignment of 216 sequences, built with 121 (mtCOI), 142 (18S) and 183 (28S) sequences had a length of 2666 nucleotides; 81 and 69 taxa were represented by the combination of three and two markers, respectively (Supplementary Table 1). Alignments are available at http://purl.org/phylo/treebase/phylows/study/TB2:S20079.

The mtCOI inter-specific K2P pairwise distances varied between 14 and 21% (72-116 nucleotide sites) for the MPPA, the lowest distance was observed between *A. besseyi* and *A. ritzemabosi*. Inter-specific distances for the encoded amino acids sequences ranged between 6-13%; intra-specific values reached 5% in *A. ritzemabosi*. The rDNA genes registered inter-specific distances between 11-17% (18S) and 17-40% (28S) while the intra-specific distances reached up to 2% (*i.e* 1-9 nucleotides) in the analyzed sequences of *A. besseyi* (mtCOI and 18S) and *A. fragariae* (28S) (Table III.2). Based on these distances and the congruence among the phylogenetic trees (see discussion), the potential of COI for the diagnosis for these PPA is underlined.

Table III. 1. Details and corresponding accession numbers of *de novo* sequences *per* taxa, reference code and genetic marker

Reference code	Country of origin	Substrate/ host	Genbank accession numbers		
			COI	18S	28S (D2D3)
Aphelenchoides					
A. besseyi	China	Rice	KX356862	KX356705	KX356774
	Italy	Rice	KX356863	KX356706	KX356775
	Turkey	Rice	KX356864	KX356707	KX356776
	Costa Rica	Beans	-	KX356697	KX356753
			KX356845	KX356698	KX356755
			-	KX356699	KX356756
			-	KX356702	-
			-	KX356703	KX356765
	Costa Rica	Rice	KX356844	-	KX356754
			KX356846	-	KX356757
			-	-	KX356758
			KX356847	-	KX356759
			KX356848	KX356700	KX356760
			KX356849	KX356701	KX356761
			KX356850	-	KX356762
			-	-	KX356763
			-	-	KX356764
			-	-	KX356766
			KX356851	-	KX356767
			KX356852	-	KX356768
			-	-	KX356769
			KX356853	-	KX356770
			-	-	KX356771
			KX356854	-	KX356772
			KX356855	-	-
			KX356856	KX356704	KX356773
A. fragariae	The Netherlands	Peony	-	KX356708	KX356778
		Anemone	KX356857	KX356709	KX356779
A. ritzemabosi	The Netherlands	Onion	KX356906	-	KX356837
A. subtenuis	The Netherlands	Crocus	KX356859	KX356710	KX356781
		Onion	KX356860	KX356711	KX356782
		Crocus	KX356861	KX356712	KX356783
A. sp. Cu1	Portugal	Wood	-	-	KX356777
A. sp. Cu2	The Netherlands	Pinus	KX356858	KX356740	KX356780
ApheBarkBE1	Belgium	Bark3	KX356866	-	KX356792
ApheBarkBE2	Belgium	Pine tree	KX356894	-	-
		Pine tree	KX356895	-	-
		Pine tree	KX356896	-	-
		Pine tree	KX356897	KX356739	KX356826

Table III.1 *Continued*

ApheBarkBE3	Belgium	Bark6	-	KX356715	KX356789
ApheBarkBE4	Belgium	Bark3 Bark3	- -	KX356722 KX356723	KX356804 -
ApheBarkBE5	Belgium	Bark9 Bark10 Bark10	- - -	KX356714 (a) - KX356728 (b)	KX356787 (a) KX356808 (b) KX356811 (b)
ApheBarkCR1	Costa Rica	Bark1 Bark1 Bark1	KX356890 KX356891 KX356892	- KX356737 KX356738	- KX356825 -
ApheBarkCR2	Costa Rica	Bark4 Bark4 Bark4	KX356886 KX356887 KX356888	KX356736 - -	KX356822 KX356823 -
ApheBarkCR3	Costa Rica	Bark4 Bark4 Bark5	KX356883 KX356885 KX356889	- - -	KX356820 KX356821 KX356824
ApheBarkCR4	Costa Rica	Bark4	KX356884	-	-
ApheBarkCR5	Costa Rica	Bark7	KX356893	-	-
ApheDivBE	Belgium	Fungi0 Bark8 Bark3	- - -	KX356718 - KX356727	KX356793 KX356803 -
ApheDivEu	Belgium Germany	Bark6 Bark2 Bark2 Bark3 Soil Soil FungiA Soil	- KX356873 - KX356874 KX356875 KX356876 KX356901 KX356879	KX356713 KX356724 KX356725 KX356726 - - - KX356732	KX356786 KX356805 KX356806 KX356807 KX356809 KX356810 KX356830 KX356815
ApheFungiBE1	Belgium	FungiB	KX356899	KX356742	KX356828
ApheFungiBE2	Belgium	Fungi2 Fungi2	KX356900 -	KX356743 -	KX356829 KX356831
ApheFungiBE3	Belgium	Fungi3 Fungi3 Fungi3 Fungi3 Fungi3	- KX356902 KX356903 KX356904 KX356905	- KX356744 - KX356745 KX356746	KX356832 KX356833 KX356834 KX356835 KX356836
ApheFungiBE4	Belgium	Fungi1 Fungi1	KX356881 KX356882	KX356734 KX356735	KX356818 KX356819
ApheMossBE	Belgium	Moss Moss Moss Moss	KX356867 KX356868 KX356869 KX356870	- - - -	- - KX356794 KX356795

Table III.1 *Continued*

		Moss Moss	KX356871 -	KX356719 -	KX356796 KX356802
ApheSoilBE	Belgium	Soil	KX356872	-	KX356799
ApheSoilGE	Germany	Soil	KX356878	KX356731	KX356814
ApheWoodW1	Taiwan Brazil SouthAfrica	Wood Soil Wood Wood	KX356840 KX356841 KX356842 KX356843	KX356693 KX356694 KX356695 KX356696	KX356749 KX356750 KX356751 KX356752
ApheWoodW2	China	Wood	KX356839	KX356692	KX356748
Aphelenchus					
Aphelenchus sp.	Rwanda	Soil	-	KX356730	KX356813
Bursaphelenchus					
B. mucronatus	The Netherlands	Wood	KX356898	KX356741	KX356827
Bursaphelenchus sp.	Belgium Rwanda	Soil Soil Soil Soil Soil	- - - - KX356877	- KX356720 KX356721 - KX356729	KX356797 KX356798 KX356800 KX356801 KX356812
Laimaphelenchus					
L. pannocaudus	The Netherlands	Pine tree	KX356907	KX356747	KX356838
LaimaBarkBE1	Belgium	Bark6	-	-	KX356788
LaimaBarkBE2	Belgium	Bark9 Bark9 Bark3 Bark3	- - - KX356865	- - KX356716 KX356717	KX356784 KX356785 KX356790 KX356791
Neodiplogaster (OG)					
Neodiplogaster sp.	Belgium	FungiC FungiC	- KX356880	- KX356733	KX356816 KX356817

Notes: Bark1: *Syzygium* sp. (Myrtaceae), Bark2: *Betula* sp. (Betulaceae), Bark3: *Quercus* sp. (Fagaceae), Bark4: *Bauhinia* sp. (Fabaceae), Bark5: *Annona* sp. (Annonaceae), Bark6: *Taxus* sp. (Taxaceae), Bark7: *Persea americana* (Lauraceae), Bark8: *Cedrus* sp. (Pinaceae), Bark9: *Gingko biloba* (Ginkgoaceae), Bark10: *Prunus laurocerasus* (Rosaceae). "Wood" refers to packaging wood, "Fungi" samples were not identified, OG: Out Group.

Phylogenetic analyses

The mtCOI, 18S and concatenated topologies strongly support two major clades, labeled as I and II (Figs III.1 and III.2), while the 28S tree only resolves the latter (Fig. III.1d). All topologies show two clusters containing PPA species (denoted as 1 and 2; PPA in bold); the first cluster comprises *A. besseyi* and *A. ritzemabosi,* the other contains *A. fragariae* and *A.*

blastophthorus in the rDNA-based trees (Fig. III.1c and Fig. III.2). The fourth MPPA, *A. subtenuis*, shows an early branching position within Clade II in the rDNA-based topologies and the concatenated tree (Fig. III.1c-d and Fig. III.2). *A. subtenuis* appeared closely related to *Robustodorus megadorus* in the rDNA trees (Fig. III.1c and 1d) and remarkably close to *Martininema* and two free-living samples in the amino acids topology (Fig. III.1b).

Table III.2. Kimura-two-parameter (K2P) differences between the four major plant-parasitic *Aphelenchoides* species, obtained from one mitochondrial and two rDNA markers. The number of differences in the corresponding encoded amino acids of mtCOI sequences is given between brackets

Amplified region and number of sequences (N) per *Aphelenchoides* species					
mtCOI [Sequence length 526]					
N	Species	*A. besseyi*	*A. fragariae*	*A. ritzemabosi*	*A. subtenuis*
18	*A. besseyi*	0-9 (0)	106-111 (15)	72-75 (7-8)	108-111 (20)
1	*A. fragariae*	106-111 (15)	-	113-116 (14-15)	97 (22)
3	*A. ritzemabosi*	72-75 (7-8)	113-116 (14-15)	1-3 (0-1)	107-109 (19-20)
3	*A. subtenuis*	108-111 (20)	97 (22)	107-109 (19-20)	0
SSU RNA (18S) [Sequence length 462bp]					
N	Species	*A. besseyi*	*A. fragariae*	*A. ritzemabosi*	*A. subtenuis*
11	*A. besseyi*	0-7	55-88	17-25	68, 71
6	*A. fragariae*	55-58	1-3	60-63	52-58
2	*A. ritzemabosi*	17-25	60-63	3	73-77
8	*A. subtenuis*	68, 71	52-58	73-77	0, 5
D2D3 expansion region of the LSU RNA (28S) [Sequence length 470bp]					
N	Species	*A. besseyi*	*A. fragariae*	*A. ritzemabosi*	*A. subtenuis*
19	*A. besseyi*	0-3	170-176	80-87	156-165
4	*A. fragariae*	170-176	3-9	183-189	153-166
2	*A. ritzemabosi*	80-87	183-189	5	152-161
3	*A. subtenuis*	156-165	153-166	152-161	0, 1

A sister relationship of *A. besseyi* with *A. ritzemabosi* was only supported in the 28S analysis and the concatenated topology (Fig. III.1d and Fig. III.2). In all analyses, several *Aphelenchoides* taxa are found to be closely related to the PPA (Fig. III.1 and III.2), for

example *A. fujianensis* in cluster 1 and free-living species from diverse habitats such as soil, fungi and bark in the cluster 2 (see Fig. III.1 and Table III.1). The majority of newly generated *Aphelenchoides* sequences are part of Clade II (Fig. III.1 and III.2) while only less than 10 taxa clustered in Clade I. This distribution, especially in Clade II, indicates a remarkably high undiscovered diversity in *Aphelenchoides,* 66% of the sequences in this clade correspond to non-identified taxa and represent at least 20 putative new species, estimated by setting the minimum inter-specific differences value to 4% (see code names, Table III.1). This study also confirms the paraphyly of *Aphelenchoides,* as *Ficophagus*, *Laimaphelenchus*, *Martininema*, and *Schistonchus* are embedded within *Aphelenchoides* sequences (Fig. III.1a-c and Fig. III.2). *Schistonchus* s.s. is placed in Clade I while *Ficophagus* and *Martininema* are positioned within Clade II in all topologies (Fig. III.1 and Fig. III.2). *Laimaphelenchus* is polyphyletic and is placed within Clade I, except for *L. belgradiensis* (based on mtCOI data) and *L. pannocaudus* (based on mtCOI and rDNA analyses) (Fig. III.1 and III.2).

The phylogenetic position of the PPA species based on the three single genes and the concatenated trees (Fig. III.1 and III.2) suggest that plant parasitism had arisen at least three times in *Aphelenchoides* (see leaves in Fig. III.1 and III.2): *A. besseyi* and *A. ritzemabosi* in Clade I (yet not supported by the 28S topology), *A. blastophthorus* and *A. fragariae* within Clade II (cluster 2) and *A. subtenuis* early branching in Clade II (Fig. III.1c-d and Fig. III.2). *A. bicaudatus* and *A. saprophilus* could represent two additional origins, but only one gene has been sequenced for these species (*i.e.* 18S) and the phylogenetic relationships are not resolved (Fig. III.1c and III.2). Based on our results, several GenBank sequences are likely to be misidentified. Some entries that are labeled as *A. besseyi* form a different group in all topologies (see "*A. besseyi"* in Fig. III.1) and several "*A. ritzemabosi"* 28S sequences clustered within the clade of *A. fragariae* (see Supplementary Table III.1). The 28S K2P pairwise distances of these "*A. ritzemabosi*" sequences were 39-40% compared to *A. ritzemabosi* but only 0-2% compared to *A. fragariae*, while "*A. besseyi*" and *A. besseyi* had pairwise distances from 4 to 28% based on the three markers (not shown).

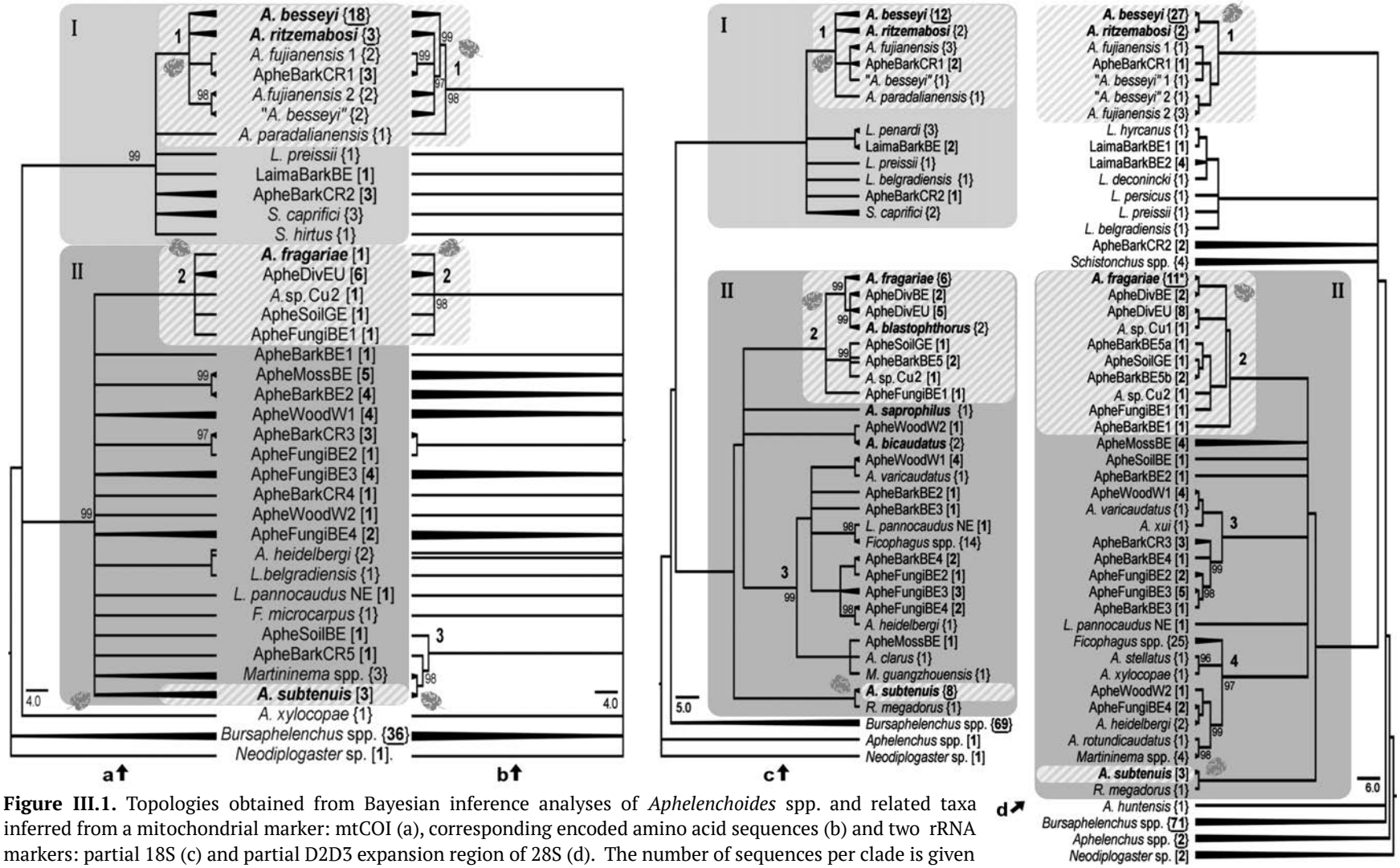

Figure III.1. Topologies obtained from Bayesian inference analyses of *Aphelenchoides* spp. and related taxa inferred from a mitochondrial marker: mtCOI (a), corresponding encoded amino acid sequences (b) and two rRNA markers: partial 18S (c) and partial D2D3 expansion region of 28S (d). The number of sequences per clade is given in bold if new; between curly brackets if taken from GenBank; underlined when combined. Plant-parasitic *Aphelenchoides* species in bold; branches are proportionally presented; posterior probabilities >95% are plotted; assume 100% if not depicted. Leaves indicate points where plant-parasitism may have arisen.

Discussion

mtCOI and the molecular identification of plant-parasitic *Aphelenchoides*

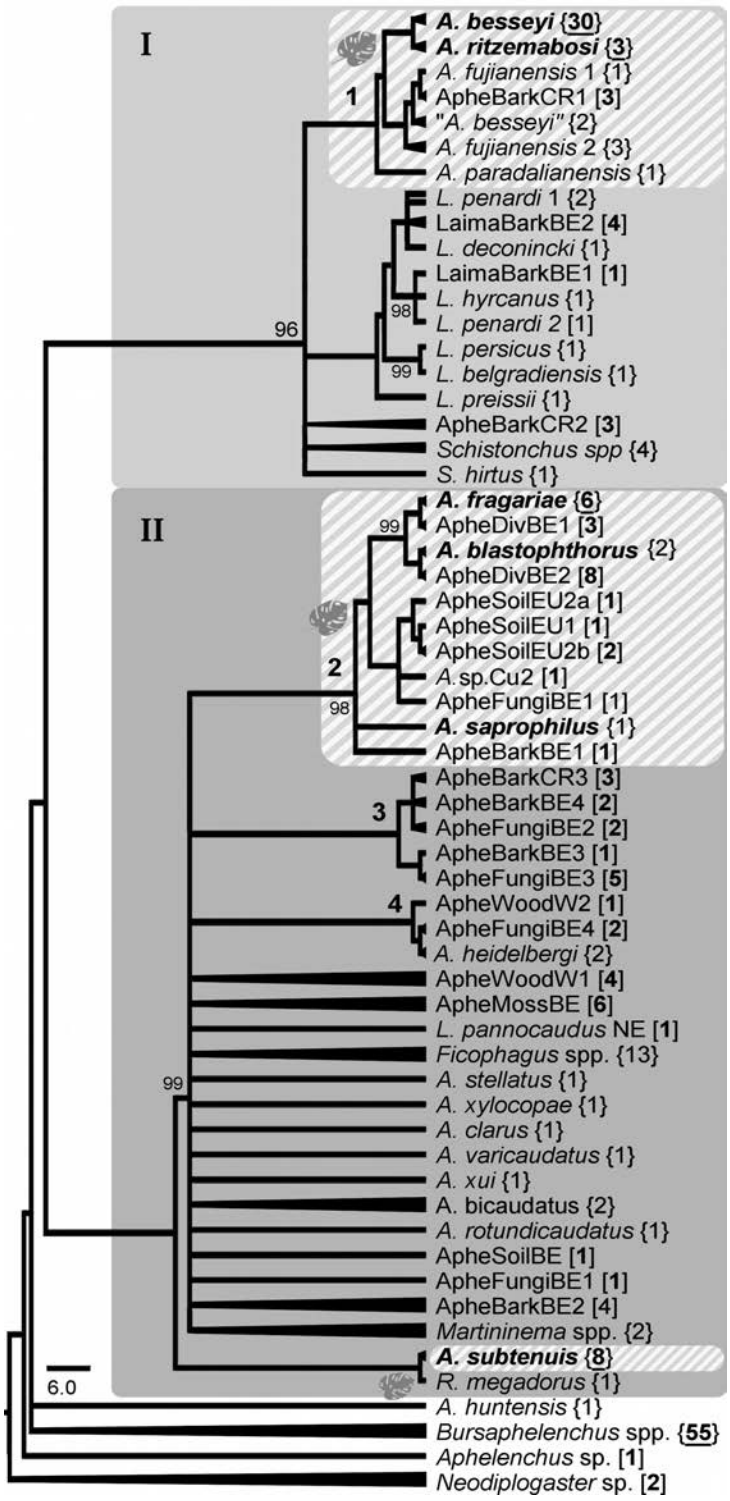

Figure III.2. Topology obtained from the Bayesian inference analysis of *Aphelenchoides* spp. and related taxa inferred from a concatenated file of one mitochondrial marker (COI) and two rDNA markers (partial 18S and D2D3 region of 28S). The number of sequences *per* clade is given in bold if new; between curly brackets if taken from GenBank; underlined when combined. Plant-parasitic *Aphelenchoides* species in bold; branches are proportionally presented; posterior probabilities >95% are plotted; assume 100% if not depicted. Leaves represent points where plant-parasitism may have arisen.

The inter-specific variation, as exemplified for the MPPA species (Table III. 2), is large enough for any of the single genes evaluated in this paper (*i.e.* mtCOI, 18S rDNA and 28S rDNA) to enable a robust identification of *Aphelenchoides* species. The intra-specific K2P distances of the mtCOI are similar to those known for marine species (Armenteros *et al.* 2014; Derycke *et al.* 2007; Fonseca *et al.* 2008) and other plant-parasitic nematodes, *e.g.* 1.1% in *Pratylenchus zeae* (Troccoli *et al.* 2016) and 4.1% in *Scutellonema brachyurus* (van den Berg *et al.* 2013). However, the maximum inter-specific distance within the MPPA (17-40%) is twice the maximum value reported for closely-related parasitic species (10-20%) (Blouin 2002) and roughly two or three times greater

than those reported between some *Scutellonema* species (10.2-13.4% between two types of *S. brachyurus*, and 20-24% between *S. bradys* and *Scutellonema* sp.) (van den Berg *et al.* 2013).

Although the COI region is notoriously difficult to amplify, with a success rate falling far below 50% in some groups of Nematoda (De Ley *et al.* 2005; Derycke *et al.* 2010), the PCR success rate in *Aphelenchoides* (65%) using the primers of Kanzaki and Futai (2002) is very important for its use as a DNA barcode gene.

In general, the higher mutation rate of mitochondrial sequences provides a better differentiation of closely related species or populations, and is particularly useful for the identification and description of hybrid or cryptic species (Kanzaki and Giblin-Davis 2012; Palomares-Rius *et al.* 2014a; Powers 2004; Shaw *et al.* 2013); and therefore, is especially convenient for groups with constrained morphological features such as *Aphelenchoides* (de Jesus *et al.* 2016; Rodrigues Da Silva *et al.* 2010; Zhuo *et al.* 2010). Despite the COI is widely used as a barcode in many animal groups (Derycke *et al.* 2010; Lin *et al.* 2015), several flaws have been reported, for example, an extreme diversity in nucleotide composition, the effect of symbionts (*i.e. Wolbachia*), anomalous properties (e.g. recombination, insertion, multipartitioning) and particularly the Nuclear Mitochondrial Sequences (NUMTS), which are copies of mtDNA sequences into nuclear chromosomes (Derycke *et al.* 2010; Frézal and Leblois 2008; Lin *et al.* 2015; Rodrigues Da Silva *et al.* 2010; Shaw *et al.* 2013). However, NUMTS are said to be rare in nematodes (Derycke *et al.* 2010) and to our knowledge they have not been yet recorded in Aphelenchoididae. Furthermore, in this chapter we have demonstrated the overall congruence between tree topologies and species delimitation provided by the mtCOI, 18S rDNA and 28S rDNA genes, which, together with the number of inter-specific differences (Table III.2), confirms the validity of COI as a barcode for *Aphelenchoides*. We therefore acknowledge the mtCOI and the D2D3 region as suitable options for *Aphelenchoides* diagnosis alongside 18S, which is already documented as being species-specific for this genus (Rybarczyk-Mydłowska *et al.* 2012).

Phylogenetic relationships and diversity of *Aphelenchoides*

As expected, the rDNA-based topologies obtained here are largely in agreement with earlier

studies (Esmaeili *et al.* 2016; Kanzaki *et al.* 2014a; b; Oro 2015; Rybarczyk-Mydłowska *et al.* 2012). Remarkably, the mtCOI topology shows a partial correspondence with the rDNA trees, especially regarding the two supported clades that contain free-living and PPA species (clades 1 and 2, Fig. III.1). Also, the early branching position of *A. subtenuis* in Clade II, according to the rDNAs and the concatenated tree (Fig. III.1 and III.2), is consistent with the available topologies (Esmaeili *et al.* 2016; Rybarczyk-Mydłowska *et al.* 2012; Ryss *et al.* 2013), while the close relationship with *Martininema* in the amino acid-based tree appears to be merely tentative.

Our results clearly confirm the paraphyly of *Aphelenchoides*, as suggested by previous works (Azizi *et al.* 2015; Cardoza *et al.* 2008; Esmaeili *et al.* 2016; Kanzaki *et al.* 2014a; Zhao *et al.* 2008); and indicates that the generic delimitation within Aphelenchoididae and the diversity of *Aphelenchoides* are far from settled. The genus *Schistonchus* has recently been divided into three distinct genera: *Schistonchus* (s.s), *Ficophagus* and *Martininema* based on a combined analysis of molecular (*i.e.* phylogenetic relationships) and morphological data (particularly the secretory-excretory pore position) (Davies *et al.* 2015); however, according to the mtCOI and concatenated topologies (Fig. III.1 and III.2), *Schistonchus* s.s. remains unresolved. The genus *Laimaphelenchus,* appearing in both clades I and II (Fig. III.1) is still polyphyletic despite *L. heidelbergi* was recently amended as *Aphelenchoides heidelbergi* (Carta *et al.* 2016). *L. pannocaudus* remains within Clade II while the position of *L. belgradiensis* is different depending on the analyzed region, *i.e*. found within Clade II in the mtCOI analysis but outside this clade in the rDNA and concatenated trees (Fig. III.1a *vs* Fig. III.1c, 1d and Fig. III.2). Similar conflicts can also be found for *A. huntensis* (based on 28S) and *A. xylocopae* (based on mtCOI) in that they did not cluster with any *Aphelenchoides* taxa in our analyses but are also found within Clade II (based on 18S) in their original descriptions (Esmaeili *et al.* 2016; Kanzaki 2006).

Additionally, problematic phylogenetic placements are apparent at species level, most likely owing to misidentification, *i.e*. "*A. besseyi"* and those "*A. ritzemabosi"* sequences within the clade of *A. fragariae* (see Fig. III.1 and Supplementary Table III.1). Such taxonomical conundrums are common in *Aphelenchoides* due to its difficult morphology-based

identification and the co-existence of similar species in the same sample (de Jesus *et al.* 2016; Hockland 2001; Sánchez-Monge *et al.* 2015). The identification of our representatives of these species was confirmed by their key morphological features (Hockland 2004) and by comparison to other sequences on databases, so we were able to pinpoint the genuine *A. besseyi* and *A. ritzemabosi* with confidence.

Evolution of plant parasitism in *Aphelenchoides*

According to recent molecular phylogenies, plant parasitism has arisen at least four times in Nematoda, in the clades Longidoridae, Triplonchida, and twice in Tylenchomorpha, *i.e.* tylenchs and aphelenchs (Sultana *et al.* 2013). In this chapter we confirm that plant-parasitism (denoted by leaves pictograms in the topologies) has also evolved more than once in *Aphelenchoides* (Bird *et al.* 2014; Rybarczyk-Mydłowska *et al.* 2012).

Our phylogenetic analyses clearly show that PPA and fungivorous species are closely related in strongly supported clades (see the PPA clusters 1 and 2, Fig. III.1) similar to the way that plant-parasitic *Bursaphelenchus* species are interspersed among mycophagous species (Bird *et al.* 2014). This indicates an independent origin of plant-parasitism from fungivorous ancestors on multiple occasions (Ye *et al.* 2007). Moreover, the ability of both, *Aphelenchoides* and *Bursaphelenchus* to feed on fungi, and the broad range of associated plants of the former (Chapter II) highlights their physiological plasticity to switch towards plant-parasitism. This fungal origin is also underpinned by molecular analyses that indicate a Horizontal Gen Transfer (HGT) of cellulase genes (family GH45) from fungi to plant-parasitic aphelenchids in an early ancestral species in Aphelenchoidea (Haegeman *et al.* 2011; Palomares-Rius *et al.* 2014b). However, this is based on the analysis of only few *Bursaphelenchus* spp. and the Clade I species *A. besseyi* (Jones *et al.* 2005; Kikuchi *et al.* 2014; Sun *et al.* 2014). Interestingly, the only cellulase gene identified for *A. fragariae* (Clade II) is a member of the GH5 family sharing more similarities with other plant-parasitic tylenchs than with *Bursaphelenchus* spp. (Fu *et al.* 2012), and the corresponding transcripts were not found in *A. besseyi*'s transcriptome (Wang *et al.* 2014). However, a GH5-candidate (Abe-GH5-1), showing the same origin with other nematodes' GH5 yet different from *A. fragariae*'s (Afr-ENG-1, 2 and 3), was recently reported in certain isolates of *A. besseyi* (Wu *et al.* 2016), hence, the hypothesis of

mutually exclusive families among taxa (Danchin *et al.* 2010; Helder *et al*. 2015) needs further analysis. Evidently, it would be worthwhile to characterize the cellulase genes from other plant-parasitic lines in *Aphelenchoides* in order to foster a more comprehensive insight into nematodes' plant-parasitism and evolution.

Conclusion

The overall congruence of the three analyzed markers, mtCOI, 18S and 28S, demonstrated that all three are suitable for the diagnosis of *Aphelenchoides* species, with similar PCR success rates among the markers. The presence of four genera, *Ficophagus*, *Laimaphelenchus, Martininema* and *Schistonchus* imbedded within *Aphelenchoides,* confirms the paraphyly of the genus *Aphelenchoides*. The present multigene approach is the first for *Aphelenchoides* and supports the idea of multiple independent origins of plant-parasitism, most likely from mycophagous ancestors. The newly-generated sequences in this survey, 69, 56 and 91 of mtCOI, 18S and 28S markers respectively, include the first mtCOI data for *A. fragariae* and *A. subtenuis,* and the first 28S sequences of the latter. These data not only benefit the molecular diagnosis of *Aphelenchoides* taxa but also contribute strongly to a more comprehensive framework for phylogenetic and biodiversity studies. It must be noted, however, that the remarkable number of undescribed species in this study underlines that the biodiversity of *Aphelenchoides* remains largely unexplored. This knowledge gap could be filled by increasing the number of comprehensively analyzed taxa, including molecular, morphological and ecological data to achieve a better understanding of the evolution and biodiversity of *Aphelenchoides*.

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Supplementary Table III.1. Sequences used in the single- and multi- gene analyses per taxa, reference code and genetic marker. 'Additional sequence(s)' were used only in the corresponding single gene analysis

Taxa	Genbank accession numbers		
Aphelenchoides			
Species \ Marker	COI	18S	28S (D2D3)
A. besseyi	KX356862	KX356705	KX356774
	KX356863	KX356706	KX356775
	KX356864	KX356707	KX356776
	-	KX356697	KX356753
	KX356845	KX356698	KX356755
	-	KX356699	KX356756
	-	KX356702	-
	-	KX356703	KX356765
	KX356844	-	KX356754
	KX356846	-	KX356757
	-	-	KX356758
	KX356847	-	KX356759
	KX356848	KX356700	KX356760
	KX356849	KX356701	KX356761
	KX356850	-	KX356762
	-	-	KX356763
	-	-	KX356764
	-	-	-
	-	-	KX356766
	KX356851	-	KX356767
	KX356852	-	KX356768
	-	-	KX356769
	KX356853	-	KX356770
	-	-	KX356771
	KX356854	-	KX356772
	KX356855	-	-
	KX356856	KX356704	KX356773
	HQ540530	JQ957877	HQ540538
	HQ540531	-	DQ328684
	-	-	KP757369
"A. besseyi"	EU983281	AY508035	(1) AY508109
	HQ540532	-	(2) HQ540534
A. bicaudatus	-	AY284643	-
	-	JN887885	-
A. blastophthorus	-	JQ957879	-
	-	AY284644	-
A. clarus	-	AY911887	-
A. fragariae	-	KX356708	KX356778
	KX356857	KX356709	KX356779
	-	JQ957880	AB368540
	-	AB067755	EU325684
	-	JQ957895	-
	-	DQ901551	-

Supplementary Table III.1. *Continued*

A. fujianensis	(1) KT782802 (1) FJ520226 (2) KT782809 (2) KT782809 -	- - (2) KT692678 (2) KT692673 (2) KT692674-	(1) KT692695 - (2) KT692701 (2) KT692702 (2) KT692697
A. huntensis	-	-	KR864862
A. paradalianensis	GU367865	GU337993	-
A. ritzemabosi	KX356906 KT82812 GU367869	- JQ957881 JQ957882	KX356837 KT692713 -
"A. ritzemabosi"	-	-	KR261601, KR261603 KP835687, KT261769 KT835683, KT261768 KT261770
A. rotundicaudatus	-	-	KF772859
A. saprophilus	-	FJ040408	-
A. stellatus	-	-	KF638651
A. subtenuis	KX356859 KX356860 KX356861 - - - - -	KX356710 KX356711 KX356712 JQ957893 JQ957890 JQ957888 JQ957891 JQ957892	KX356781 KX356782 KX356783 - - - - -
A. varicaudatus	-	HQ283351	HQ283353
A. xui	-	-	FJ643488
A. xylocopae	AB252222	-	AB434933
A. sp Cu2	KX356858	KX356740	KX356780
ApheBarkBE1	KX356866	-	KX356792
ApheBarkBE2	KX356894 KX356895 KX356896 KX356897	- - - KX356739	- - - KX356826
ApheBarkBE3	-	KX356715	KX356789
ApheBarkBE4	- -	KX356722 KX356723	KX356804 -
ApheBarkBE5	- - -	KX356714 (a) - KX356728 (b)	KX356787 (a) KX356808 (b) KX356811 (b)
ApheBarkCR1	KX356890 KX356891 KX356892	- KX356737 KX356738	- KX356825 -

Supplementary Table III.1. *Continued*

ApheBarkCR2	KX356886 KX356887 KX356888	KX356736 - -	KX356822 KX356823 -
ApheBarkCR3	KX356883 KX356885 KX356889	- - -	KX356820 KX356821 KX356824
ApheBarkCR4 (Add. sequence)	KX356884	-	-
ApheBarkCR5 (Add. sequence)	KX356893	-	-
ApheDivBE	- -	KX356718 -	KX356793 KX356803
	-	KX356727	-
ApheDivEu	- KX356873 - KX356874 KX356875 KX356876 KX356901 KX356879	KX356713 KX356724 KX356725 KX356726 - - - KX356732	KX356786 KX356805 KX356806 KX356807 KX356809 KX356810 KX356830 KX356815
ApheFungiBE1	KX356899	KX356742	KX356828
ApheFungiBE2	KX356900 -	KX356743 -	KX356829 KX356831
ApheFungiBE3	- KX356902 KX356903 KX356904 KX356905	- KX356744 - KX356745 KX356746	KX356832 KX356833 KX356834 KX356835 KX356836
ApheFungiBE4	KX356881 KX356882	KX356734 KX356735	KX356818 KX356819
ApheMossBE1	KX356867 KX356868 KX356869 KX356870 KX356871 -	- - - - KX356719 -	- - KX356794 KX356795 KX356796 KX356802
ApheSoilBE	KX356872	-	KX356799
ApheSoilGE	KX356878	KX356731	KX356814
ApheWoodW1	KX356840 KX356841 KX356842 KX356843	KX356693 KX356694 KX356695 KX356696	KX356749 KX356750 KX356751 KX356752
ApheWoodW2	KX356839	KX356692	KX356748
Aphelenchus			
Species \ Marker	COI	18S	28S (D2D3)
Aphelenchus sp.	-	KX356730	KX356813

Supplementary Table III.1. *Continued*

Additional sequence	-	-	JQ378400
Bursaphelenchus			
Species \ Marker	COI	18S	28S (D2D3)
B. abietinus	AY508037	AY508011	AY508074
B. africanus	JF317265	JF317266	HM623784
B. anatolius	AY508056	AY508025	AY508093
B. antoniae	-	AM279709	AM279710
B. arthuri	-	AM397010	AM396564
B. arthuroides	HQ599191	HQ599188	HQ599190
B. borealis	AY508038	AY508012	AY508075
B. braaschae	JF317264	GQ845409	GQ845408
B. clavicauda	-	AB299221	AB299222
B. cocophilus	-	AY509153	KT156769
B. corneolus	HQ407407	JQ765872	HQ407405
B. debrae	EF488816	-	EF488813
B. doui	FJ520228	AB299223	AB299226
B. eggersi	AY508040	AY508013	AY508078
B. fraudulentus	AY508042	AB067758	AY508081
B. fungivorus	AY508045	AY508016	AY508082
B. gerberae	AY508055	AY508024	AY508092
B. hellenicus	AY508046	AY508017	AY508083
B. hildegardae	-	AM397013	AM396569
B. hofmanni	AY508047	AY508018	AY508084
B. hylobianum	AY508048	AY508019	AY508085
B. kevini	EU325687	-	AY753532
B. luxuriosae	AB097863	AB097864	AB650013
B. mazandaranense	-	JN153102	J153103
B. mucronatus	AB646227	AB067759	AB932857
B. mucronatus (new)	KX356898	KX356741	KX356827
B. paraburgeri	HQ727728	HQ727727	HQ727726
B. paracorneolus	AY508058	AY508027	AY508095
B. paraluxuriosae	JF966207	JF966206	JF966204
B. paraparvispicularis	JF317263	GQ421483	GQ429010
B. parvispicularis	-	AB218829	AB368537
B. penai	-	AB901293	AB901292
B. pinasteri	-	AM397016	AM396574
B. platzeri	AY508057	AY508026	AY508094
B. poligraphi	AY508059	AY508028	AY508096
B. populi	HQ699854	HQ699855	HQ699856
B. rufipennis	AB368527	AB368529	AB368530
B. sakishimanus	-	LC027461	LC027462
B. seani	AY508060	AY508029	AY508097
B. sexdentati	AY508065	AY508032	AY508102
B. tusciae	AY508067	AY508033	AY508104
B. ulmophilus	-	KR011752	KP331049
B. vallesianus	-	AM397020	AM396578
B. willibaldi	-	AM397021	AM396579
B. xylophilus	JF317257	GU206792	AY508108
	KF025330	KF025319	DQ364687
	JF317250	AY508034	JF317239
Bursaphelenchus sp.		-	KX356797
		KX356720	KX356798

Supplementary Table III.1. *Continued*

	- - KX356877	KX356721 - KX356729	KX356800 KX356801 KX356812
Additional sequences (used in single-gen analyses)	AB368528	AM397011, AB067757 JQ765873, HQ407406 AM397012, AB650015 KF496907, JX154585 AY284648, AM397015 AB358983, AB918706 AM397017, KF978103 AB232162, AB901291 AM397019, AY508033 FJ768947, KJ636306 AM397023	FJ768949, EU107359 KT156779, J903921 KF496910, LC087117 JQ287495, EU295494 AM396573, EU295493 DQ364688, EU295492 AM396575, EU295496 KF978102, AY508098 AM396577, EU295497 FJ998283, AM396581
Ficophagus			
Species \ Marker	COI	18S	28S (D2D3)
F. altermacrophylla *F. aureus* *F. benjamina*	- - -	- DQ912922 KJ638355	AB535534 DQ912925 AB535542
   F. centerae *F. laevigatus* *F. microcarpus* *F. virens*	- - - - - GU392235 - - -	KJ638352 KJ638351 - DQ912923 DQ912921 GU229645 - - -	AB535553 AB535557 AB535558 DQ912928 DQ912926 GU392234 AB535543 EU018052 AB535564
Additional sequences (used in single-gene analyses)		KJ638361, KJ638368 KJ638364, KC526928 KJ638362, KJ638360 KJ638349	KJ638377, KJ638384 KJ638376, KJ638380 KJ638378, KJ638386 KJ638375, KJ638385 AB535565, AB535566 KJ638381, KC526929, KJ638372
Laimaphelenchus			
Species \ Marker	COI	18S	28S (D2D3)
L. belgradiensis *L. deconincki* *L. heidelbergi* *L. hyrcanus* *L. pannocaudus* *L. penardi*	KF881747 - EU287592 KJ564292 - KX356907 - - -	KF881745 - EU287587 - - KX356747 AY593919 AY593918 EU306346	KF881746 KF998578 EU287595 KJ564293 KJ567061 KX356838 - - -

Supplementary Table III.1. *Continued*

L. persicus	-	-	J006987
L. preissii	EU287594	EU287590	EU287598
LaimaBarkBE1	-	-	KX356788
LaimaBarkBE2	-	-	KX356784
	-	-	KX356785
	-	KX356716	KX356790
	KX356865	KX356717	KX356791
Martininema			
Species \ Marker	COI	18S	28S (D2D3)
M. baculum	-	-	AB535541
M. guangzhouensis	EU419757	DQ912924	DQ912927
Additional sequences (used in single-gene analyses)	KM817191 KC250364	-	KM817190 KC250363
Schistonchus			
Species \ Marker	COI	18S	28S (D2D3)
S. caprifici	EU287604 EU287607 EU287608	FN564940 GU190763	FN564936 GU190765
S. hirtus	GQ849474	-	-
S. macrophylla	-	-	AB535530
	-	-	AB535533
Others			
Species \ Marker	COI	18S	28S (D2D3)
Neodiplogaster sp.	-	-	KX356816
	KX356880	KX356733	KX356817
Robustodorus megadorus	-	KC687094	KC687095

Note: Misidentified sequences of PPA species are given between quotation marks as "*A. besseyi*" and "*A. ritzemabosi*". Accession numbers starting with "KX" correspond to newly generated sequences.

CHAPTER IV
Morphological traits in *Aphelenchoides*' phylogeny: a reverse taxonomic approach

In preparation

Sánchez-Monge, A.[1,2], Janssen, T.[1], Couvreur M.[1] & Bert, W.[1] (201*) Exploring morphological traits in *Aphelenchoides*' phylogeny (Aphelenchoidea: Nematoda): a reverse taxonomic approach with an amendment to the genus diagnosis. *In prep.*

[1] *Nematology Research Unit, Department of Biology, Ghent University, Campus Ledeganck, B-9000, Belgium.*
[2] *Universidad de Costa Rica, Escuela de Estudios Generales, 2060, Costa Rica*

Abstract:

The genus *Aphelenchoides* is highly complex both on morphological and molecular grounds. The combination of low inter-specific and high intra-specific morphological variability makes morphology-based identification extremely difficult, and molecular tools are indispensable for accurate diagnosis. However, most taxa are not yet associated with discriminating molecular data, moreover, some entries of *Aphelenchoides* spp. in public databases appear to be completely wrong. Furthermore, species of the genera *Ficophagus*, *Laimaphelenchus*, *Martininema* and *Schistonchus* are embedded within those clades of *Aphelenchoides*, evincing a non-monophyletic origin and a big gap in the knowledge of their phylogenetic relationships. It is especially unclear which morphological traits are phylogenetically informative and/or informative to diagnose *Aphelenchoides* taxa. In order to elucidate the taxonomy and phylogeny of this genus, we collected and analyzed several specimens of *Aphelenchoides* and related taxa, including free-living specimens from soil, bark, fungi as well as plant-parasites. Subsequently, we constructed a molecular framework based on two different molecular markers (18S rDNA and 28S rDNA D2D3 expansion region), on which morphological and biological features were plotted and discussed. Based on this integrative work, the tail terminus and the position of the secretory-excretory pore relative to the median bulb are regarded as phylogenetically-relevant characters. Furthermore, we provide new data for phylogenetic and morphological studies and propose an amendment of the genus diagnosis together with a tentative species-grouping system based on tail-terminus features. Practical constraints and additional comments on related genera are discussed.

Keywords: *Ficophagus*, foliar nematodes, *Laimaphelenchus*, *L. pannocaudus*, *Martininema*, rDNA, *Schistonchus*

Resumen:

El género *Aphelenchoides* es altamente complejo morfológica y molecularmente. La combinación de baja variabilidad morfológica a nivel inter-específico y alta a nivel intra-específico hace que la identificación basada en rasgos morfológicos sea extremadamente difícil, y que se requieran herramientas moleculares para un diagnóstico certero. Sin embargo, la mayoría de species no están asociados con datos moleculares para su identificación, más aún, algunas secuencias de *Aphelenchoides* spp. en bases de datos públicas parecen estar erradas. Además, species de los géneros *Ficophagus*, *Laimaphelenchus*, *Martininema* y *Schistonchus* están embebidas entre los grupos de *Aphelenchoides*, demostrando un origen no monofilético y un vacío en el conocimiento de sus relaciones filogenéticas. Se desconoce también que rasgos morfológicos son filogenéticamente informativos o útiles para diagnosticar especies de *Aphelenchoides*. A fin de dilucidar la taxonomía y filogenia de este género, se recolectaron y analizaron varios especímenes de *Aphelenchoides* y taxa relacionados, incluyendo especies de vida libre de suelo, corteza y hongos así como fitoparásitas. Seguidamente se construyó un marco de referencia molecular basado en dos diferentes marcadores moleculares (ADNr 18S y la región de expansión D2D3 del ADNr 28S), y sobre éste se analizaron y discutieron características morfológicas y biológicas. Basado en este trabajo, las estructuras de la punta de la cola y la posición del poro secretor-excretor respecto al bulbo medio son reconocidos como caracteres filogenéticamente relevantes. Adicionalmente, se generaron nuevos datos para estudios filogenéticos y morfológicos, y se propone un arreglo a la diagnosis del género junto con un sistema tentativo de grupos basado en rasgos de la cola. Se discuten también limitaciones prácticas y comentarios adicionales sobre otros géneros cercanos.

Palabras clave: ADNr, *Ficophagus*, *Laimaphelenchus*, *L. pannocaudus*, *Martininema*, nematodos foliares, *Schistonchus*

Introduction

Nematodes are the most abundant group of invertebrates (Heip *et al.* 1985; Ye *et al.* 2007). They have colonized almost all terrestrial habitats as well as aquatic environments, showing complex lifestyles and extreme adaptations in some cases (De Ley 2000). Parasitic nematodes are important both in animals and plants; in the latter, roughly 4000 species have been described as plant-parasitic nematodes (PPN). The most important genera belong to the superfamilies Tylenchoidea, Criconematoidea, Sphaerularioidea, Dorylaimoidea and Diphterophoroidea, the first two contain 19 and 7 genera, respectively, while the remaining superfamilies comprise 3 important genera each (Decraemer & Hunt 2013). Also, two other plant-parasitic genera of importance are found in Aphelenchoidea, the genus *Bursaphelenchus* with the pinewood nematode (*B. xylophilus*) and the genus *Aphelenchoides,* comprising the "foliar nematodes" (Hunt 1993; Kanzaki & Giblin-Davis 2012; Sánchez-Monge *et al.* 2015).

The latter genus harbors circa 200 species (Tables I.1, I.2), most of which were poorly described (Golhasan *et al.* 2016; Hockland 2001; Hunt 1993; Rybarczyk-Mydłowska *et al.* 2012) leading to several taxonomic conundrums (Hockland 2001). In addition to de Man's ratios, the lengths of the body, stylet, spicule and post-uterine sac; and the position of the secretory-excretory pore among others are commonly used for species identification (Hockland, 2001). However, species within this genus show low inter-specific and high intra-specific morphological variability, thus, morphology-based diagnosis of *Aphelenchoides* species is often difficult. Furthermore, nearly all species are not yet associated with discriminating molecular data (Esmaeili *et al.* 2016b; Zhuo *et al.* 2010) and most of the available sequences belong either to plant-parasitic species or to non-identified taxa whose morphological vouchers are absent (Sánchez-Monge *et al.* 2015). The combined assessment of morphological, molecular and ecological traits has only been done for some economically important species (Hsieh *et al.* 2012; de Jesus *et al.* 2016).

Next to the taxonomical problems at species level in this genus, *Aphelenchoides* is said to be polyphyletic or paraphyletic (Asghari & Eskandari 2014; Esmaeili *et al.* 2016a; Kanzaki *et al.* 2014; Kanzaki & Giblin-Davis 2012; Oro 2015; Rybarczyk-Mydłowska *et al.* 2012) and the

evolution of plant-parasitism in Aphelenchoidea is not clear (Chapter III). A variety of genetic tools and approaches has been successfully applied in nematological studies; among these, reverse taxonomy was intended to elucidate diversity in those cases where traditional approaches were not sufficient (Markmann & Tautz 2005), by relying on sequences similarity to assign anonymous taxa to known specific taxon clusters (Markmann & Tautz 2005; Randrianiaina *et al.* 2010).

The application of this approach, *i.*e. doing molecular analyses of unknown taxa prior further steps, has a broader range of applications since these anonymous sequences can be assigned to groups representing trophic levels (Markmann & Tautz 2005) or spatial distribution patterns (Janssen *et al.* 2015). Even ecological observations can be supported when the extracted sequences confirm taxa in associations with other organisms and substrates (Hazir *et al.* 2015; Kanzaki *et al.* 2012). Moreover, the reverse taxonomic approach has also lead to the description of new taxa (Kanzaki *et al.* 2009) and to deeper analyses on cryptic species complexes (Apolônio Silva de Oliveira *et al.* 2012; Derycke *et al.* 2010). Thus, with this methodology, it is possible to assess the diversity and the taxonomy of highly complex groups, which is the case for *Aphelenchoides*. However, the reverse taxonomic approach applied in this chapter was not intended to identify species nor tropic groups, but to define phylogenetic clusters of sequences, after which we explored morphological features to support the resulting clades.

Since very few *Aphelenchoides* species have molecular data (Sánchez-Monge *et al.* 2015) and most sequences in databases lack morphological vouchers, we collected molecular and morphological data of numerous specimens of *Aphelenchoides* (and related genera), including free-living taxa from soil, bark and fungi as well as plant-parasites. A molecular framework, based on two different rDNA markers (*i.e.* 18S and 28S D2D3 expansion region), was constructed, on which we plotted morphological and biological features. Based on this combination, only some morphological features can be regarded as phylogenetically informative. Additionally, we propose a new classification system based mostly on tail and tail-terminus shapes and present some comments on the taxonomy of *Aphelenchoides* and close genera, including an amendment to the genus diagnosis.

Materials & Methods

Molecular and phylogenetic analyses

Sequences were obtained and alignments were constructed as described in Chapter III. Multiple sequence alignments of partial 18S and 28S D2D3 expansion region included available GenBank sequences of *Aphelenchoides* (mainly from Chapter III), *Aphelenchus, Bursaphelenchus, Ficophagus, Laimaphelenchus, Martininema, Robustodorus, Schistonchus* and eight newly generated *Aphelenchoides* sequences.

Both single gene and concatenated trees were constructed. Two sets of alignments were prepared for each individual gene, an "inclusive" set comprised most *Aphelenchoides* sequences from GenBank (with fewer *Bursaphelenchus* representatives); and a "non-inclusive" set excluded *Aphelenchoides* entries which were not identified to species level in order to limit the amount of missing data in the multigene analysis. One concatenated dataset was based on non-inclusive alignments with the missing nucleotide positions treated as "?". The other concatenated dataset followed a more robust approach, only the taxa with data for both 18S and 28S genes were included. The COI data from Chapter III were not included in the concatenated analyses. rDNA and mitochondrial sequences are often from different taxa and we opted to reduce the number of missing sequences and/or taxa in our concatenated alignment in order to obtain phylogenetic trees with the best possible resolution.

The best-fitting base-substitution models were calculated using JmodelTest 2 (Darriba *et al.* 2012) under the Bayesian information criterion (BIC); the models K80+I+G and GTR+I+G were selected for the 18S and 28S datasets, respectively. Model calculations and Bayesian inference analyses (BIA) were performed on the CIPRES Science Gateway (Miller *et al.* 2010); BIA were ran for $3x10^7$ generations using MrBayes 3.2.6; Markov Chains were sampled at intervals of 1000 generations; burnin was chosen to be 25%. A Bayesian Inference consensus tree was created for each analysis as in Chapter III but with branches' support values equal or over 90.

Morphology and reverse taxonomy

The preparation of morphological vouchers, including temporary slides, photodocumentation and video capturing were done as described in Chapter III using an Olympus BX51 DIC Microscope (Olympus Optical), equipped with an Olympus C5060Wz camera. Vouchered specimens were analyzed and measured with the program UTHSCSA Image Tool Version 3.00 (Wilcox *et al.* 2002). Corresponding data for the known species were based on illustrations from the original descriptions or re-descriptions. The resulting conventional and complementary morphometric parameters (see Table IV.1) were tabulated *per* major *Aphelenchoides* phylogenetic clades rather than diagnostic characters for species diagnosis because the exceptionally high diversity of the retrieved populations resulted in low numbers of representatives *per* putative species (see Chapter V), hence, intra-specific variation could not be assessed. To test the agreement of morphological traits with the different clades, all morphometrics (except for the number of lateral lines and mucro shape), were standardized by subtracting the mean and dividing the value by its standard deviation (SD). The *F*-values were calculated for all features by means of the one-way analysis of variance (ANOVA); as indicators of variability of measurements, *F*-values reflect which traits are most informative for each clade. Differences in morphometrics between clades were *post-hoc* tested using the Tukey Honest Significant Difference test (TukeyHSD). Both analyses were ran online (Lowry 2016). Phylogenetically-relevant features were plotted in a schematic tree based on the concatenated analyses using Mesquite (Mesquite Project Team 2014). Image edition and schematic drawings were done with GIMP 2.8.16 (Kimball *et al.* 2014).

Results

Phylogenetic analyses

The non-inclusive and inclusive 18S alignments comprised, respectively, 179 and 171 sequences that were 826 and 851 base pairs (bp) in length. 28S alignments comprised 232 and 202 sequences for the non-inclusive and inclusive datasets, respectively, and were 881 and 831 bp in length. The resulting Bayesian inference consensus trees are depicted in Fig. IV.1 and IV.2 (non-inclusive 18S- and D2D3-based) and Fig. IV.3 and IV.4 (inclusive 18S- and

D2D3-based); inclusive and non-inclusive datasets resulted in largely similar topologies on which newly generated sequences are highlighted in bold. The concatenated alignments and resulting trees including and excluding single-gene sequences (*i.e.* Fig. IV.5 and Fig. IV.6, respectively) were correspondingly based on 282 and 127 sequences, with a total number of characters of 1711 bp.

All obtained topologies include two major clades (Figs IV.1-6). Clade I contains only four identified *Aphelenchoides* species, namely *A. besseyi*, *A. fujianensis* and *A. ritzemabosi* in the 28S-based trees plus *A. paradalianensis* in the 18S-based topologies. Several non-identified *Aphelenchoides* spp., and species of *Laimaphelenchus* and *Schistonchus* complement the taxa found in this clade. Clade II on the other hand, consists of a more diverse group of *Aphelenchoides* sequences (identified or not to species level) together with species of the genera *Ficophagus, Martininema* and *Robustodorus*. The tree topology was better supported if only the taxa with information of both genes were included (Fig. IV.6), many relationships were not resolved in the first concatenated analysis due to missing data in single-gene sequences (Fig. IV.5). Both concatenated trees and 28S topologies support monophyly of the available sequences of *Ficophagus*, *Laimaphelenchus, Martininema and Schistonchus*, and in general, 28S-based trees were better resolved than 18S topologies (Fig. IV.1-4).

A. fuchsi appeared in an ambiguous position, out of the major clades in the 18S topologies (Fig. IV.1 and IV.3) but belonging to Clade II in the 28S-based tree. *A. huntensis* and *A. stammeri* appeared out of the main *Aphelenchoides* clades and *A. composticola* (KJ636363) is placed in the *Bursaphelenchus* clade (Fig. IV.1). Finally, several *A. ritzemabosi* sequences, probably misidentified, clustered in clade II-3 in the 18S-based trees (Fig. IV.1 and IV.3) in contrast to other representatives of this species in clade I-1 (Fig. IV.1-6).

Morphological features and reverse taxonomy

More than 200 specimens, mostly *Aphelenchoides* but also several *Bursaphelenchus* and *Laimaphelenchus* representatives, were analyzed using a microscope prior molecular work. Unfortunately, not all morphologically analyzed specimens were successfully DNA-amplified. Morphological vouchers, showing key features, were deposited at

http://nematodes.myspecies.info (contact the author for better quality pictures and videos). Since males were uncommon in free-living species, all analyses were focused on females. In addition to de Man's ratios, some unexplored, potentially useful characters for *Aphelenchoides* diagnosis were considered (see Table IV.1); special attention was paid to the median bulb as the most distinctive feature in Aphelenchoidea.

Most morphometric features (68%) were significantly different ($P<0.05$) among clades (Table IV.2). Traits with no significant differences between the clades were: the relative length of the pharyngeal gland (b'); measurements related to the vulva position (V, V' and V°); ratios of the knobs, lips, medial bulb, median bulb's valves and their position (KnR, LpR, VaR, VaP); length of the isthmus, pharyngeal glands and post uterine sacs (PGA, PUS); relative size of the PUS to the distance between vulva and anus (PUS/VuAn); and the anal body with (ABW). The highest *F*-value, *i.e.* the most different character among clades, was obtained for the position of the median bulb divided by the position of the secretory-excretory pore (LMBA/EPA), followed by the c value, lip region maximum width (LpMW), knobs width (KnW) and the position of the secretory-excretory pore (Table IV.2). Clade II-2, composed by *A. subtenuis* and *R. megadorus*, was the most different from all other clades, showing between 12 and 28 significantly different traits in respect to the other groups (Table IV.2). Clades II-2 and II-6 are most different from each other with 28 significantly different traits, while clade I vs clades II-2 and II-3, and II-3 vs II-5 had no significant differences (Table IV.2).

However, despite the significant differences, measurements of these significantly different features overlapped among clades (Table IV.1). Only two features related to the position of the secretory-excretory pore, *i.e.* EPA and LMBA/EPA, showed significant differences and non overlapping values between clade II-6 and the other clades (Tables IV.1 and IV.2). Thus, these two secretory-excretory pore traits are the only values that clear-cut delineate clade II-6 from the other clades. For the morphological traits that could not be statistically analyzed, only the mucro shape showed a remarkable correspondence with the clades in the phylogenetic trees (Fig. IV.7), this prompted us to forward the tail-terminus morphology in a grouping scheme presented in Table IV.3. Table IV.4 provides a complete categorized list of *Aphelenchoides* species based on original descriptions and other available literature,

including *species inquerendae*. Following this arrangement, Clade I is composed by species from groups 3.1, 3.2 and one species from 2.1.1 (*i.e*. *A. paradalianensis*), whereas most taxa in clade II-3 belong to group 2.2.1 except for one specimen from the 2.2.2 group (*i.e*. *A*. sp 021). The remaining groups are less related to the clades, for example, clade II-6 has representatives from groups 1.1 and 2.1.2; and clade II-5 contains at least one representative from each group except number 4 (Fig. IV.7).

Discussion

Phylogenetic relationships of *Aphelenchoides* and close genera

The obtained tree topologies agree with recent studies in the division of *Aphelenchoides* species into two major clades, alongside with several closely related genera embedded within *Aphelenchoides* sequences (Fig. IV.1-6); thus, supporting the paraphyly of this genus (Asghari & Eskandari 2014; Esmaeili *et al.* 2016a; Kanzaki *et al.* 2014; Kanzaki & Giblin-Davis 2012; Oro 2015; Rybarczyk-Mydłowska *et al.* 2012). The further division of clade II-1 into two well defined branches (except for II-3 see below) was already evinced in previous studies (Esmaeili *et al.* 2016a; b; Zhuo *et al.* 2010). Clade II-3 comprises *A. fragariae* and related sequences including *A. blastophthorus*; while most *Aphelenchoides* sequences plus *Ficophagus* and *Martininema* reside in the other branch. The position of the clade composed by *A. subtenuis* and *Robustodorus megadorus*, *i.e.* II-2, is also consistent with previous studies (Esmaeili *et al.* 2016b; Rybarczyk-Mydłowska *et al.* 2012; Ryss *et al.* 2013).

The 28S-based topologies are partially in agreement with the 18S-based trees and the differences do not represent actual conflicts for the MPPA species, as mentioned in Chapter III. However, topologies based on inclusive and non-inclusive datasets have differences even for the same molecular marker. For example, the sister relationship between *A. besseyi* and *A. ritzemabosi* is not supported in the inclusive 18S-based tree but it is supported in the non-inclusive 18S and in the inclusive 28S dataset. Similarly, clade II-3 (*i.e. A. fragariae* and others) is not supported based on the non-inclusive 18S-tree, but had a high supporting value in the inclusive data set for the same gene, and is also strongly supported by the mtCOI analyses (*i.e.* based on nucleotides and amino acids) in Chapter III (Fig. III-1a, 1b).

Also within this clade, a sister relationship between *A. fragariae* and *A*. sp 021 and *A*. sp. 069 is only supported in the inclusive 28S-based tree (Fig. IV.4). Apart from clade II-2, the only supported clade in the COI analysis was Clade I-1. However, the internal resolution of this clade remains poor, only the relation of *A. fujianensis* and *A*. sp. 142, and a second group of *A. fujianensis* and "*A. besseyi*" sequences appear to be supported (Fig. III.1). These clades, *i.e.* I-1 and II-2, were also obtained in the rDNA-based trees in Chapter III and Chapter IV, however, with the exception of II-3 in the non-inclusive 18S tree (Fig. IV.1).

The phylogenetic position of some *Aphelenchoides* representatives depends on the analysis, for example, *A. heidelbergi* is sister to *A*. sp. 108 (*i.e.* ApheFungiBE4 in Fig. III.1c, d) in the rDNA trees from Chapter III, but this relationship was not supported in the 18S trees in Chapter IV (Fig. IV.1 and IV.3). Other relationships are more consistent, for instance the cluster containing *A*. sp. 13, *A*. sp. 15, *A*. sp. 16 and *A*. sp. 17 (*i.e.* ApheWoodW1 in Fig. III.1), is sister to *A. varicaudatus* in all rDNA-based trees (Fig. III.1c, d; Fig. IV.1-IV.6). The placement of *A. huntensis* and *A. stammeri* outside the major clades in the rDNA trees is consistent with previous studies (Esmaeili *et al.* 2016a). However, the positioning of *A. fuchsi* outside the major clades (see concatenated-based topologies: Fig. IV.5 and IV.6), is different from its placement within Clade II according to Esmaeili *et al.* (2016b). Some problematic phylogenetic placements at species level, for example the seeming polyphyly of *A. ritzemabosi* and *A. besseyi*, are most likely owed to misidentification (see de Jesus *et al.* 2016) and Chapter III). Also the sequences of *A. bicaudatus* and *A. saprophilus* clustered in two different positions (Fig. III-1, Fig. IV.1 and Fig. IV.3), but their genuine placement remains to be investigated.

The position of the non-*Aphelenchoides* genera, except for *Robustodorus*, is not yet fully settled. *Martininema*'s position in clade II is consistent with recent works by Golhasan *et al*. (2016) and Esmaeili *et al*. (2016b) but not with Esmaeili *et al*. (2016a) in which *M. guangzhouensis* is sister to all other taxa, except *A. subtenuis*, in the 18S topology. However, this genus presents three possible placements in the obtained topologies in this study (Fig IV.1-IV.4): sister to *A*. sp 027 plus *A. clarus* or *A. rotundicaudatus* in the non-inclusive 18S- and 28S- tress, respectively; unresolved in the inclusive 18S topology; and sister to *A.*

rotundicaudatus, within a clade containing *A*. sp. 12, *A*. sp. 108, *A*. sp. 110, *A. heidelbergi*, *A. stellatus* and four unknown sequences from Genbank, in the inclusive 28S tree. Clade I-2, comprising *Laimaphelenchus* species, is absent in both 18S-based trees but strongly supported in the 28S and concatenated topologies (Fig IV.1-IV.6). The few representatives of *Schistonchus* s.s. formed a monophyletic cluster within Clade I-1 in all rDNA-based trees from Chapters III and IV but not in the COI-based tree (Fig. III.1a). *Ficophagus* is also monophyletic and belongs to clade II.1 in all analyses; however, its exact position remains unresolved; only a close relationship with *L. pannocaudus* (now *A. pannocaudus*) was obtained in the 18S tree (Fig. III.1c). Finally, *Robustodorus*' sister relationship with *A. subtenuis*, as described by Ryss *et al*. (2013) based on 18S, was confirmed based on both, 18S and 28S analyses (Chapters III and IV).

Compared to the mtCOI-based tree (Fig. III.1), the phylogenetic resolution obtained with rDNA genes (Figs IV.1-IV.4) is definitely better to reconstruct relatedness among taxa. However, the mtCOI region provides a better differentiation of closely related species or populations which is especially important for studies on hybrid or cryptic species and groups with limited diagnostic morphological features (de Jesus *et al.* 2016; Kanzaki & Giblin-Davis 2012; Palomares-Rius *et al.* 2014; Powers 2004; Rodrigues Da Silva *et al.* 2010; Zhuo *et al.* 2010). Nonetheless, several flaws have been reported for the use of this region (see also Chapter III), such as an extreme diversity in nucleotide composition, the effect of symbionts, phenomena of recombination, insertion, multipartitioning, and the Nuclear Mitochondrial Sequences (NUMTS), that although rare in nematodes (Derycke *et al*. 2010), could be present in Aphelenchoididae but not yet informed.

The topological variation between analyses as described in Chapters III and IV, is commonly found in aphelenchids' studies, as mentioned by Zhao *et al.* (2008). The differences in the phylogenetic relationships can be attributed to different phylogenetic histories told by the datasets, resulting from several biological events in the evolution of the species, genes or groups, such as different DNA divergence times, alone or in combination with incomplete fixation of gene/species lineages and the persistence of ancestral alleles in more recent diverging lineages (Leliaert *et al*. 2014; Wiens 1998). Therefore, the accumulation of

sequences of different genes representing a broader range of populations and geographical locations is needed to build a more complete framework to understand the relationships between *Aphelenchoides* and its closely-related genera.

Morphological traits and phylogeny

Characters of relatively high value for taxonomic studies in Aphelenchoididae, listed by Ryss *et al.* (2013), include the secretory-excretory pore position, the female tail tip appendages, the number of ridges of the cephalic disc, the length of the post uterine sac, the number of incisures in the lateral field, the female vulval lip and the structure of spicules' condylus. These features, excluding the vulval lip and spicules' condylus, were also recommended by Hockland to identify *Aphelenchoides* species together with the tail shape and the stylet length (Deimi *et al.* 2006). Also the shape and size of the spicules can be occasionally informative at species level (Davies *et al.* 2015; Hockland 2001; Hockland 2004; Ryss *et al.* 2013). We evaluated these features (except for the spicules' shapes, vulval lip and the number of ridges) but a congruence with the phylogenetic clades was not observed (Table IV.1 and IV.2, Fig. IV.7). Moreover and according to our data, the length on the post-uterine sac (PUS) relative to the distance vulva-anus is a highly conserved morphological feature in *Aphelenchoides*, *i.e.* without statistically significant differences and low *F*-values (Tables IV.1 and IV.2). Also conserved are the vulval position and the dimensions of the knobs', valves and median bulb, however, the overlapping values among the obtained phylogenetic clades do not judge their species specific value. Hockland (2001) for example, considered the tail terminus, length of PUS, lateral lines, position of the secretory-excretory pore, body length and "a" values as either high or medium rating features for *Aphelenchoides* species diagnosis, therefore, morphology-based diagnosis of species is possible with a combination of these characters.

The position of the secretory-excretory pore (EP) relative to the nerve ring is, for Ryss *et al.* (2013) and Hockland (cited by Deimi *et al.* 2006), an important feature for *Aphelenchoides* identification. However, Ryss *et al.* (2013) argued that taxa in Clade I and II (corresponding to clade 2b and clade 2a in Ryss *et al.* 2013) have the secretory-excretory pore respectively, posterior to the nerve ring or at the level (or anterior) to the nerve ring. Yet, this difference

between clades is not that clear cut, for example the EP can be posterior in *A. ritzemabosi* and *A. paradalianensis* but anterior in *A. fujianensis* (Clade I), and posterior or at the level of the nerve ring in *A. fragariae* and *A. heidelbergi* but anterior in *A. bicaudatus* and *A. blastophthorus* (Clade II). Furthermore, *A. ensete* and *A. haguei* illustrate the intra-specific variability of the EP position, *i.e.* anterior, at the middle or posterior to the level of the nerve ring (Swart *et al.* 2000; Swart & Heyns 1997).

The unusual location of the secretory-excretory pore relative to the median bulb in clade II-6, *i.e.* anterior, is, however, not showed by all members of this clade in the single gene trees (Fig. IV.1 and IV.3); *i.e. A. clarus* (AY911887) and *A. bicaudatus* (JN887885 and AY284643), as the EP is never anterior to the median bulb in these species (Siddiqui 1976; Thorne & Malek 1968). Such feature has also been proven taxonomically relevant for *Bursaphelenchus'* groups classification (Ryss *et al.* 2005) but to our knowledge, it is uncommon in *Aphelenchoides* taxa. Aside *A. rotundicautus*, only *A. parabicaudatus* and *A. submersus* show this condition (Shavrov 1967; Truskova 1973), but associated molecular data is absent for these two species.

Despite there is little variation within *Aphelenchoides*, the number of lateral lines is not only difficult to estimate (Hockland 2001) but also an example of how the specimens' preparation can affect the interpretation of external morphology using light microscopy (Fig. IV.8); moreover, this number can vary, and usually decreases towards the head and tail region (Hockland 2001). Different to what we found in *Aphelenchoides*, in *Bursaphelenchus* species the number of lateral lines is known to be not only useful for species diagnosis but also congruent with phylogenetic clades (Braasch 2001; Ye *et al.* 2007). However, variation in the number of lateral lines and the diversity and spatial distribution of lateral structures, such us alae, ridges, striae and other outgrowths (Fig. IV.8) can be attributed in some cases to the compression or depression of the lateral fields during mounting (Hockland 2001; Myers & Hooper 1971); depressions create folds or creases that may look like striae, augmenting the interpreted number or lines. By contrast, this number can be underestimated when fine striae are present but light microscopy is unable to show them (Deimi *et al.* 2006; Hockland 2001; Hooper & Ibrahim 1994; Qing *et al.* 2015).

Notwithstanding the congruence between the tail shape and terminus (Table IV.3) and the phylogenetic clades (Fig. IV.7), not all registered tail shapes are associated with rDNA sequences in our analyses and exceptions (highlighted with an asterisks "*" in Fig. IV.7), are present within Clade II. In her diagnostic compendium of the genus *Aphelenchoides,* Shahina (1996) evaluated 141 species and recognized four groups of tail shapes: 1. tail simple without any outgrowth or mucronate structure; 2. tail with one or sometimes two mucronate structures on tail terminus; 3. tail with tetramucronate spine or star shape and 4. tail outgrowth other than spine or star. Such classification has been widely used in comparisons and descriptions of new species (*e.g.* Esmaeili *et al.* 2016a; b; Golhasan *et al.* 2016; Wang *et al.* 2013; Zhuo *et al.* 2010). Here we show for the first time, in combination with data from original descriptions and recent literature, that this classification partially resembles natural groups.

However, several unknown/multi-state tail morphologies remain to be investigated, furthermore, for several species no tail tip morphology could be assigned because either the description or the illustrations were not detailed (Table IV.4). The proposed categorization (Table IV.3) is basically a molecularly-supported refinement of the four-groups tabulation of Shahina (1996) with slight modifications, for example, group one (no mucro) is subdivided into tail shape: round (1.1) or pointed (1.2); and group two (one or two mucronate structures) is split into one (2.1) or two (2.2) structures. Further subdivisions within groups are possible and would help *Aphelenchoides* diagnosis, however, although tail shape and terminus are shown here to be valuable, this feature should be used with moderation (Nickle 1970). Intra-specific variation, such as the presence/absence of the mucro, is possible and sexual dimorphism can often be found (B'Chir 1979, Hockland 2001). For example, around six morphological variations in the tail terminus have been reported for *A. varicaudatus* and *A. rutgersi* (Ibrahim & Hooper 1994; Myers & Hooper 1971) and *A. besseyi* and *A. ritzemabosi* present at least three morphotypes depending on the number of mucro protrusions (Crozzoli *et al.* 2008; Khan *et al.* 2012; Hockland 2004). Furthermore, morphological changes, including the shape of the tail-terminus, have been detailed for other aphelenchids (*B. xylophilus*) during the transition between feeding phases *i.e.* mycetophagous-phytophagous

(Tsai *et al.* 2015), although not yet described, similar transitions are also possible for *Aphelenchoides*.

Amendment to the diagnosis of the genus *Aphelenchoides*

The genus *Aphelenchoides* was erected by Fischer (1894) with *A. kuehnii* as the type species; currently, this genus harbors circa 200 species and several taxonomic conundrums (Tables I.1 and I.2). Given the morphological features and variations recorded in this study (Fig. IV.7), an amendment of the genus is proposed as follows, changes are underlined:

Diagnosis

Genus *Aphelenchoides* Fischer, 1894
syn. *Asteroaphelenchoides* Drozdovsky, 1967
Chitinoaphelenchus Micoletzky, 1922
Paraphelenchoides Haque, 1967
Pathoaphelenchus Cobb, 1927
Pseudaphelenchoides Drozdovsky, 1967

Bionomics: mycophagous; phytophagous; insect associate; soil, wood and bark inhabiting

Diagnosis: (modified after Hunt, 1993). Aphelenchoidinae. Small to long nematodes, usually between 0.4 to 1.2 mm in length. Heat relaxed females die straight to ventrally arcuate whereas the males assume a 'walking stick' shape with the tail region sharply curled ventrally. Cuticle transversely striated. Lateral fields often with four incisures but may be between two and five, rarely six[1]. Cephalic region usually rounded in form and slightly offset. There are six equally sized lips and the cephalic skeleton is weak. Stylet slender, usually with knobs or basal swellings, often about 10-12µm long and usually less than 20µm long. Procorpus cylindrical, leading to a well developed ovoid, squarish or spherical median bulb with central valve plates. Isthmus rudimentary, short or absent, pharyngeal gland lobe well developed and lying dorsally to the intestine. Secretory-excretory pore below the level of the stylet's base, mostly posterior or at the level of the median bulb but can be also anterior to the median bulb. Nerve ring posterior to the median bulb, the secretory-excretory pore may be anterior or posterior to the nerve ring. Vulva postmedian, usually at between 60 to 75% of the body length, only very exceptionally more posterior. Genital tract monoprodelphic

typically outstretched, buy may be anteriorly reflexed. Developing oocytes in one or more rows. Post-uterine sac usually present and often containing spermatozoa, but may be absent. Tail conoid with a variable terminus that may be bluntly or finely rounded, digitate or bifurcate or with a ventral projection. Terminus often differs between sexes. One or more mucronate structures of various shapes usually present, some may bear microprotuberances (micropapillae). Mail tail strongly hooked ventrally to form the characteristic 'walking stick' form, conoid in shape and tapering to a variable terminus. Spicules thorn-shape, paired and separate. The rostrum and appex are usually well developed, but may be almost absent. Typically there are three pairs of caudal papillae, one pair adanal, one pair subterminal and the other in between. Bursa absent.

[1] three lateral lines are observed in *A. bicaudatus* (Jen *et al.* 2012), *A. iranicus* (Golhasan *et al.* 2016) and *A. subtenuis* (Deimi *et al.* 2006); five in *A. shamimi* (Khera 1970) and six have been described for *A. allius* (Feng 2012), *A. besseyi* (Khan *et al.* 2012), *A. nechaleos* (Hooper & Ibrahim 1994), *A. paranechaleos* (Hooper & Ibrahim 1994); *A. parasexlineatus* (Kulinich 1964) and *A. sexlineatus* (Eroshenko 1967).

The position of *Aphelenchoides* and other genera within Aphelenchoididae

The systematics of *Aphelenchoides* has been unstable (Hunt 1993, 2008; Kanzaki & Giblin-Davis 2012; Nickle 1970) and several new genera have been proposed and later synonymized. Among these, the proposition of the genera *Asteroaphelenchoides* and *Pseudaphelenchoides* for *A. besseyi* and *A. ritzemabosi*, respectively, is of particular interest given current new evidence. Both genera were proposed by Drozdovsky (1967) based on their mucro shapes, *i.e.* star-like and crown shape (Table IV.3), and the presence of five or six blastomeres in early embryonic divisions. Later, both genera were synonymized with *Aphelenchoides* by Nickle (1970) as the character of the embryonic divisions "did not convince" him, and the difference in mucro shapes could be accounted for within *Aphelenchoides*. However, since Clade I (containing these two species) and Clade II (comprising most *Aphelenchoides* species) show remarkable independent lineages, molecularly and morphologically supported (Fig. IV.7, Table IV.3), a reappraisal of Drozdovsky's observations could become a valuable input for the upcoming taxonomic discussions.

Logically, because *Aphelenchoides* is paraphyletic, the morphological diagnosis of the other genera, is more straight forward thanks to distinctive discriminating features, except for *Laimaphelenchus*. Based on the analysis of diagnostic characters and their states from described species and morphospecies in combination with molecular phylogenetic inferences using three molecular markers (*i.e.* mtCOI, 18S rDNA and D2D3 expansion region of the 28S rDNA), Davies *et al.* (2015) split *Schistonchus* s.l. into *Schistonchus* (s.s), *Ficophagus* and *Martininema*, making most genera in Aphelenchoidea monophyletic. *Schistonchus* s.l. are associated with fig sycones and fig-pollinating wasps, and unlike *Aphelenchoides* and *Laimaphelenchus*, they have a robust stylet (between 10 and 40µm long) with strong basal knobs (Davies *et al.* 2015). The three genera can be morphologically differentiated by the secretory-excretory pore position, which can be very near the cephalic region (*Ficophagus*); at the anterior end of the median bulb (*Martininema*); or in the region of, or posterior to, the median bulb (*Schistonchus* s.s.) (Davies *et al*. 2015). The monospecific genus *Robustodorus* can be easily identified by its strong stylet with narrow lumen, massive knobs and guiding apparatus, which are unique characteristics within Aphelenchoididae (Ryss *et al.* 2013).

In the most recent taxonomical change in *Laimaphelenchus* (by Carta *et al.* 2016) *L. heidelbergi* was transferred to the genus *Aphelenchoides*, based on the divergent position of this species from other *Laimaphelenchus* species. This change, already suggested by Ryss *et al.* (2013), was actually done by Hirling (1986), but this was not followed by other authors. Based on our morphological analysis this transfer can be supported, as the mucro shape of *Aphelenchoides heidelbergi* is similar to related taxa in clade II-5 (Fig. IV.7). Furthermore, scanning electron microscopy reveals that the tail terminus of several species in this clade have microprotuberances like those documented in the description of *L. heidelbergi* (Zhao et al. 2007), e.g. *A.* sp 13 and *A*. xui (Fig. IV.7 [25], Wang *et al.* 2013).

Also *L. pannocaudus*, which is missing a vulval flap and tetra-pedunculated tail-terminus, resides in a clade with no relation to *Laimaphelenchus* species (II-5, Fig. IV.1-4). These two features are not always present in the genus, but to our knowledge, no *Laimaphelenchus* species lacks both of them. For example, *L. helicosoma*, *L. preissii* and *L. unituberculus* have a vulval flap but bear only one tubercle, while *L. australis*, *L. patulus*, *L. phloesi* and *L pini* have

three or four tubercules but lack the vulval flap (Zhao *et al.* 2007). Hirling (1986) proposed to transfer all *Laimaphelenchus* spp. without a vulval flap to *Aphelenchoides*, this change was acknowledged by Ebsary (1991) but not by Hunt (2008) nor later works on *Laimaphelenchus* e.g. Maleita *et al.* 2015; Miraeiz *et al.* 2015; Oro 2015 and Zhao *et al.* 2006; 2007. Given the combination of its phylogenetic position and *Aphelenchoides*-like morphology, the transfer of *L. pannocaudus* to *Aphelenchoides* is accepted.

With the placement of *A. heidelbergi* and *A. pannocaudus* in *Aphelenchoides*, the monophyly of *Laimaphelenchus* is supported in the 28S and concatenated trees, although not in 18S topologies (Figs IV.1-6). Besides, according to COI analyses (Chapter III), only *L. belgradiensis* (KF881747) remains outside *Laimaphelenchus* (Fig. III.1) but this is possibly due to a misidentification. Nonetheless, *Aphelenchoides* and *Laimaphelenchus* lack robust morphological characters and several traits overlap between them (Davies *et al.* 2015; Nickle 1970). Hence, further research should deepen in these taxonomic delimitations through a combination of morphological and molecular data.

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Table IV.1. Morphometrics (in µm) of *Aphelenchoides* species and *Robustodorus megadorus per* phylogenetic clade in Fig IV.7. (Mean ± Standard deviation, Coefficient of variation)

Feature or ratio	I (5♀, 3♂)	II (33♀, 2♂)			
		II-2 (2♀)	II-3 (10♀, 1♂)	II-5 (18♀)	II-6 (3♀,1♂)
L	574.8 ± 224.5, 39.0	700 ± 282.8, 40.4	603.7 ± 140.3, 23.3	573.6 ± 152.5, 26.6	325.1 ± 73.9, 22.7
L’	537.6 ± 216.8, 40.3	679.4 ± 276.2, 40.6	549.1 ± 131.5, 24.0	533.1 ± 149.5, 28.1	298.2 ± 73.8, 24.7
L°	55.0 ± 17.8, 32.4	80.4 ± 27.4, 34.1	59.8 ± 14.2, 23.8	58.3 ± 18.7, 32.1	45.8 ± 7.2, 15.7
a°	38.1 ± 14.0, 40.3	67.8 ± 46.3, 68.3	45.1 ± 12.4, 27.5	38.4 ± 7.4, 19.3	34.2 ± 8.0, 23.3
b°	10.0 ± 3.0, 30.1	16.5 ± 10.3, 62.3	10.4 ± 1.6, 15.7	10.4 ± 2.3, 22.2	9.0 ± 2.2, 24.3
b’	5.0 ± 1.0, 20.7	7.3 ± 5.3, 72.6	5.4 ± 1.8, 33.8	4.7 ± 0.8, 17.5	4.2 ± 1.1, 25.8
c	15.2 ± 3.6, 23.8	33.4 ± 2.9, 8.6	15.3 ± 1.6, 10.7	14.3 ± 3.6, 25.3	12.1 ± 2.8, 22.8
c’	55.0 ± 17.8, 32.4	80.4 ± 27.4, 34.1	59.8 ± 14.2, 23.8	58.3 ± 18.7, 32.1	45.8 ± 7.2, 15.7
V	68.0 ± 7.7, 11.3	73.3 ± 0.1, 0.1	69.4 ± 1.5, 2.1	67.4 ± 2.6, 3.9	69.1 ± 1.4, 2.0
V’	78.3 ± 7.5, 9.6	78.7 ± 0.4, 0.5	81.3 ± 2.0, 2.4	81.0 ± 3.4, 4.2	84.7 ± 3.4, 4.1
V°	37.6 ± 8.1, 21.6	30.5 ± 2.1, 7.0	37.4 ± 9.1, 24.2	33.4 ± 7.1, 21.3	34.1 ± 8.1, 23.8
Sty	11.5 ± 2.2, 19.1	15.0 ± 5.6, 37.7	10.8 ± 2.2, 19.9	10.8 ± 1.5, 13.8	7.7 ± 0.7, 8.9
KnW	1.9 ± 0.3, 17.1	2.9 ± 0.7, 25.3	1.8 ± 0.3, 17.5	1.8 ± 0.3, 18.8	1.3 ± 0.2, 16.6
KnH	1.7 ± 0.6, 33.8	1.9 ± 0.8, 41.0	1.6 ± 0.5, 29.8	1.6 ± 0.3, 21.7	0.9 ± 0.1, 9.2
KnR	1.3 ± 0.4, 33.6	1.6 ± 0.3, 16.5	1.3 ± 0.4, 29.3	1.2 ± 0.5, 38.3	1.4 ± 0.3, 21.3
LpWB	6.0 ± 1.4, 22.7	7.1 ± 0.1, 0.8	5.6 ± 0.6, 11.5	5.7 ± 0.7, 12.8	3.9 ± 0.1, 2.7
LpH	2.7 ± 0.8, 29.2	3.2 ± 0.9, 27.6	2.8 ± 0.5, 16.0	2.5 ± 0.5, 20.3	1.7 ± 0.3, 17.6
LpMW	6.4 ± 1.4, 21.5	8.0 ± 0.7, 8.3	5.7 ± 0.7, 11.5	5.9 ± 0.8, 13.9	3.7 ± 0.4, 11.8
LPR	0.5 ± 0.1, 22.4	0.5 ± 0.1, 26.8	0.5 ± 0.1, 12.2	0.4 ± 0.1, 20.3	0.4 ± 0.1, 18.0
VaL	3.3 ± 1.1, 33.3	2.6 ± 0.4, 14.0	3.9 ± 0.9, 22.4	3.6 ± 0.8, 21.8	2.1 ± 0.1, 6.0
VaW	2.5 ± 0.6, 22.2	2.1 ± 0.9, 41.4	2.6 ± 0.5, 17.9	3.0 ± 0.6, 19.0	1.5 ± 0.3, 17.1
VaR	1.3 ± 0.2, 17.9	1.3 ± 0.4, 28.1	1.5 ± 0.4, 29.3	1.2 ± 0.2, 19.3	1.4 ± 0.3, 18.4
LVaAMB	7.4 ± 1.5, 19.6	6.6 ± 1.4, 20.9	7.9 ± 0.6, 7.5	7.5 ± 1.2, 16.0	4.8 ± 0.8, 17.2
LVaPMB	6.5 ± 2.5, 38.1	5.3 ± 0.9, 17.4	6.3 ± 1.2, 19.0	6.3 ± 1.2, 18.6	3.9 ± 0.3, 7.9
VaP	1.2 ± 0.3, 23.0	1.2 ± 0.0, 3.5	1.3 ± 0.2, 17.2	1.2 ± 0.1, 12.3	1.3 ± 0.3, 23.4
MBL	14.6 ± 3.2, 21.7	11.8 ± 2.6, 22.0	14.4 ± 1.1, 7.8	13.8 ± 2.1, 15.1	9.5 ± 0.5, 5.4
MBW	10.1 ± 3.2, 31.4	9.2 ± 2.9, 31.6	10.1 ± 1.2, 12.0	10.9 ± 1.7, 15.7	6.9 ± 0.7, 9.6
MBR	1.5 ± 0.2, 15.6	1.3 ± 0.1, 10.0	1.4 ± 0.2, 16.2	1.3 ± 0.1, 9.2	1.4 ± 0.2, 13.0
LMBA	56.6 ± 12.1, 21.4	46.0 ± 11.5, 25.0	58.0 ± 7.6, 13.1	54.7 ± 6.4, 11.7	36.1 ± 1.5, 4.1
BWMB	15.3 ± 4.2, 27.4	11.6 ± 3.8, 32.4	13.7 ± 2.4, 17.7	14.9 ± 2.7, 18.2	9.5 ± 0.2, 1.6
BWMB/LpWB	2.6 ± 0.5, 19.6	1.6 ± 0.5, 31.6	2.4 ± 0.3, 11.0	2.6 ± 0.2, 9.2	2.5 ± 0.1, 3.8
Isthmus length	6.5 ± 3.3, 50.4	8.2 ± 4.9, 59.8	4.2 ± 5.3, 126.5	3.8 ± 3.8, 97.9	2.4 ± 3.9, 164.1
PGA	135.6 ± 37.6, 27.8	110.9 ± 41.8, 37.7	131.8 ± 34.9, 26.5	120.7 ± 26.1, 21.6	81.2 ± 16.8, 20.7
EPA	79.7 ± 23.1, 50.4	60.9 ± 2.1, 3.5	76.4 ± 13.3, 17.4	69.0 ± 10.8, 15.6	25.7 ± 4.0, 15.6
EPMB	23.0 ± 13.9, 60.2	14.9 ± 9.3, 62.9	20.5 ± 9.3, 45.3	14.1 ± 6.2, 43.6	11.3 ± 1.1, 9.3
EP°	14.7 ± 3.1, 21.1	9.5 ± 4.2, 43.6	12.9 ± 2.1, 16.4	12.7 ± 2.6, 20.8	7.9 ± 1.8, 23.2
LMBA/EPA	72.9 ± 9, 12.4	75.3 ± 16.2, 21.6	74.3 ± 9.1, 12.2	80.1 ± 6.5, 8.1	145.8 ± 8.3, 5.7

Table IV.1. *Continued*

BWV	18.9 ± 5.3, 27.8	23.4 ± 10.9, 46.8	16.5 ± 3.4, 20.6	17.5 ± 4.2, 24.1	10.0 ± 0.2, 1.5
VuAn	176.3 ± 54.9, 31.1	165.9 ± 68.1, 41.0	146.7 ± 39.4, 26.5	148.3 ± 42.0, 28.3	77.1 ± 21.4, 27.7
ABW	10.3 ± 1.9, 18.9	8.6 ± 0.6, 6.7	10.0 ± 2.1, 21.0	10.1 ± 2.1, 20.3	7.1 ± 0.8, 11.1
PUS	53.8 ± 45.6, 84.8	78.2 ± 66.2, 64.6	74.4 ± 35.1, 47.0	60.2 ± 22.8, 37.9	30.5 ± 4.1, 13.4
PUS/VuAn, as %	27.2 ± 13.7, 50.4	42.5 ± 22.4, 52.7	44.0 ± 12.3, 27.9	39.5 ± 12.3, 31.1	41.9 ± 14.6, 34.9
Tail length (TL)	37.2 ± 9.6, 25.9	20.7 ± 6.7, 32.4	38.1 ± 5.5, 14.5	40.5 ± 7.5, 18.6	26.9 ± 2.3, 8.5
Length of mucro	2.2 ± 0.5, 22.0	0.7 ± 1.0, 141.4	2.3 ± 0.5, 20.8	2.5 ± 0.8, 30.9	1.2 ± 0.8, 68.4
Tail terminus type	3,4	0,1	1,2	1-4	1,2
No. of lateral lines	3,4	3,4	4,5	3-5	3

L: total body length | **L':** L - TL | **L°:** L/ABW | **a°:** L/body width at middle of MB | **b°:** L/distance from anterior end to middle of the MB | **b':** L/distance from anterior end to posterior end of pharyngeal glands | **c:** L/tail length | **c':** tail length/anal-cloacal aperture width | **V:** position of vulva as % of L | **V':** position of vulva as % of distance from anterior end to anus | **V°:** L/body width at vulva | **Sty:** stylet length | **KnW:** Knobs width | **KnH:** Knobs height | **KnR:** Knobs ratio (KnW/KnH) | **LpWB:** Lips width at base | **LpH:** Lips height | **LpMW:** Lips maximum width | **LpR:** Lips ratio (LpMW/LpH) | **VaL:** MB valves length | **VaW:** MB valves width | **VaR:** MB valves ratio (VaL/VaW) | **MBL:** Median bulb length | **MBW:** Median bulb width | **MBR:** Median bulb ratio (MBL/MBW) | **LVaAMB:** Length from the middle of the valves to the anterior end of the MB | **LVaPMB:** Length from the middle of the valves to the posterior end of the MB | **VaP:** Valves position in the MB (LVaAMB/LVaPMB) | **LMBA:** length from the middle of the MB to the anterior end | **BWMB:** Body width at MB | **BWMB**/LpWB | P**GA:** Length of the tip of the phrayngeal gland lobe to the anterior end | **EPA:** distance of the secretory-excretory pore to the anterior end | **EPMB:** distance of the secretory-excretory pore to the middle of the MB | **EP°:** position of EP as % of L | **LMBA/EP**A | **BWV:** Body width at vulva | **VuAn:** Length from Vulva to anus | **ABW:** Anal body width | **PUS:** Post uterine sac length | **Tail terminus:** 0. no mucro; 1. single; needle/finger-like mucro; 2. single mucro with micro protuberances, 3. mucro with two proyections; 4. star-like mucro

Table IV.2. Tukey HSD Test results after one-way Analysis of Variance (ANOVA) for several morphological features of *Aphelenchoides* species and *Robustodorus megadorus per* phylogenetic clade in Fig. IV.7

Feature (ratio)	N	F	P	I vs II-6	I vs II-5	I vs II-3	I vs II-2	II-6 vs II-5	II-6 vs II-3	II-6 vs II-2	II-5 vs II-3	II-5 vs II-2	II-3 vs II-2
L	43	3.43	0.017275							b			
L’	42	3.56	0.014867							b			
L°	42	2.65	0.048445							a			
a°	43	5.49	0.001372				b			b		b	
b°	43	5.53	0.001310				b			b		a	a
b’	31	2.43	0.073050				b			b		a	a
c	42	36.56	<.0001				b			b		b	b
c’	42	2.65	0.048445							a			
V	37	1.89	0.136324							a			
V’	37	1.83	0.147339									a	
V°	38	0.63	0.644552							a			
Sty	43	8.37	<.0001	a						b		a	a
KnW	43	14.82	<.0001	a			b			b		b	b
KnH	43	3.65	0.013022							a			
KnR	43	0.59	0.671900							a			
LpWB	43	9.69	<.0001	b				a	a	b			a
LpH	43	4.91	0.002727	a					a	b			
LpMW	43	15.02	<.0001	b				b	b	b		b	b
LpR	43	0.45	0.771718	b				b	b	b		b	b
VaL	43	3.71	0.012062						a				
VaW	43	5.62	0.001180	a				b	a				
VaR	43	0.83	0.514497							a			
LVaAMB	43	6.03	0.000737	b				b	b				
LVaPMB	43	2.66	0.047353										
VaP	43	0.12	0.974527										
MBL	43	5.27	0.001776	b				a	b				
MBW	43	3.02	0.029454					a					
MBR	43	1.18	0.335082					a					
LMBA	43	6.45	0.000460	b				b	b				
BWMB	43	3.41	0.017727	a				a					
BWMB/LpWB	43	8.07	<.0001				b			b		b	b
Isthmus length	43	1.58	0.199432	b				b	b				
PGA	31	2.03	0.119581	b				b	b	b			
EPA	35	10.24	<.0001	b				b	b	b			
EPMB	39	9.61	<.0001	b				b	b	b			
Ep°	35	5.02	0.003220	b			a						

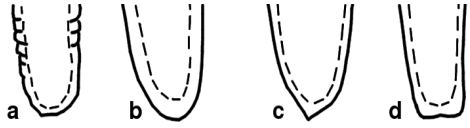
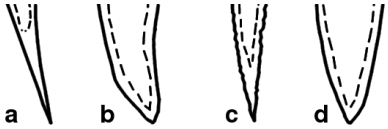
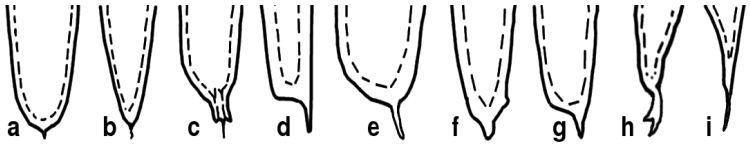
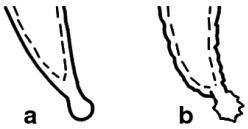
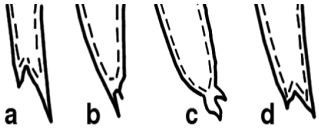
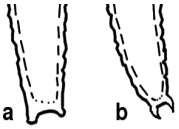
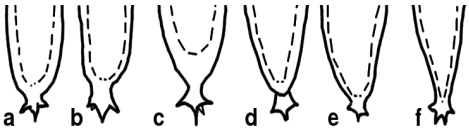
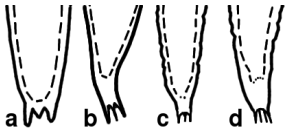
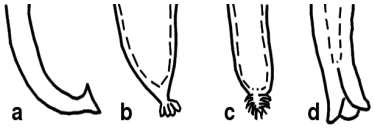
Table IV.2. *Continued*

LMBA/EPA	35	65.2	<.0001	b				b	b	b			
BWV	38	5.08	0.002657							b			
VuAn	42	3.48	0.016466	a						a			
ABW	42	2.41	0.066540				b	a				b	b
PUS	32	1.39	0.263864				b			b		b	b
PUS/VuAn, as %	32	1.08	0.385920				b			b		b	b
Tail length (TL)	42	6.67	0.000380				b	a				b	b
Length of mucro	42	6.14	0.000681				a	a				b	b
Total of statistically-significant features (ratios)				18	0	0	12	18	14	28	0	15	14

a: P<.05, b: P<.01

L: total body length | **L':** L - TL | **L°:** L/ABW | **a°:** L/body width at middle of MB | **b°:** L/distance from anterior end to middle of the MB | **b':** L/distance from anterior end to posterior end of pharyngeal glands | **c:** L/tail length | **c':** tail length/anal-cloacal aperture width | **V:** position of vulva as % of L | **V':** position of vulva as % of distance from anterior end to anus | **V°:** L/body width at vulva | **Sty:** stylet length | **KnW:** Knobs width | **KnH:** Knobs height | **KnR:** Knobs ratio (KnW/KnH) | **LpWB:** Lips width at base | **LpH:** Lips height | **LpMW:** Lips maximum width | **LpR:** Lips ratio (LpMW/LpH) | **VaL:** MB valves length | **VaW:** MB valves width | **VaR:** MB valves ratio (VaL/VaW) | **MBL:** Median bulb length | **MBW:** Median bulb width | **MBR:** Median bulb ratio (MBL/MBW) | **LVaAMB:** Length from the middle of the valves to the anterior end of the MB | **LVaPMB:** Length from the middle of the valves to the posterior end of the MB | **VaP:** Valves position in the MB (LVaAMB/LVaPMB) | **LMBA:** length from the middle of the MB to the anterior end | **BWMB:** Body width at MB | **BWMB**/LpWB | P**GA:** Length of the tip of the phrayngeal gland lobe to the anterior end | **EPA:** distance of the secretory-excretory pore to the anterior end | **EPMB:** distance of the secretory-excretory pore to the middle of the MB | **EP°:** position of EP as % of L | **LMBA/EP**A | **BWV:** Body width at vulva | **VuAn:** Length from Vulva to anus | **ABW:** Anal body width | **PUS:** Post uterine sac length

Table IV.3. *Aphelenchoides* groups according to female tail shape and terminus *sensu* Shahina (1996) (left) and proposed subgroups based on tail and mucro shapes (right); modifications in bold

<table>
<tr><td>Sensu Shahina (1996)</td><td colspan="2">This document</td></tr>
<tr><td>1. Tail simple without any outgrowth or mucronate structure</td><td>1.1 Round/truncated end
 
e.g. (a) limberi; (b) obtusus; (c) rotundicaudatus; (d) rutgersi</td><td>1.2 Bluntly pointed/acute end
 
e.g. (a) microstylus; (b) pytiokteini; (c) taraii; (d) tenuidens</td></tr>
<tr><td rowspan="5">2. Tail with one or sometimes two mucronate structures on tail terminus</td><td colspan="2">2.1 One mucronate structure</td></tr>
<tr><td colspan="2">2.1.1 Pointed mucro, from short to hair-like, can bear two processes
 
e.g. (a) arachidis; (b) blastophthorus; (c) centralis; (d) cibolensis; (e) clarus; (f) hamatus; (g) sacchari; (h) paradalianensis; (i) parasaprophilus</td></tr>
<tr><td>2.1.2 Mucro bearing small protuberances or papillae, leaf-like shape
 
e.g. (a) appendurus; (b) fuchsi; (c) haguei; (d) huntensis; (e) vaughani; (f) xui</td><td>2.1.3 Clavate or semi-spherical mucro
 
e.g. (a) gynotylurus; (b) seiachicus</td></tr>
<tr><td colspan="2">2.2 Two mucronate structures</td></tr>
<tr><td>2.2.1 Structures not equally sized
 
e.g. (a) bicaudatus; (b) bimucronatus; (c) silvester; (d) varicaudatus</td><td>2.2.2 Structures equally sized
 
e.g. (a, b) kungradiensis</td></tr>
<tr><td>3. Tail with (tri or) tetramucronate spine or star shape</td><td>3.1 Protrusions oriented to multiple directions (star-shape)
 
e.g. (a) aligarhiensis; (b) besseyi; (c) goodeyi; (d) hylurgi; (e) jonesi; (f) nonveilli</td><td>3.2 Protrusions oriented to the same direction (crown-shape)
 
e.g. (a) hylurgi; (b) pannocaudus; (c, d) ritzemabosi</td></tr>
<tr><td>4. Tail outgrowth other than spine or star, exceptional mucro shapes</td><td colspan="2"> 
e.g. (a) disccaudatus; (b) iranicus; (c) parabrushmucronatus; (d) sphaerocephalus</td></tr>
</table>

Note: schematic illustrations redrawn from original descriptions or redescriptions at 100x magnification

Table IV.4. List of *Aphelenchoides* species *per* group of tail and tail-terminus shapes according to the arrangement proposed in Table IV.3

1	**1.1:** *A. capsuloplanus*; *A. confusus*; *A. helicus*; *A. jacobi*; *A. limberi*; *A. obtusicaudatus*; *A. obtusus*; *A. rosei*; *A. rotundicaudatus*; *A. sparsus* (?); *A. tuzeti*	
	1.2: *A. brevicaudatus* (?); *A. helicus*; *A. involutus*; *A. longiurus* (?); *A. marinus* (?); *A. microstylus*; *A. pityokteini*; *A. polygraphi*; *A. subparietinus*; *A. taraii*; *A. zeravschanicus* (?)	
2	**2.1*:** *A. agarici*; *A. coffeae*; *A. conimucronatus*; *A. cyrtus*; *A. dubitus*; *A. helicosoma*; *A. myceliophagus*; *A. naticochensis*; *A.neocomposticola*; *A. parabicaudatus*; *A. parasexlineatus*; *A. swarupi* *A. varicaudatus*	**2.1.1:** *A. absari*; *A. abyssinicus*; *A. aerialis*; *A. allius*; *A. angusticaudatus*; *A. arachidis*; *A. arcticus*; *A. blastophthorus*; *A. brassicae*; *A. brevionchus*; *A. centralis*; *A. chalonus*; *A. chamelocephalus*; *A. chinensis*; *A. cibolensis*; *A. clarolineatus*; *A. clarus*; *A. composticola*; *A. conophthori*; *A. curiolis*; *A. dactylocercus*; *A. dalianensis; A. daubichaensis*; *A. delhiensis*; *A. dhanachandi*; *A. echinocaudatus*; *A. editocaputis*; *A. eltayebi*; *A. emiliae*; *A. ensete*; *A. eradicitus*; *A. ferrandini*; *A. fragariae*; *A. franklinae*; *A. goeldii*; *A. graminis*; *A. hamatus*; *A. helophilus*; *A. hypotris*; *A. indicus*; *A. jodhpurensis*; *A. lagenoferrus*; *A. lanceolatus*; *A. lilium*; *A. longistylus*; *A. loofi*; *A. lucknowensis*; *A. macromucrons*; *A. macronucleatus*; *A. meghalayensis*; *A. minimus*; *A. montanus*; *A. nechaleos*; *A. neoechinocaudatus*; *A. neominoris*; *A. orientalis*; *A. panaxi*; *A. paradalianensis; A. paranechaleos*; *A. parasaprophilus*; *A. parascalacaudatus*; *A. parasubtenuis*; *A. parietinus*; *A. petersi*; *A. platycephalus*; *A. pusillus*; *A. rarus*; *A. resinosi*; *A. rhytium*; *A. richardsoni*; *A. rutgersi*; *A. sacchari*; *A. sanwali*; *A. saprophilus*; *A. scalacaudatus*; *A. sexlineatus*; *A. shamimi*; *A. singhi*; *A. sinodendroni*; *A. spasskii*; *A. spicomucronatus*; *A. spinohamautus*; *A. spinosus*; *A. submersus*; *A. subtenuis*; *A. suipingensis*; *A. tagetae*; *A. trivialis*; *A. tsalolikhini*; *A. tumulicaudatus*; *A. varicaudatus*; *A. vigor*; *A. xylocopae*
		2.1.2: *A. appendurus*; *A. fuchsi*; *A. haguei*; *A. heidelbergi*; *A. huntensis*; *A. vaughani*; *A. xui*
		2.1.3: *A. gynotylurus*; *A. seiachicus*
	2.2*: *A. varicaudatus*	**2.2.1:** *A. bicaudatus*; *A. bimucronatus*; *A. hainanensis*; *A. silvester*
		2.2.2: *A. kungradiensis*
3	** A. menthae*	**3.1:** *A. aligarhiensis*; *A. andrassyi*; *A. asterocaudatus*; *A. asteromucronatus*; *A. besseyi*; *A. brevistylus*; *A. coffeae*; *A. fujianensis*; *A. goldeni*; *A. goodeyi*; *A. hylurgi*; *A. jonesi*; *A. lichenicola*; *A. nonveilleri*; *A. panaxofolia*; *A. siddiqii*; *A. silvester; A. stellatus*; *A. unisexus*; *A. wallacei*
		3.2: *A. hylurgi*; *A. pannocaudus; A. ritzemabosi*
4	*A. brushimucronatus*; *A. disccaudatus; A. iranicus; A. parabrushmucronatus*; *A. sphaerocephalus*	
(*)	*A. africanus*; *A. bengalensis*; *A. breviuteralis*; *A. chauhani*; *A. depressospicularis*; *A. elongatus*; *A. exilis*; *A. fluviatilis*; *A. graminophilus*; *A. hessei*; *A. kuehnii*; *A. longiuteralis*; *A. microspermi*; *A. minoris*; *A. paramonovi*; *A. pinusi, A. pyri*; *A. retusus*; *A. richtersi*; *A. robustus*; *A. sapinus*; *A. sinensis*; *A. sylvaticus*; *A. tenarius*; *A. ternarius*; *A. villosus*	

* these species were not allocated in any group or subgroup due to lack of accurate translation/illustration, or descriptions unavailable/incomplete. Some were confusing or contradictory among literature, and more information should be provided before classification. '(?)' species are likely to be in the reported group according to the original description, but confirmation is needed.

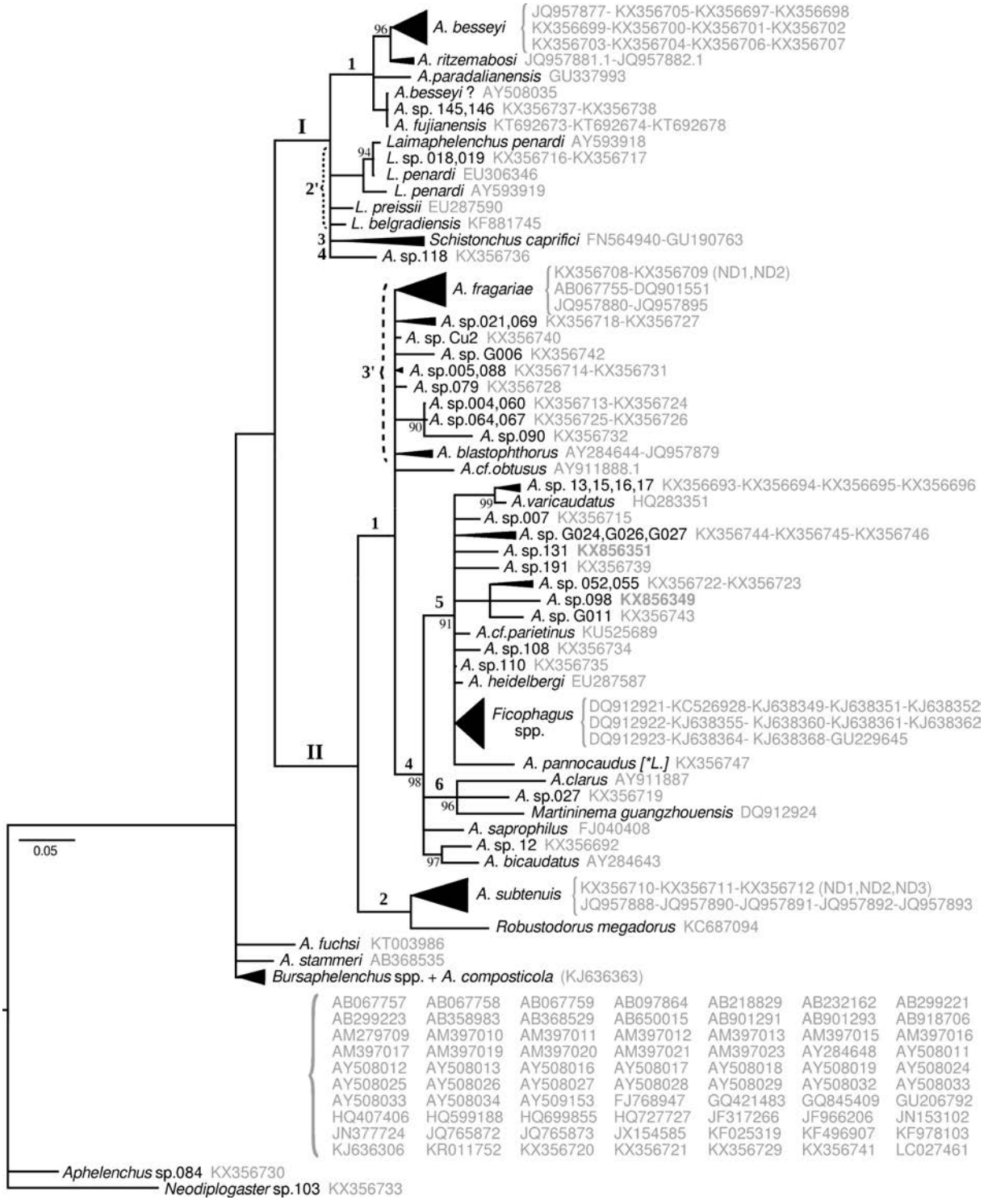


Figure IV.1. Bayesian inference consensus tree of *Aphelenchoides* spp. and related taxa inferred from partial 18S sequences, bold if newly generated. The dataset ("non-inclusive") excluded entries from public databases that were not identified to species level. Only relationships with supporting values ⩾90% are plotted; supporting values = 100% are not depicted.

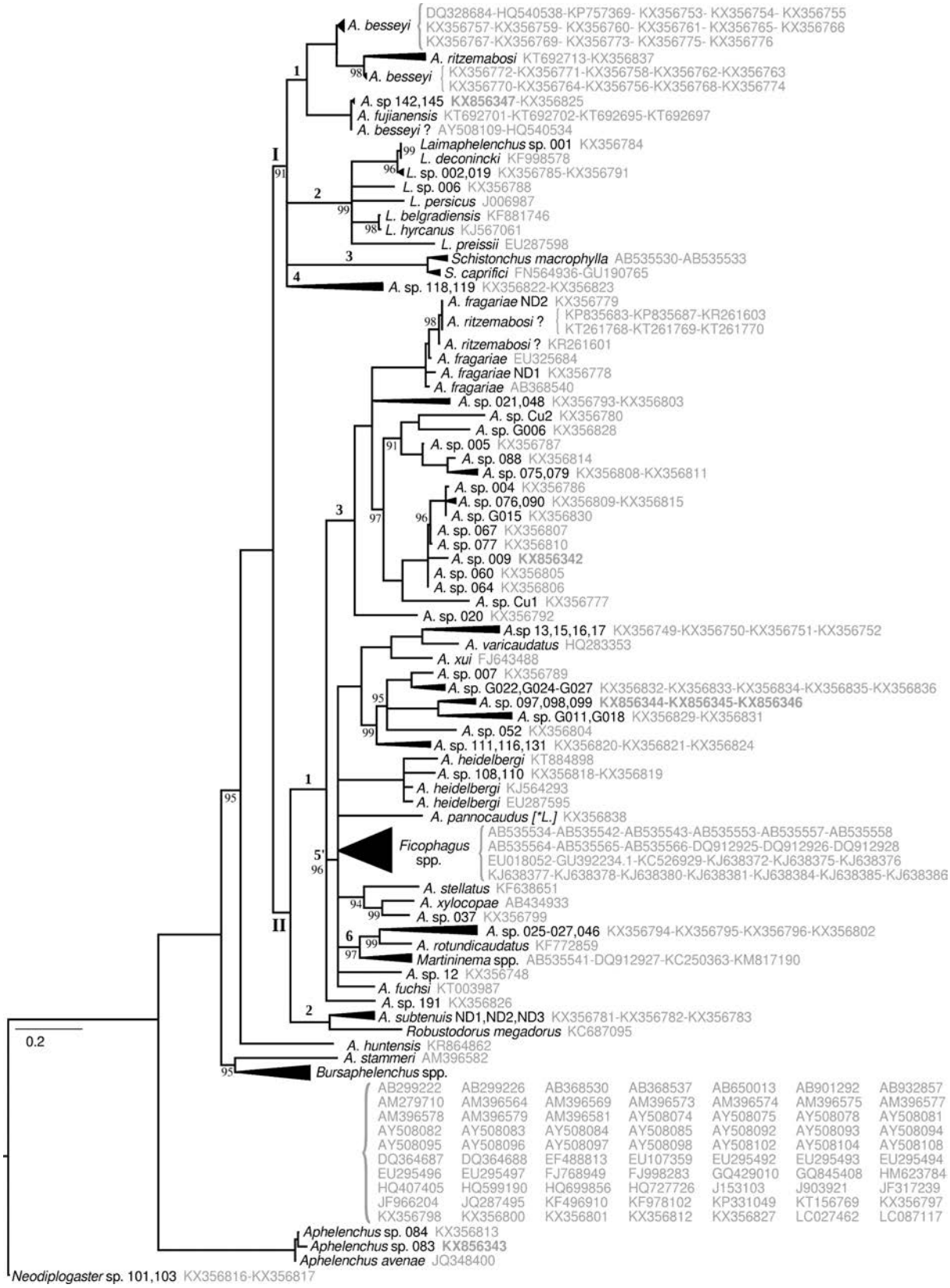


Figure IV.2. Bayesian inference consensus tree of *Aphelenchoides* spp. and related taxa inferred from partial 28S (D2D3 expansion region) sequences, bold if newly generated. The dataset ("non-inclusive") excluded entries from public databases that were not identified to species level. Only relationships with supporting values ⩾90% are plotted; supporting values = 100% are not depicted.

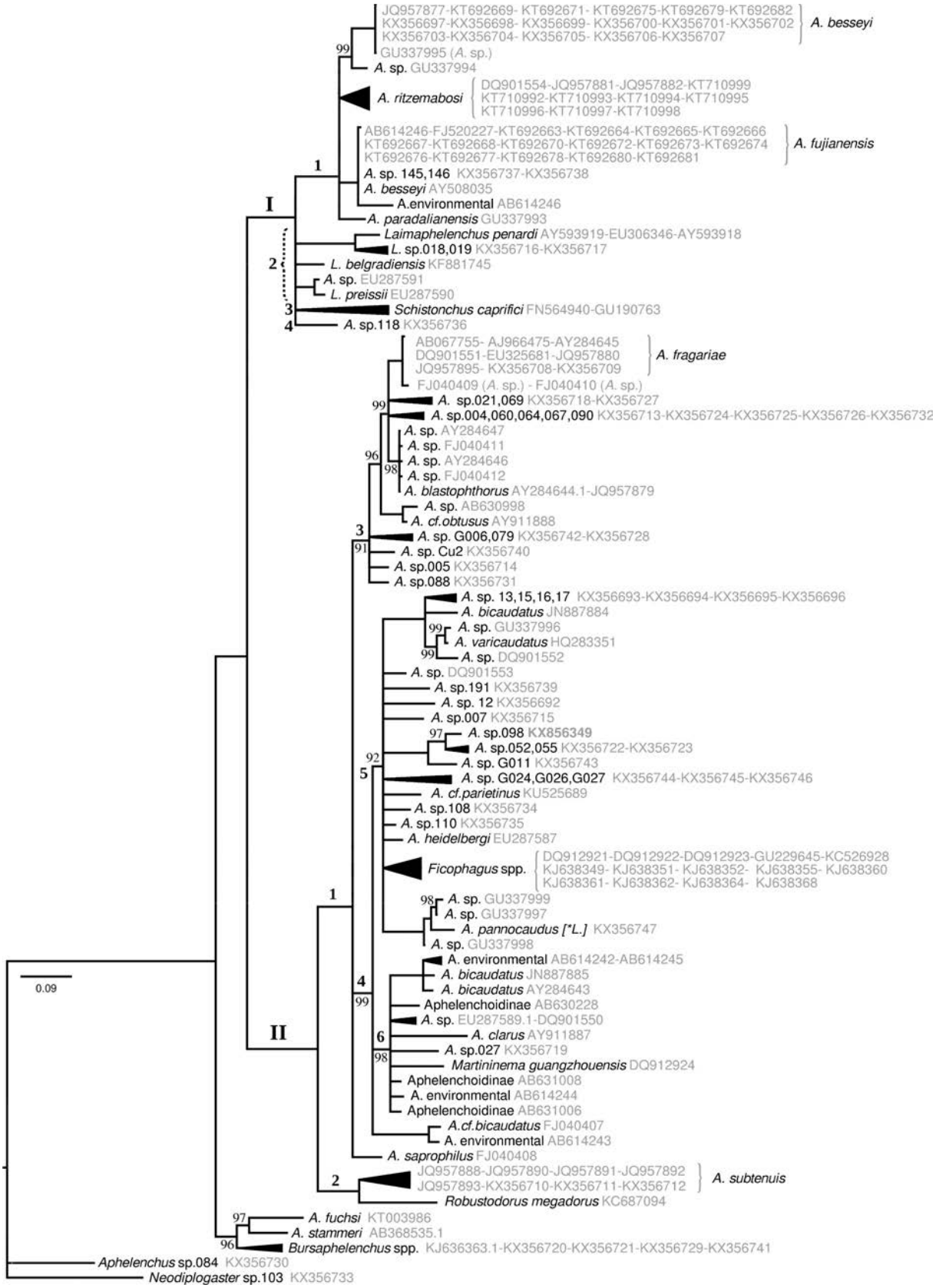


Figure IV.3. Bayesian inference consensus tree of *Aphelenchoides* spp. and related taxa inferred from partial 18S sequences, bold if newly generated. Most *Aphelenchoides* entries from databases were included in the analysis ("inclusive" dataset); only relationships with supporting values ⩾90% are plotted; supporting values = 100% are not depicted.

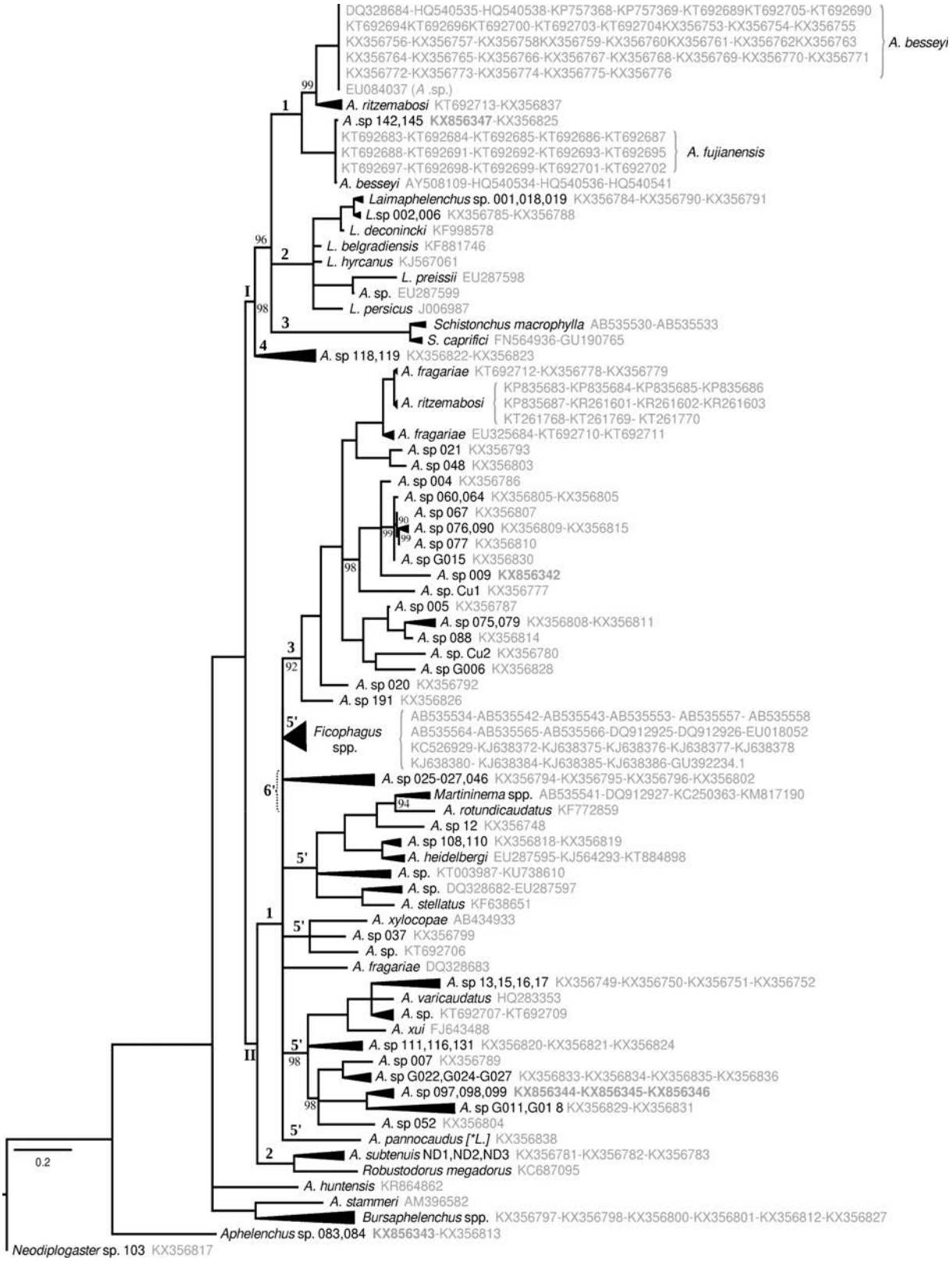


Figure IV.4. Bayesian inference consensus tree of *Aphelenchoides* spp. and related taxa inferred from partial 28S (D2D3 expansion region) sequences, bold if newly generated. Most *Aphelenchoides* entries from databases were included in the analysis ("inclusive" dataset); only relationships with supporting values ⩾90% are plotted; supporting values = 100% are not depicted.

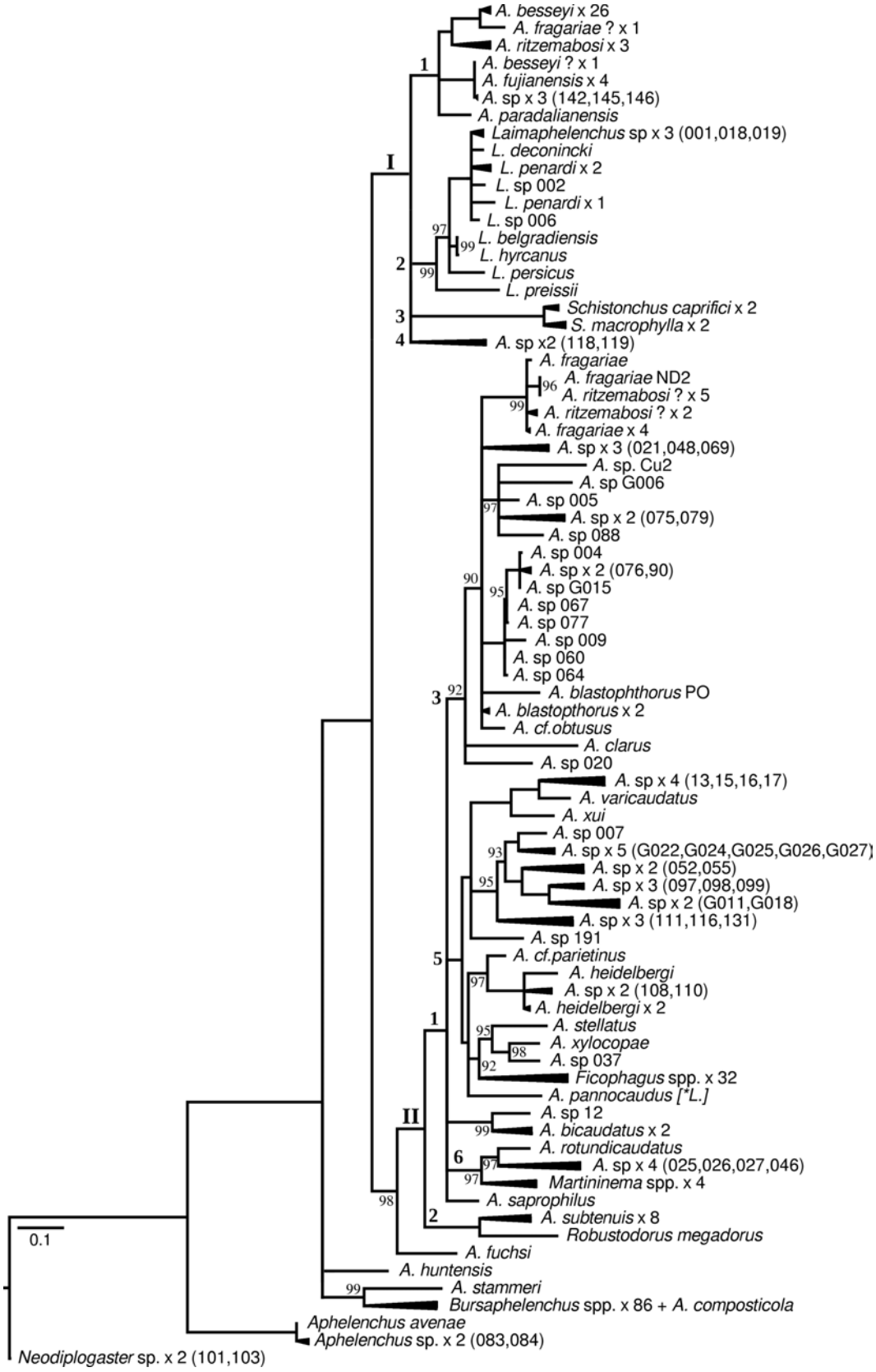


Figure IV.5. Bayesian inference consensus tree of *Aphelenchoides* spp. and related taxa inferred from a concatenated file of partial 18S and D2D3 region of 28S rDNA markers. Only relationships with supporting values ⩾90% are plotted; supporting values = 100% are not depicted

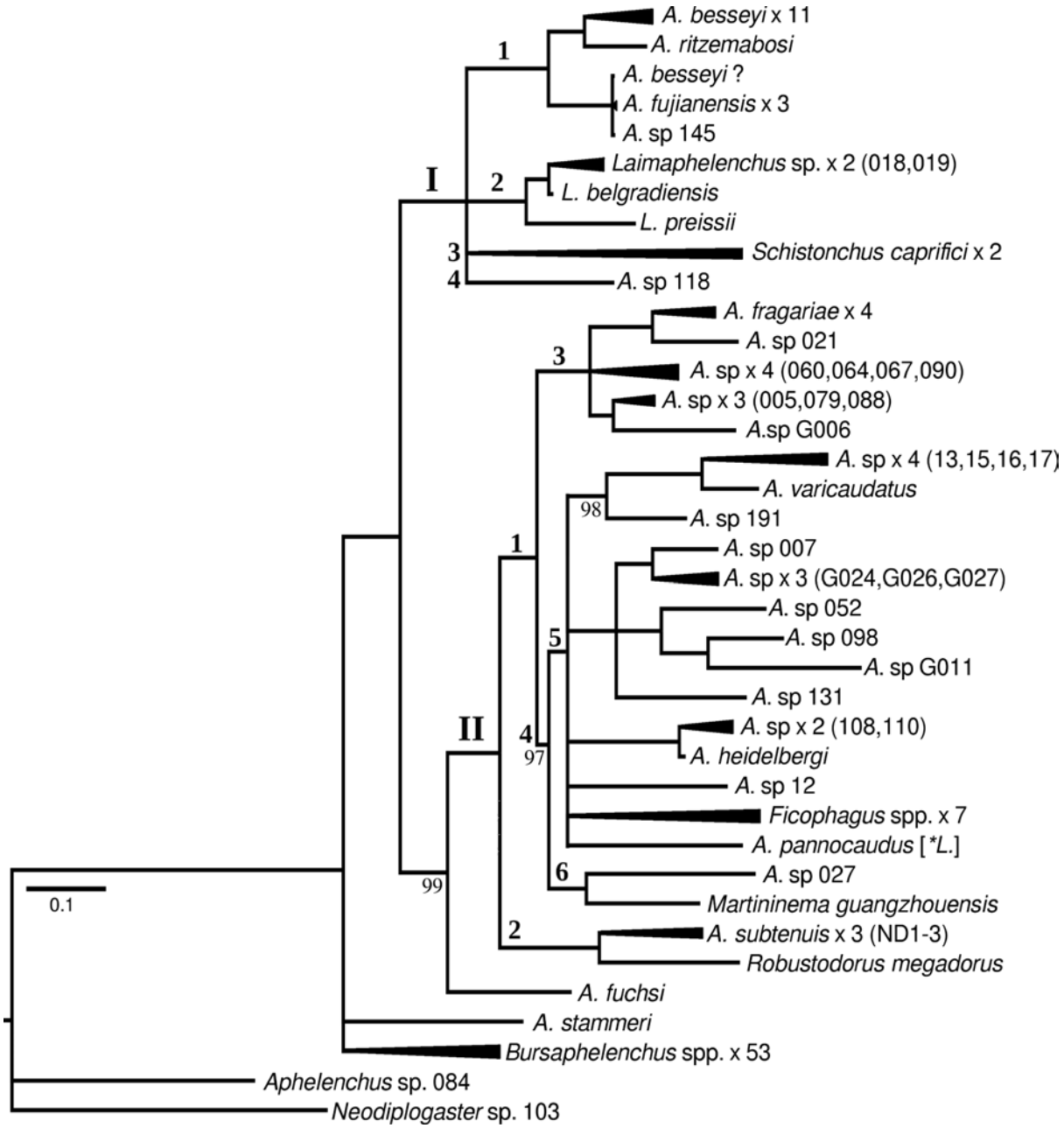


Figure IV.6. Bayesian inference consensus tree of *Aphelenchoides* spp. and related taxa inferred from a concatenated file of partial 18S and D2D3 region of 28S rDNA markers, only entries with data for both genes were included. Relationships with supporting values ⩾90% are plotted; supporting values = 100% are not depicted

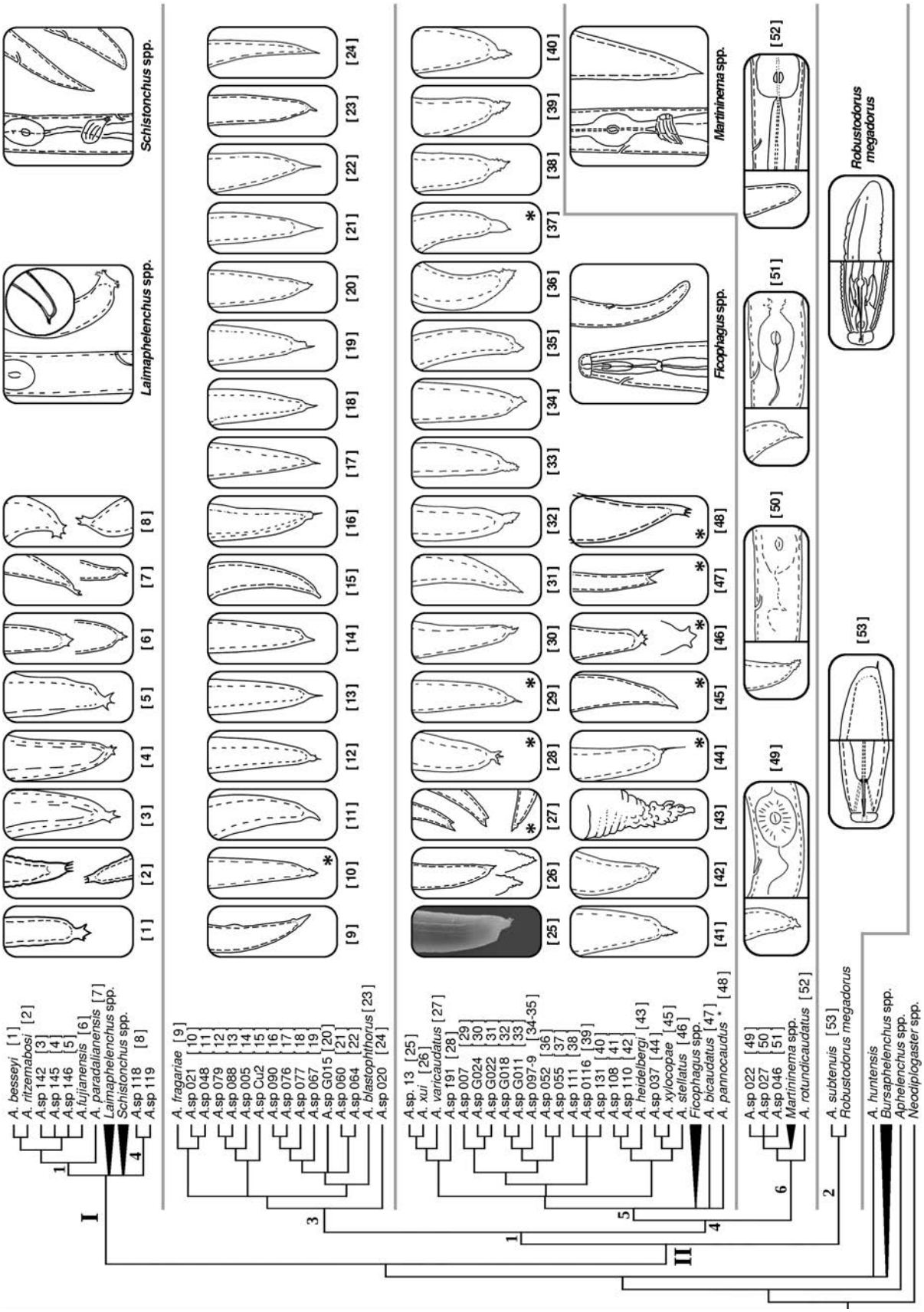


Figure IV.7. Tail terminus shapes of *Aphelenchoides* spp. and related taxa per phylogenetic clade. Schematic tree manually reconstructed based on two consensus topologies (Fig. IV.5 and Fig. IV.6). Known species and genera were redrawn from original descriptions or redescriptions

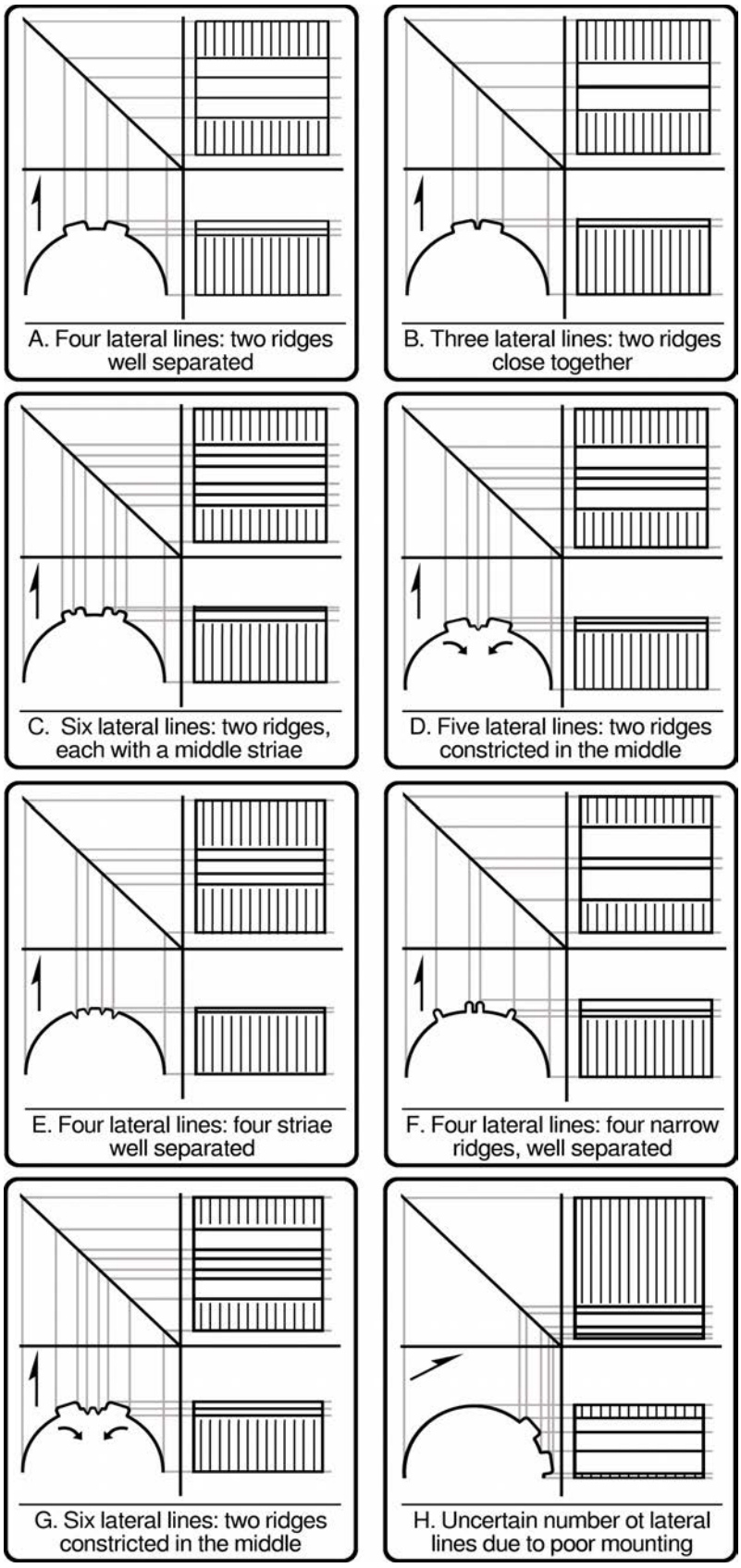


Figure IV.8. Isometric projections of assorted nematode lateral sides and corresponding possible interpretations of the lateral field's morphology under light microscopy

CHAPTER V
General discussion

The main aim of this thesis was to investigate and update several aspects of the genus *Aphelenchoides,* a relatively unexplored group within Aphelenchoidea. This research showed that plant-parasitic *Aphelenchoides* species are associated with a broader range and a higher number of plants than previously informed, although their parasitic relationships needs further confirmation. We also demonstrated the potential of the Cytochrome Oxidase I region (mtCOI) for molecular diagnosis of this genus. The phylogenetic analyses obtained during this study largely agree with recent literature on two main *Aphelenchoides* clades, together with four other genera, namely *Ficophagus*, *Laimaphelenchus*, *Martininema* and *Schistonchus* (see Chapter III and IV). Additionally, the correlation of several morphological features with the obtained phylogenetic clades was explored, and based on this we proposed a new grouping system focused on tail and tail terminus morphology. In this last chapter, the main findings are briefly discussed, future research prospects are mentioned and additional observations and comments on *Aphelenchoides* diversity are provided. Finally, the limitations in this study are given.

Plant parasitic *Aphelenchoides*: generalist and specialist species

Out of 118 nominal species of *Aphelenchoides,* plus 79 of uncertain status (Tables I.1 and I.2), only 14 have been reported as plant-parasitic species (PPA), see Table II.1. As expected, the most commonly reported species were the foliar nematodes, *i.e. A. besseyi*, *A. ritzemabosi* and *A. fragariae*, that together with *A. subtenuis* are associated with more than 1000 plant species. Because of the broad range of reported hosts, these four species and also *A. arachidis, A. bicaudatus* and *A. blastopthorus*, are regarded as generalist parasites. The remaining plant-parasitic species are only known from one or two hosts, being regarded therefore, as specialist species (Table I.1). Remarkably, despite the number of reported associations is high, it is not common for a plant species to have more than one main plant-parasitic *Aphelenchoides,* and only six have reports of the three foliar species (Fig. I.2); similarly, combinations with the other minor plant-parasites are unusual (Fig. I.3).

Notwithstanding the compilation of plant-associations is long (available at http://nematodes.myspecies.info/), the list represents only a fraction of the potential ranges.

Additionally, there is uncertainty in many cases not only about the parasitic relationship but also on the identification of nematodes or hosts. Equally limited is the knowledge on interactions of the plant-parasitic species and other invertebrates and microorganisms, and further research should be conducted on this topics to elucidate the importance of these associations and the actual status of parasitic relationships.

mtCOI diagnosis of plant-parasitic *Aphelenchoides* and related taxa

Compared to rDNA, mitochondrial Cytochrome Oxidase I gene (COI) was rather unexplored for its effectiveness to diagnose *Aphelenchoides*. This molecular marker is said to be difficult to amplify in some groups of nematodes (De Ley et al. 2005), however, we achieved 65% success rate in our samples, which is similar to the rDNA markers'. In this study we contribute to the actual use of COI for diagnosis with 69 new mtCOI sequences of *Aphelenchoides* taxa, including the first COI sequences of *A. fragariae* and *A. subtenuis*; and the first 28S sequences of the latter (represented by three populations). Such sequences will improve the assessment of a more complete framework for phylogenetic studies, and provide a broader number of taxa to compare for molecular diagnosis.

Additionally, we were able to conclude that the inter-specific variation of the MPPA species (Table III.2), is large enough for any of the three molecular markers, *i.e.* 18S, 28S and mtCOI, to robustly diagnose *Aphelenchoides* species. Moreover, the higher rate of evolution of mitochondrial sequences provide a better differentiation of closely related species, which is especially convenient in groups with constrained morphological features such as *Aphelenchoides*. Yet, this higher mutation rate lowers the number of clades retrieved in the mitochondrial-based topologies (Fig. III.1), hence, mtCOI's value to reconstruct phylogenetic relationships is limited compared to rDNA markers.

By combining the sequences from the three markers (see methodology overview in Fig. V.1), we constructed the first concatenated analysis of *Aphelenchoides*. This tree is in agreement with the single gene topologies and confirms that PPA do not form a monophyletic group, also discussed in previous works (Rybarczyk-Mydłowska *et al*. 2012). Furthermore, all PPA species are close to free-living taxa, suggesting that plant parasitism had arisen from

fungivorous ancestors at least three times in *Aphelenchoides*: *A. besseyi* and *A. ritzemabosi* in Clade I; *A. blastophthorus*, *A. fragariae* and *A. saprophilus* within Clade II and *A. subtenuis* early branching in Clade II (Fig V.1).

Phylogenetic relationships and usefulness of morphological data in *Aphelenchoides*

As explained throughout this thesis, *Aphelenchoides* poses a challenge to nematologists because of its generally conserved morphology, poor species descriptions and scarce number of molecular data, making this genus especially complicated considering its enormous diversity (see below). Moreover, *Aphelenchoides* and *Laimaphelenchus* do not have diagnostic morphological features and overlap in several characters (Davies *et al.* 2015), while *Ficophagus, Martininema* and *Schistonchus s.s*. are distinguished by a robust stylet with strong basal knobs and can be differentiated by the secretory-excretory pore position (Davies *et al.* 2015). *Robustodorus* can be diagnosed by the characteristic strong stylet with narrow lumen, massive knobs and guiding apparatus (Ryss *et al.* 2013).

The compiled morphological data of *Aphelenchoides* suggest that most morphometrical features and ratios are highly variable within clades and did not correspond with molecularly-defined groups. Only the mucro shape (*i.e.* tail terminus) and the position of the secretory-excretory pore relative to the median bulb showed a remarkable correspondence with the clades in the phylogenetic trees (Fig IV.7). All other evaluated morphometrical data did not show a correspondence with molecularly defined clades, *i.e.* body length, length of the post-uterine sac, stylet length, lateral lines, position of the secretory-excretory pore (relative to the nerve ring), tail shape, tail length, vulval position, body length/tail length, tail length/anal body width, position of the nerve ring, body length/anal body width, body length/body width at middle of median bulb, body length/distance from anterior end to the middle of the median bulb, body length/body width at vulva, knobs width, knobs height, knobs ratio, lips width at base, lips height, lips maximum width, lips ratio, median bulb valves length, width of median bulb valves, median bulb valves ratio, median bulb length, median bulb width, median bulb ratio, length from the middle of the valves to the anterior end of the median bulb, length from the middle of the valves to the posterior end of the median bulb, valves position in the median bulb, length from the middle of the median bulb

to the anterior end, body width at the median bulb, body width at the median bulb/lips width at base, length from the tip of the pharyngeal gland lobe to the anterior end, distance of the secretory-excretory pore to the anterior end, distance of the secretory-excretory pore to the middle of the median bulb, position of EP as % of the body length, length from the anterior end to the middle of the median bulb/distance of the secretory-excretory pore to the anterior end, body width at vulva, and length from vulva to anus.

High F-values obtained in Chapter IV, *i.e.* appointing the most different traits among clades, correspond to the following features: position of the median bulb divided by the position of the secretory-excretory pore (LMBA/EPA), "c" value, maximum width of the lip region and knobs width. Therefore, further research should analyze if these features are valuable as species diagnostic characters. Conversely, measurements related to the vulva position; ratios of the knobs, lips, medial bulb, median bulb's valves and their position; length of the isthmus, pharyngeal glands and post uterine sac; relative size of the latter to the distance between vulva and anus, and the anal body width, have less value for species diagnosis based on their low F-values and thus high intra-specific variation.

However, the above calculations were made at supra-specific levels because most putative species were represented by only few specimens (see "ecology and diversity" below). Thus, intra-specific variation *vs* inter-specific variation could not be formally estimated. Yet, although morphometrical features and ratios are too variable to correspond with phylogenetic clades, they can be valuable for species diagnosis. For example, the position of the secretory-excretory pore relative to the nerve ring and the number of lateral lines are measurements that turned out to be not phylogenetically informative but are relevant and informative for species diagnosis in several cases according to Hockland (2001; 2004). Furthermore, these two publications, which are comprehensively illustrated, exemplify how morphology-based diagnosis of the MPPA can be accurate by using conventional measurements and polytomous keys for species identification. As new information, *i.e.* molecular, physiological and behavioral data, and potentially-useful characters such as distinct tails and tail terminus shapes, are generated, complementary data can be added to such keys for more accurate diagnosis of a larger number of species.

Finally, and because the tail morphology does partially agree with the phylogenetic topology, the proposed classification scheme (Table IV.3) forms largely natural groups (Fig. IV.7). However, further studies should address several pending statuses and accurately represent multi-state species (Table IV.4).

Ecology and diversity of *Aphelenchoides*

Biologically, the family Aphelenchoididae comprises mycophagous and lichen-associated species, predators and facultative or obligated ecto- and endo- parasites of insects and plants (Hunt 1993; Kanzaki & Giblin-Davis 2012). Except for predators, all these feeding behaviors can be found in *Aphelenchoides* species, which are predominantly mycophagous.

Despite reported plant-parasitic *Aphelenchoides* represent less than 10% of the nominal species (14 species), their impact and host ranges can surpass those of other major plant-parasitic nematodes (Jones *et al.* 2013; Chapter I). Furthermore, given the flexibility of feeding behaviors, a high number of associations with invertebrates and pathogens can also be expected in this genus, but this remains largely unexplored (Chapter III). Bacteria or fungi could also have an impact in the plant infection process as the interaction with pathogens can seriously aggravate plant damage. As several aphelenchs have a relation with insects, e.g. *Bursaphelenchus* spp. and their vector beetles; and *Schistonchus* s.l. and the fig pollinating wasps, insects may also play an important role for dispersal of *Aphelenchoides*. However, little is known about insect phoresy in this genus, even for the extensively-studied plant-parasitic taxa, and only few cases of associations with insects have been reported (Cardoza *et al.* 2008; Kaisa 2000; Kanzaki 2006).

Biodiversity of *Aphelenchoides* species on the other hand, is far from being settled; only in the first half of 2016 four new species were described, namely *A. fuchsi* (Esmaeili *et al.* 2016b), *A. huntensis* (Esmaeili *et al.* 2016a), *A. iranicus* (Golhasan *et al.* 2016) and *A. meghalayensis* (Bina Chanu & Mohilal 2016). This number is likely to increase in the coming years as bark and wood samples have recently become rich substrates for new *Aphelenchoides* species resulting from the protocols to detect the pinewood nematodes *i.e. B. xylophilus* (Esmaeili *et al.* 2016a; Sánchez-Monge *et al.* 2015). In agreement with Rybarczyk-Mydłowska

et al. (2012) and Chapter IV, Clade II shows a remarkable number of unidentified clusters (putative new species) whereas Clade I has relatively few new unknown *Aphelenchoides* representatives (Fig. V.1), all of which were collected from a limited number of Costa Rican samples.

A recent study comparing nematode diversity between one temperate and one tropical rainforest showed that the total species richness in the latter was 300% more (Porazinska *et al.* 2012). This is consistent with Kerfahi *et al.* (2016), who found that beta and gamma diversity, *i.e.* differences in communities among sites and diversity of the entire area, respectively, were higher in the tropics, despite alpha diversity showed no difference between the evaluated areas, *i.e.* a high arctic region *vs* Malaysian rainforests. Both studies underline the undiscovered richness hidden in tropical environmental heterogeneity (e.g. vertical stratification, insects' biodiversity and diversity of biomes); this, together with the inherent plasticity of nematodes to exploit resources and conditions, enable a high diversity of nematodes (Hunt 1993; 2008; Manzanilla-López & Hunt 2005; Powers *et al.* 2009). Nonetheless, subtropical and tropical areas are still far less studied than temperate regions (Manzanilla-López & Hunt 2005; Powers *et al.* 2009). The high diversity of taxa from European samples in our data is mainly due to a higher success rate during DNA amplification (data not shown), but also to a larger systematic sampling and a greater number of evaluated substrates than tropical samples. But at the same time, the limited samples from Costa Rica allowed already to uncover putative new species in Clade I, which has otherwise a relative poor diversity (Fig V.1).

To illustrate a rough estimate on the number of species based on the inclusive 28S dataset in Chapter IV, we used the Kimura-two-parameter (K2P) model to measure the intra- and inter-specific differences among sequences (see Chapter III) based on a trimmed alignment of circa 500bp in length. The intra-specific K2P distances were between 0% and 7% for the known species (*A. fujianensis*, see Fig. V.1) and based on this, we estimated the unidentified specimens as separate putative species when distances were 9% or higher. Even following this very conservative estimation we obtained a total of 29 putative species within the two major clades (Fig. V.1) based on only 47 newly generated and 8 GenBank sequences of

unknown species. Remarkably, only two of the free-living retrieved specimens of *Aphelenchoides* (see Chapter III), could be assigned to an identified species, *i.e*. *A*. sp. 142 and *A*. sp. 145 to *A. fujianensis* (Clade I, Fig. V.1). These sequences represent the first report of this species in Costa Rica and the second in South America after Brazil (de Jesus *et al.* 2016).

The fact that, based on sequences' differences, nearly all analyzed samples result in putative new species underlines the enormous diversity in *Aphelenchoides*. Furthermore, our data suggests that *A. besseyi* is probably a complex of cryptic species, as one subclade (composed mostly by samples from beans) reached 9% of differences compared to the other sequences of this species (Fig. V.1). This potentially-ongoing cryptic speciation is also supported by additional findings and observations, such as 1) its exceptionally broad range of associated plants (Chapter II) that indicates possible separate lineages (this could also be the case of *A. fragariae*); 2) the differential abilities to infect the same host (Hsieh *et al*. 2012), 3) the differential viability of some crosses (Hsieh *et al*. 2012), 4) the ability to parasitize a specific host after crossing isolates from two different hosts (Hsieh *et al*. 2012), 5) the confirmation of two modes of reproduction in different isolates, *i.e.* cross fertilization and parthenogenesis (Hsieh *et al*. 2012) and 6) the more recent discovery of a GH5-candidate in *A. besseyi*'s genome, only found in bird's nest isolates and absent in rice populations, which suggests independent evolutionary pathways (Wu *et al*. 2016). This information should be considered in future research on this species.

Finally, despite the main scope of this research was not a quantitative analysis of diversity, the number of specimens *vs* the number and type of samples obviously suggest that neglected substrates hide an important number of aphelenchoidids (Fig. V.1). Bark represented 59% of the total number of samples, followed by fungi (21%), soil (14%), and moss (7%), from which 99, 28, 15 and 10 specimens, respectively, were recovered. Considering that 10, 8, 2 and 1 putative species were found in bark, fungi, soil and moss, respectively (Fig. V.1), the diversity foreshadowed in the phylogenetic trees is, evidently, just a glimpse of the real amount of species waiting to be discovered, particularly on bark and wood samples. However, such task should be oriented towards an understanding of the

relatedness among species rather than the mere description of taxa in this already constipated genus (De Ley 2000).

Issues and limitations in *Aphelenchoides* studies; remarks for future work

Species and molecular delimitation

Nematodes have, in general, several aspects that hinder their study: microscopic size, conserved morphology, poor inventories, inadequate descriptions and a depleting number of morphology-based taxonomists (Ahmed *et al.* 2015; Ye *et al.* 2007). *Aphelenchoides* is an excellent example of the challenges associated with nematology because of its overwhelming diversity, abundance in diverse substrates and possible associations, but especially because most species are poorly described and nearly all lack associated molecular data. Given the fact that the taxonomic situation in *Aphelenchoides* is already problematic, the description of the numerous new species, particularly if only morphologically described or based on few specimens, does not lead to a better understanding of the group, on the contrary, they only perpetuate the difficulty of species diagnosis (see below) and can be even useless especially if there is not relevance to an ongoing research (De Ley 2000).

The identification of *Aphelenchoides* species is a complicated task due to the high intra-specific and low inter-specific morphological variability that has led to numerous taxonomical conundrums and potential cryptic species complexes (Kanzaki & Giblin-Davis 2012; Hockland 2001). The high diversity found in this study is based on the barcode-gap approach (Hebert *et al.* 2004), *i.e*. on the comparison of intra- and inter-specific differences to discriminate possible independent lineages (see putative species in Fig. V.1). Since these putative taxa are represented by only few specimens each, morphological features were only considered at supra-specific level, but also the evaluation of molecular differences between populations (metapopulation level) is hampered. However, a combination of phylogenetic and population genetic analyses is needed to work with the more recent advanced molecular species delimitation methods, which was not possible based on the available data. The methodology applied in this thesis, implying the estimation of thresholds based on established species according to the barcode-gap approach, may not be accurate for delineating closely related species in groups that are taxonomically understudied (Meyer &

Paulay 2005). This is certainly the case for *Aphelenchoides* and given the fact that no morphological support at species level was provided, the number of putative species should be regarded only as a rough estimation that does not follow a particular species' concept. Regardless of these constraints, the enormous diversity within Aphelenchoides is evident and this highlights the pending taxonomical work on the genus.

The use of molecular data in Nematology have resulted in numerous improvements and new possibilities, including its use as an alternative dataset to reconstruct phylogenies, independent from morphology, ecology or life stories (Ye *et al.* 2007); here we have demonstrated that also the sequences not identified to species level are informative in phylogenetic studies (Fig. IV.3, Fig. IV.4 and Fig. V.1). We were also able to support one species transfer and propose one more (*A. heidelbergi* and *A. pannocaudus*, respectively) thanks to a combination of molecular and morphological data. However, the use of molecular techniques in nematode taxonomy -if not combined with other data- have also generated considerable criticism (Abebe *et al.* 2013; Will *et al.* 2005), moreover, these techniques have serious limitations (Abebe *et al.* 2013), particularly in *Aphelenchoides*. Molecular diagnosis of unknown *Aphelenchoides* by simply comparing sequences in databases (*i.e.* blasting) usually retrieves both, unidentified and identified entries, which should be considered carefully. Several sequences are misidentified and some species' placements are different depending on the analyzed gene (see de Jesus *et al.* 2016 and Chapter IV). Moreover, aphelenchoidids' phylogenies are subjected to change each time a new sequence is added due to the limited number of taxa (Zhao *et al.* 2008), and evidently, molecular identification do not solve all nomenclatorial problems (Zhang *et al.* 2015) as diversity is only partially represented in databases (De Ley 2000).

Morphological data and interpretation

Alternatively, the right combination of expertise and traditional parameters for diagnosis, *i.e.* morphology-based taxonomy, has been proven effective for decades (Abebe *et al.* 2013; Hockland 2004) despite the limitations of light microscopy in terms of resolution (De Ley 2000, Chapter IV). However and especially for *Aphelenchoides*, it is extremely difficult to

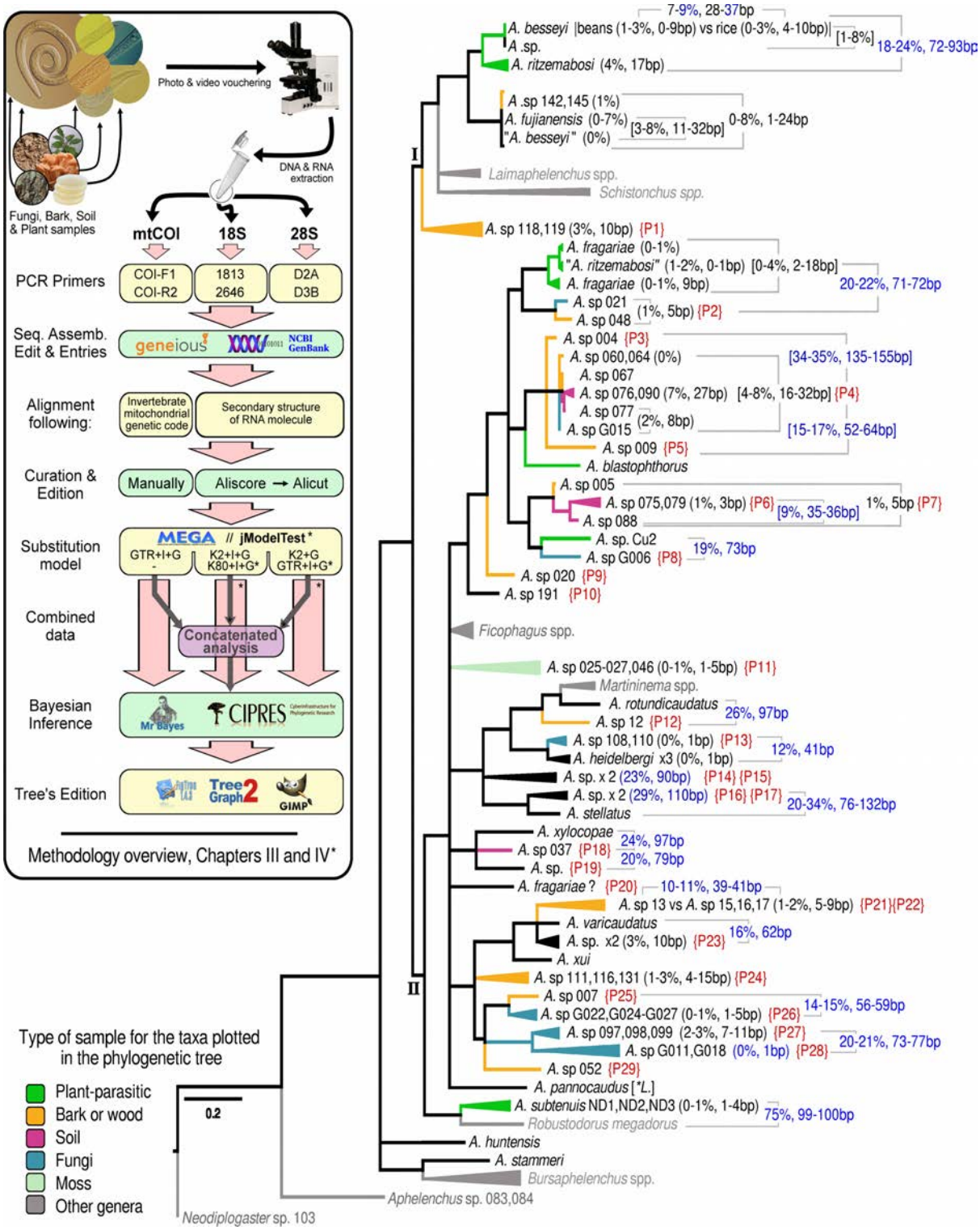


Fig. V.1 Bayesian inference consensus tree of *Aphelenchoides* spp. and related taxa inferred from partial 28S (D2D3 expansion region) sequences from the "inclusive" dataset (Chapter IV). Percentages of differences and number of different base pairs among sequences were calculated under the Kimura-two-parameter (K2P) model; putative species ({Pn}) differ 9% or more from their closest sister taxon; color codes represent the sampled substrates. Framed: Overview of the different steps in the analysis and reconstruction of single and multigene phylogenetic trees in Chapters III and IV.

diagnose species at morphological level, thus, poor descriptions (based only on morphology) are to some extent doubtful, moreover, many alike species may in fact be the same species or a complex of species (Hockland 2001). Some structures and artifacts can be misleading for the untrained eye; even the difference between knobbed and thickened stylets depends on the observer's conception (Franklin 1955). Fig. IV.8 schematically shows how light microscopy is often unable to discern the structures associated with lateral lines, moreover, a single nematode can have a variable number of lines and cuticle formations through its entire body (Hockland 2001) and this is only verifiable or revealed under scanning electron microscopy (SEM) (Deimi *et al.* 2006; Ryss *et al.* 2013).

Consequently, diagnosis based on poorly mounted material should be avoided and the description of the number of lateral lines together with their associated structures e.g. ridges, striae, cuticle incisures, etc, is advised (Fig. IV.8). To assist this situation, complementary tools and techniques such as SEM and confocal scanning laser microscopy (CSLM) have been implemented in several studies (Hockland 2001; Mašová *et al.* 2009; Zhao *et al.* 2007) and are gradually becoming more common in nematological research and taxa descriptions, particularly SEM (Deimi *et al.* 2006; Doucet 1992; Golhasan *et al.* 2016; Hooper & Ibrahim 1994; Jen *et al.* 2012; Qing *et al.* 2015; Ryss *et al.* 2013; Zhao *et al.* 2008). Moreover, the combination of light microscopy (LM) and SEM images enables now a relatively easy and fast 3D reconstruction and modelling method (see Appendixes). Despite these tools and reconstructions can be incorporated as a complement to pictures and drawings of (new) nematode descriptions, it should be stressed that fine drawings detailing particular features are always needed for diagnosis (Hockland 2001) since access and information to new technologies are limited in some cases. Moreover, SEM should be used carefully, as it can be misleading due to artifacts (see previous discussion on *A. heidelbergi*, Chapter III).

Taxonomically-relevant parameters for *Aphelenchoides* such as the body length, body length divided by the maximum body width, secretory-excretory pore position, the female tail tip appendages, the number of ridges of the cephalic disc, the length of the ovary and the post uterine sac, the vulval position, the number of incisures in the lateral field, the structure of

spicules' condylus, the tail shape and length and the stylet length (Davies *et al*. 2015; Deimi *et al*. 2006; Hockland, 2001; Ryss *et al*. 2013; Chapter I and IV) together with additional parameters suggested in this thesis, *i.e*. position of the secretory-excretory pore relative to the median bulb, and the body length divided by the body width at the middle of the median bulb or by the distance from the anterior end to the middle of the median bulb (see Tables IV.1 and IV.2), should receive distinct attention in the (re)description of *Aphelenchoides* species. Nonetheless, as stated by Sanwal (1965) in a review of the "*parietinus* group": "a taxonomical character which proves to be reliable and constant for a group of species in a genus may not necessarily have the same validity and reliability for the other section of the genus". Therefore, studies on the intra-specific variation are essential in order to assess distinguishing characters for their degree of constancy and stability (Hockland 2001).

Unfortunately, the analysis of variability among populations to define useful diagnostic characters are absent in most species descriptions; thus, it is likely that many species will not be recognized again (Hockland 2001). Because of the high morphological variability in *Aphelenchoides*, overlap of characteristics is always possible, even if species are described based on very detailed and comprehensive morphological analysis. This was already highlighted by Hockland (2001) while testing her proposed polytomous key. Furthermore, based on the low numbers of specimens, *i.e*. less than 20, used in the original descriptions, Hockland (2001) considered that 59% of the species were inadequately described, as features' variation was not properly represented. However, particular morphological features such as distinct tail shapes (e.g. *A. sphaerocephalus*, *A. disccaudatus*, *A. iranicus*, *A. parabrushmucronatus*, Table IV.3) could be considered "sufficient" for diagnosing these species, but these are exceptional cases; usually, traditional morphometric or morphological features have serious limitations for species identification.

Species (re)descriptions and grouping systems

Recent (re)descriptions of species include both good and bad examples. The combination with SEM images and molecular data has improved the quality of morphological observations and the possibility of diagnosis, respectively. However, some of the most recent descriptions kept a limited approach, for instance, measuring less than 15 females and not

including complementary tools and data for identification. This is the case of *A. meghalayensis* (Bina & Mohilal 2016) and *A. aerialis* (Bina-Chanu *et al.* 2013), which are based respectively on 13♀ plus 7♂ and 6♀ plus 3♂, and both descriptions lack associated molecular data. Despite *A. meghalayensis*' description includes SEM and LM micrographs, detailed drawings of some important features such as the stylet and the mucro-shape are missing, in addition, no comments were provided about the differential position of the secretory-excretory pore between sexes, *i.e.* at the level of the median bulb in males and posterior to it in females. Drawings of *A. aerialis* are also not detailed and LM pictures are unclear, moreover this species' differential diagnosis mentions that "The species differed from all other species of *Aphelenchoides* in having a tail without bifurcation with strong ventral mucro with single ventro-sublateral caudal papillae in male", yet, the mucro is not detailed in the drawings nor in the description.

The description of *A. fuchsi, A. huntensis* (Esmaeili *et al.* 2016a; b), *A. iranicus* (Golhasan et al. 2016) and to certain extent the re-description of *A. subtenuis* by Deimi *et al.* (2006) can be forwarded as good examples of taxonomic works in this genus. The descriptions of Esmaeili *et al.* were based on 15♀ and 5♂, and provided good-quality LM images complemented with detailed drawings. *A. iranicus'* description was based on less specimens (11♀ and 5♂) but the mucro shape, as a distinctive feature, was illustrated with LM, SEM and detailed line drawings. These three descriptions are complemented with molecular data and phylogenetic reconstructions based on two molecular markers. Finally, the re-description of *A. subtenuis* (based on 10♀ and 10♂) provides excellent SEM images of this species and useful illustrations and morphological data. However, the authors did not generate molecular data which is considered to be essential, especially also for a re-description.

Comprehensive descriptions of new taxa must allow a comparison of key morphological characters (see Chapter IV), which is, according to Sanwal (1965) and Hockland (2001), the best approach for accurate diagnosis when based on morphological traits. In agreement with Hockland (2001), future descriptions should be based on measurements of at least 15 females to represent species' variability; males, if available, can be described with five specimens. Molecular data on the other hand, eases molecular diagnosis and complements

several of these descriptions, moreover, they become essential in groups with limited morphological diagnostic traits (such as *Aphelenchoides*) and cryptic species complexes (Palomares-Rius *et al*. 2014). Furthermore, the added value of data associated with sequences is that they can be fitted within a molecular framework and this facilitates several approaches such as reverse taxonomy. However, based on current markers and methods, the resulting tree topologies are not always consistent. For example, *A. huntensis* is within Clade II in the 18S analysis but sister to all *Aphelenchoides* according to 28S (Esmaeili *et al*. 2016a); while *A. iranicus* is positioned inside Clade II in the 28S topology but in an early diverging position in the 18S tree (Golhasan *et al*. 2016). These topological differences could be attributed to different phylogenetic histories told by the datasets (Leliaert *et al*. 2014; Wiens, 1998; see also Chapter IV).

From the morphological point of view, comparisons and descriptions of new species routinely refer to Shahina's (1996) compendium and grouping system based on 141 described species. However, according to Hockland (2001) this publication is unreliable because not all original descriptions were used in the compendium; females' and males' characters were not distinguished from one another, the number of specimens *per* description, scale bars in the drawings were neglected and data for several species were measured directly from drawings, which were drawn by different authors. Nevertheless, Shahina's classification partially resembles the natural groups obtained in our analyses; although the proposed subdivision of such groups (Table IV.3) do better correspond to the supra-specific phylogenetic clades (Fig. IV.7).

Despite the proposed grouping appears to reflect natural groups, it also has limitations. For example several species have multiple character states or could not be assigned to the categories of Table IV.3 because information was insufficient in the original descriptions; also some mucro shapes such as clavate or semi-spherical (2.1.3) or two equally sized mucronate structures (2.2.2) were not associated with molecular data (Fig. IV.7). Hence, further research based on additional more-representative data is needed to properly evaluate the proposed grouping system, since, similar to Shahina (1996), this study partially relies on data measured directly from drawings from original descriptions due to limitations

of time and access to identified material, *i.e.* material that allows sequencing and/or type material.

Notwithstanding these limitations, the obtained morphological and species' diversity, although only estimated in this study, are doubtlessly extraordinary. Additionally, the morphological diversity of aphelenchs's can be further illustrated by some unusual and interesting examples that were not included in the analyses because the specimens showing these traits were not successfully sequenced (see Fig. V.2). These features, including embryonated eggs, unidentified large internal bodies, other types of mucro shapes and variations in the number of lateral lines and associates structures, may be valuable to consider in future research as they could be biological relevant or diagnostic at species or supra-specific level.

An integrative work

Clearly, the input provided by digital morphological vouchers thorough this work has been proven to be extremely valuable because they allowed a more comprehensive analysis of the resulting phylogenies. Via virtual vouchering it is possible to improve the process of biodiversity cataloging, help in the study of cryptic species, enhance education (De Ley & Bert 2002; De Ley *et al.* 2005) and, as presented in this thesis, enable the implementation of the reverse taxonomic approach for tentative delimitation of supra-specific clades. Digital vouchering is therefore, encouraged for future studies, as highlighted also by Ye *et al.* (2007). Furthermore, the combination of molecular data with morphology partially solves the difficulties that traditional morphology-based taxonomy presents, such as resolution limits and micro-structures interpretation, and avoids the criticized oversimplification of taxonomy to molecular work (Abebe *et al.* 2013).

Integration of molecular and morphological data have already improved the understanding of the evolutionary history of groups such as Kinorhyncha (Sorensen *et al.* 2015), fungi (Nagy *et al.* 2013), ferns (Péchon *et al.* 2016), birds (Mayr 2008) and several botanical families (Appelhans *et al.* 2011; Chen *et al.* 1999; Graham *et al.* 2005; Roque & Funk 2013; Ruhfel *et al.* 2013). In these cases, morphology-based trees were tested against molecular-based

topologies, after which a combination of datasets led to the finding of non- and monophyletic groups, synapomorphic characters, distinctive lineages and even the placement of a fossil genus. In another example, the integration of data resulted in the proposal of new phylogenetically-relevant morphological features for a particular group of springtails (Collembola), after implementing the ancestral state reconstruction (Zhang *et al.* 2015). However, most of these studies were done on macroorganisms at family level, for which morphological data, which are generally easier to observe, were gradually collected over the years.

Several integrative-approach studies can be also found in Nematoda, the following examples can be forwarded as good practices of integrated taxonomy: Fonseca *et al.* (2008) and Apolônio Silva de Oliveira *et al.* (2012) evinced diagnostic morphological features for cryptic marine nematode species after analyzing molecular data, and Ye *et al.* (2007) tested morphological features against phylogenetic clades in *Bursaphelenchus* species, finding a general congruence between the two datasets. More recently, Miljutina & Miljutin (2016) evaluated the intra-specific variability of morphological traits in a marine nematode species, noticing both, high-intraspecific variable and more conserved characters. Similar analyzes could be done for *Aphelenchoides*, but only after accumulating molecular and morphological data from several species and populations.

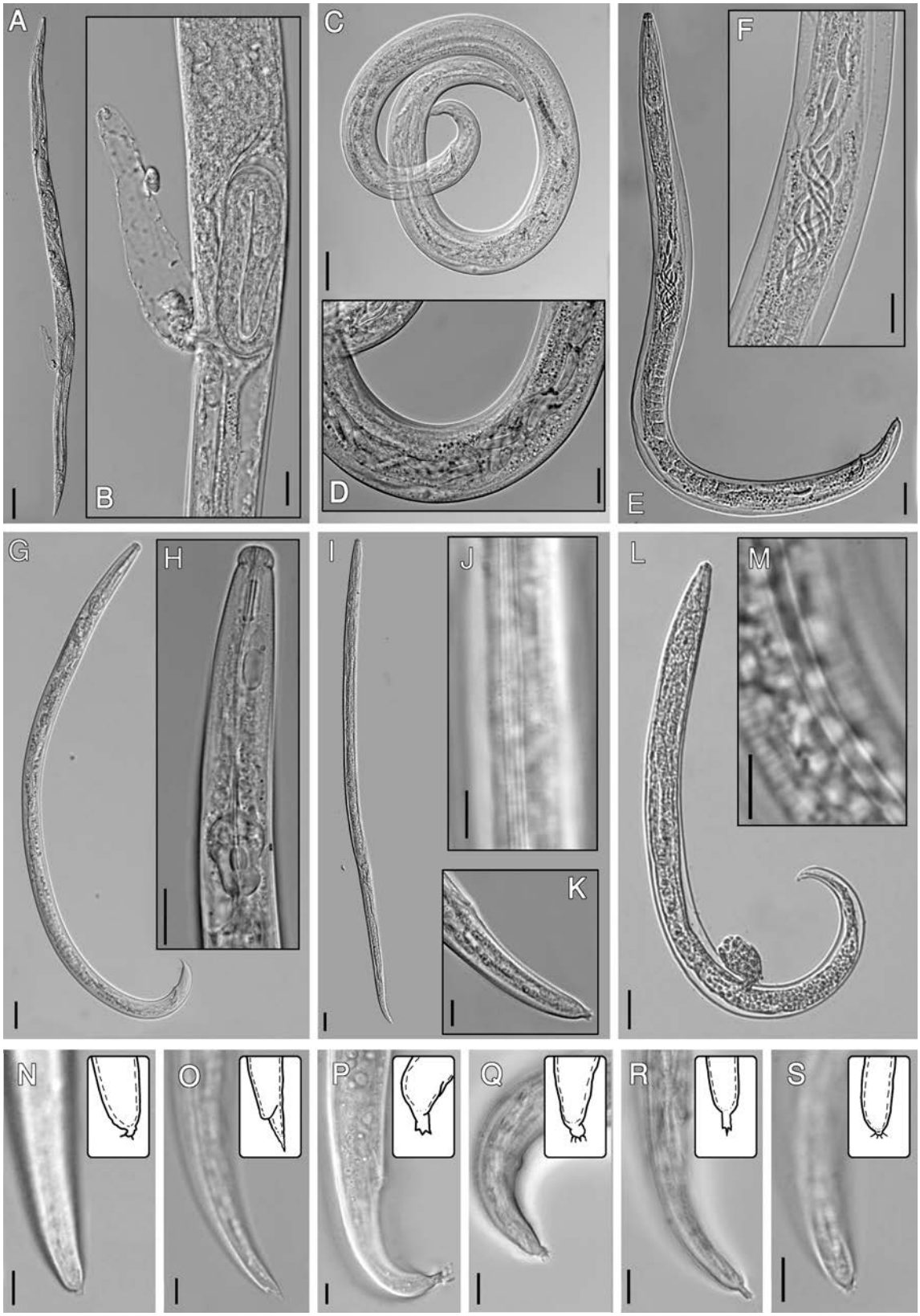


Fig. V.2 Unusual morphological features of aphelenchids recovered from several samples. Embrionated eggs (A, B); unidentified large internal bodies (C-F); deformed median bulb (G, H); lateral fields (I, J, L, M); mucro shapes: star-like (K); mucro with two hair-like protrusions (N); a tail-terminus flap (O); three pointed mucro (P, R); and spherical mucro with protrusions (Q, S). Scale bars are 5um long, except for B, D, F, H (10 µm) and A, C, G, I, L (20 µm)

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General conclusion

Aphelenchoides is, without a doubt, a complex genus in many ways, and is an excellent example of the challenges associated with Nematology; this because of its abundance in diverse substrates, possible associations, overwhelming diversity, lack of available molecular data and poor species' descriptions. In this thesis we updated the knowledge on the range of plants associated with the plant-parasitic species, we compared the phylogenetic resolution of three molecular markers and their usefulness for diagnosis and we deepened our understanding of the phylogenetic relationships and taxonomy of the genus. In brief:

1. *Aphelenchoides* comprises circa 200 nominal species, most of which were poorly described. Circa 118 can be forwarded as revised/valid species.
2. Fourteen *Aphelenchoides* species have been described as plant- parasitic species, four have a major word wide impact on agriculture, including almost 1000 records on associated plants.
3. Non-supported parasitic relationships are common in literature and should be avoided.
4. Associations with insects and microorganisms may play a crucial role in nematode-plant interactions in this genus.
5. We generated 69 mtCOI and 123 rDNA sequences *Aphelenchoides* spp. and proved the potential of the COI region for plant-parasitic *Aphelenchoides* identification.
6. We constructed the first concatenated phylogenetic analysis of the genus and a multiple origin of plant-parasitism within *Aphelenchoides* is supported.
7. Our data show that several sequences of plant-parasitic *Aphelenchoides* in databases are likely to be misidentified.
8. Based on molecular and ecological data, *Aphelenchoides besseyi* is more likely a complex of species.
9. According to our data, the transfer of *Laimaphelenchus heidelbergi* to *Aphelenchoides* is supported. We also propose the transfer of *Laimaphelenchus pannocaudus* to *Aphelenchoides*.

10. *Aphelenchoides*' morphology was found to be relatively uniform in comparison to a remarkably broad molecular diversity, almost all morphological traits did not agree with phylogenetic clades.
11. Only the position of the secretory-excretory pore relative to the median bulb and the tail-terminus shape (*i.e*. mucro shape) showed correspondence with *Aphelenchoides'* molecularly-supported supra-specific clades.
12. A grouping system, based on tail and tail-terminus shapes, was proposed for *Aphelenchoides* species. This classification largely agrees with natural groups.
13. *Ficophagus*, *Martininema*, *Laimaphelenchus*, *Schistonchus* and *Robustodorus* are all imbedded within the genus *Aphelenchoides*, pointing out that *Aphelenchoides* is paraphyletic.
14. An amendment to the genus diagnosis is proposed and includes the following observations: the number of incisures in the lateral field can vary from two to six, a rudimentary isthmus may be present, the secretory-excretory pore can be anterior to the median bulb and microprotuberances (micropapillae) can be present on the mucro.
15. New (re)descriptions of *Aphelenchoides* species should include:
    - Measurements of at least 15 females (and 5 males if possible)
    - Detailed drawings and detailed illustrations of particular features, especially for the anterior and tail regions
    - SEM images (if possible and relevant)
    - Molecular data of at least two specimens, preferably from two molecular markers, *i.e.* one rDNA and one mtCOI gene
    - Virtual morphological vouchers deposited in a public repository
16. The conserved estimation of 29 putative species (Fig. V.1), based on only 55 unknown specimens, outlines the enormous undiscovered species richness of this genus.
17. Information generated herein, *i.e.* mtCOI and rDNA sequences, the exploration of morphological traits for (re)descriptions, the proposed grouping system and the update of the genus delimitation, will be useful for morphological and molecular diagnosis of *Aphelenchoides* species.

LIST OF PUBLICATIONS AND CONTRIBUTIONS
2013-2016

Publications in SCI-indexed journals

Qing, X., **Sánchez-Monge, A**. & Bert, W. (2015) Three-dimensional modelling and printing as tools to enhance education and research in Nematology. *Nematology* 17, 1245–1248.

Qing, X., **Sánchez-Monge, A**., Janssen, T., Couvreur, M. & Bert, W. (2015) 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, 155–174.

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Other publications:

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Active contributions

67th ONTA Annual Meeting, Varadero, Cuba (2015). Poster: "3D modelling of the amphidial aperture in the genus *Malenchus* // Modelado 3D de la apertura anfidial del género *Malenchus*" Qing, X., **Sánchez-Monge, A.** & Bert, W.

67th ONTA Annual Meeting, Varadero, Cuba (2015). Oral presentation: "Reverse Taxonomy, phylogeny and DNA barcoding to illuminate the diversity of *Aphelenchoides* | Taxonomía inversa, filogenia y código de barras genético para ilustrar la diversidad de *Aphelenchoides*" **Sánchez-Monge, A.**, Janssen, T. & Bert, W.

68th International Symposium on Crop Protection, Ghent, Belgium (2015). Poster: "Plant-parasitic *Aphelenchoides* spp.: an updated list of associated plants" **Sánchez-Monge, A.**, Flores, L., Salazar L., Hockland, S. & Bert, W.

XVIII International Plant Protection Congress, Berlin, Germany (2015). Oral presentation: "An on-line and updated list of plants associated with plant parasitic *Aphelenchoides* spp., with implications for host-parasite relations within the genus" **Sánchez-Monge, A.**, Flores, L., Salazar L., Hockland, S. & Bert, W.

2nd International Academic Conference for Graduate Students, Nanjing, China (2015). Oral presentation: "Optimizing nematode taxonomy at Ghent University's Nematode Research Unit (NRU): combining morphological and molecular data for a broad range of plant-parasitic taxa" **Sánchez-Monge, A.** & Bert, W.

69th International Symposium on Crop Protection, Ghent, Belgium (2016). Poster: "Potential of mDNA -COI- as a diagnostic tool for plant-parasitic *Aphelenchoides* species" **Sánchez-Monge, A.**, Janssen, T., Fang, Y., Couvreur, M., Karssen, G. & Bert, W.

32nd ESN Symposium, Bragha, Portugal (2016). Oral presentation: "Phylogeny and diversity of the genus *Aphelenchoides*, a reverse taxonomic approach" **Sánchez-Monge, A.**, Janssen, T., Fang, Y., Couvreur, M., Karssen, G. & Bert, W.

Annexes:

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

Qing, X., Sánchez-Monge & Bert, W.
Nematology, 17, 1245–1248. (2015)

Description of *Malenchus sexlineatus* n. sp., new records of three known species of *Malenchus* Andrássy, 1968 (Nematoda: Tylenchidae) and notes on amphidial aperture development

Qing, X., Sánchez-Monge, A., Janssen, T., Couvreur, M. & Bert, W.
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Short communication

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

Xue QING [1,*], Alcides SÁNCHEZ-MONGE [1,2] and Wim BERT [1]

Three-dimensional (3D) modelling has 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, 2009; Beutel *et al.*, 2008; Ragsdale *et al.*, 2008, 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 plane 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 software widely used 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 modelling 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 freely available for 3 years for students and educators (http://www.autodesk.com/education/free-software/maya).

In the first step of this method, a SEM image is imported as reference for the exterior (View > Image Plane > Select reference image) (Fig. 1A), 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. 1C, 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. 1D, 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. 1E). Structures are created following the outline (Fig. 1E1) or by importing and modifying default polygons that resemble the structures, *via* the program tools (Fig. 1E2). 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 Figure 2(A-E); such image files 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

[1] *Nematology Research Unit, Department of Biology, Ghent University, Ledeganckstraat 35, B-9000 Ghent, Belgium*
[2] *Universidad de Costa Rica, Escuela de Estudios Generales, 2060, Costa Rica*
* Corresponding author, e-mail: xueqing4083@gmail.com
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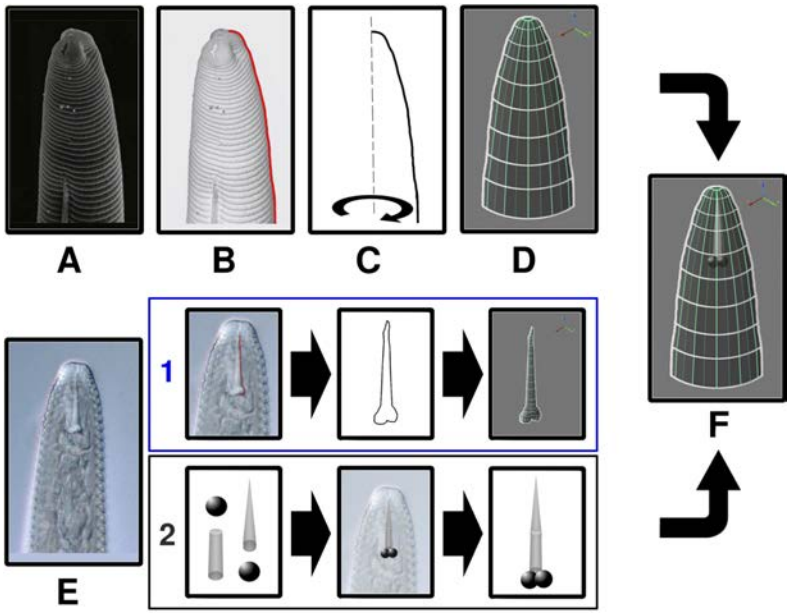

Fig. 1. Schematic representation of the process to create a 3D model of nematode structures using Autodesk® Maya®. Scanning electron microscopy (SEM) images (A) provide the base for the surface structure and the construction of the body shape; Light microscopy (LM) images (E) provide information on constructing and designing 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.

combining two separate views of the same object in a slightly-tilted position (Fig. 2F). Here, the red colour 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 a composition can be made in on-line websites or in an image editing program within a few minutes. The prepared 3D model can be also exported as an '.stl' file (File > Export All or File > Export Selection) in Autodesk® Maya® and printed on a 3D printer (Fig. 2G-K). The executable 3D printing file, the video during printing, additional high resolution 3D images and the anaglyph file of the mononchid's head are available at http://nematodes.myspecies.info.

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

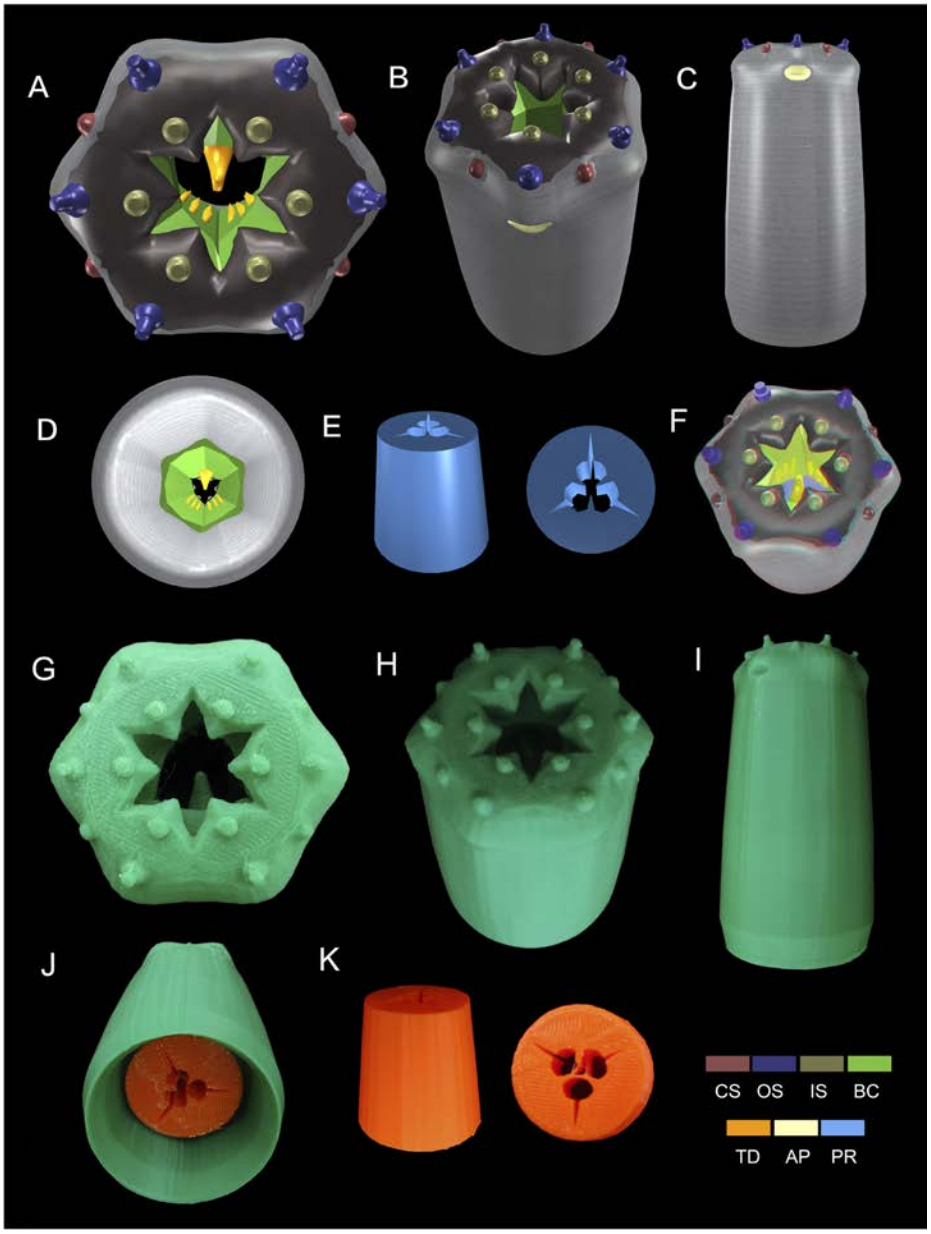

Fig. 2. 3D models of typical mononchid head region. A-E: 3D images rendering from models built by Autodesk® Maya® software. A, B: *En face* view showing six inner and outer labial sensilla, and four cephalic sensilla; C: Lateral view; D: Cross view of head shows buccal cavity; E: Different views of anterior pharynx; F: Anaglyph image of head (red/blue glasses needed to see the image in 3D). G-K: 3D prints of the models built by Autodesk® Maya® software (Printer: Makerbot® Replicator® 2, Model: 13 cm high × 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 colour bars: CS: cephalic sensilla; OS: outer labial sensilla; IS: inner labial sensilla; BC: buccal cavity; TD: teeth and denticles; AP: amphidial aperture; PR: pharynx.

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Description of *Malenchus sexlineatus* n. sp., new records of three known species of *Malenchus* Andrássy, 1968 (Nematoda: Tylenchidae) and notes on amphidial aperture development

Xue QING [1,*], Alcides SÁNCHEZ-MONGE [1,2], Toon JANSSEN [1], Marjolein COUVREUR [1] and Wim BERT [1]

[1] *Nematology Research Unit, Department of Biology, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium*
[2] *Universidad de Costa Rica, Escuela de Estudios Generales, 2060, San José, Costa Rica*

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Summary – A new species, *Malenchus sexlineatus* n. sp., discovered from the Philippines, is described based on morphological and molecular data. The new species is unusual in the genus by having six lateral lines. *Malenchus sexlineatus* n. sp. is distinguished from *M. williamsi*, the only other species in the genus with six lateral lines (based on currently available SEM data), by a shorter body of 278 (270-288) *vs* 452 (425-495) μm, shorter stylet (7.0 (6.2-7.5) *vs* 11-12 μm), narrower annulations (0.8 (0.7-0.8) *vs* 1.2-1.6 μm), lateral field comprising one elevated ridge in LM *vs* six well-separated incisures (resembling the lateral lines in *Cephalenchus*) in LM, the presence of S-shaped *vs* straight amphidial apertures, and vulval flaps absent or only one annuli long *vs* distinct. By having an exceptionally short body, *M. sexlineatus* n. sp. comes close to *M. parvus*, *M. bryanti* and *M. acarayensis*. However, there are significant differences in the lateral lines, annuli width and most morphometric ratios. Three known species, namely *M. exiguus*, *M. nanellus* and *M. pachycephalus*, all being first records and first representative from China, are characterised by 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.

Keywords – China, *Malenchus exiguus*, *Malenchus nanellus*, *Malenchus pachycephalus*, molecular, new record, new species, Philippines, phylogeny, SEM, taxonomy, Tylenchomorpha.

The genus *Malenchus* Andrássy, 1968 is one of most specious genus within Tylenchidae and has been reported worldwide (Andrássy, 1981). This genus was established by Andrássy (1968) and is characterised by prominent annulations and a dorsoventrally flattened lip region, with *M. machadoi* (Andrássy, 1963) Andrássy, 1968 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* Knobloch, 1976 and *M. truncatus* Knobloch, 1976) and the erection of *Neomalenchus* Siddiqi, 1979 with two species, respectively.

Andrássy (1981) performed a comprehensive and detailed study of *Malenchus* with the description of seven new species and proposed *Neomalenchus* as a junior synonym of *Malenchus*, an action that was followed by Geraert & Raski (1986). Later, Siddiqi (2000) considered *Neomalenchus* as a valid subgenus and introduced another subgenus (*Telomalenchus* Siddiqi, 2000) to accommodate three species with straight amphidial apertures and fewer lateral lines (four or six *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) synonymised *Malenchus* with *Fraglenchus* Siddiqi, 2000, which has a rounded lip region and a short amphidial slit. Sumenkova (1988) erected *Paramalenchus* Sumenkova, 1988 for the species *P. anthrisul-*

* Corresponding author, e-mail: xueqing4083@gmail.com

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cus Sumenkova, 1988. However, it was synonymised with *Malenchus* by Ebsary (1991), an action that was followed by Siddiqi (2000) and Geraert (2008). *Malenchus novus* Mukhina & Kazachenko, 1981 was initially assigned to *Malenchus* but later moved to *Mukzia* Siddiqi, 1986 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 differentiating character. In this study we follow Geraert (2008) who listed 35 valid species and three *nomina nuda* under two subgenera (*Malenchus* and *Telomalenchus*).

Despite the importance of the genus from a phylogenetic aspect as an early diverging branch within Tylenchomorpha (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, *Malenchus* is studied from China for the first time. A new species, *M. sexlineatus* 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

Sample collecting and processing

Samples were collected in four locations in 2012 and 2013: Mount Hamiguitan, the Philippines in August 2012; Shimen, Hunan, China; Pingwu, Sichuan, China and Mount Taibai, Shaanxi, China, in August 2013 (for additional details, see below). Nematodes were extracted from soil samples using a Baermann tray, and collected and concentrated using a 500 mesh sieve (USA standard mesh numbers, equal to 25 μm 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 deionised water and then transferred to anhydrous glycerin, following the protocol of Seinhorst (1962) modified by Sohlenius & Sandor (1987).

Morphological characterisation

Measurements and drawings were prepared manually with a drawing tube mounted on an Olympus BX51 DIC Microscope (Olympus Optical), equipped with an Olympus C5060Wz camera for photography. The holotype of the new species, examined Chinese population and paratype slides of *M. williamsi* (UGMD103427, UGMD103427, UGMD103427), *M. leiodermis* (UGMD 103431) and *M. parthenogeneticus* (UGMD103432) were recorded as a video clips mimicking a multifocal observation through a light microscope (LM) developed by De Ley & Bert (2002). The resulting digital specimen vouchers are available online at http://www.nematodes.myspecies.info.

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-fixed with 2% PFA + 2.5% glutaraldehyde in 0.1 M 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) at 12 kV.

Molecular characterisation

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 rDNA were done following the protocol of (Múnera Uribe *et al.*, 2010). *De novo* sequences were deposited in GenBank under the accession numbers KR818869 (*M. sexlineatus* 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 summarised 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 optimised 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).

Results

Malenchus sexlineatus* **n. sp.**
(Figs 1A, E, F; 2A, B, F-H, L-P; 3)

MEASUREMENTS

See Table 1.

DESCRIPTION

Female

Body very small (one of smallest known nematode species), ventrally arcuate after fixation. Body tapering slightly toward posterior end. Cuticle thick, folded between annuli, annulations exceptionally narrow (0.7-0.8 μm). Lateral field prominent, originating at 0.5 or one stylet length posterior to stylet knobs and ending at mid-tail, with six incisures in an elevated, ridge-like lateral field which is subdivided into five small sub-ridges (thereby forming four inner plus two outer incisures bordering elevation), margins relatively smooth (not crenate). Number of incisures occasionally increasing to eight by irregular short insertions of short ridges. Lip region elevated, dorsoventrally compressed, 3.5-4.1 μm wide. Oral opening surrounded by six labial papillae, set on a slightly protuberant oral plate. Amphidial apertures S-shaped, starting at labial plate. Labial framework weak. Stylet slender and delicate, cone *ca* 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 sclerotised valve. Isthmus long and slender. Terminal bulb short, pyriform. Excretory pore at level of anterior part of pharyngeal bulb. Hemizonid 2-3 annuli long and 2-3 annuli anterior to excretory pore. Deirids at 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. Crustaformeria with five cells in each row. Uterus spacious with thickened wall, uterine egg not observed (not gravid). Vulva sunken into body contour, 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 a more or less pointed, hook-shaped tip.

Male

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

TYPE HABITAT AND LOCALITY

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

* The specific epithet refers to the number of lines in the lateral field as seen under SEM and is formed from the Latin: *sex* = six and *lineatus* = lines.

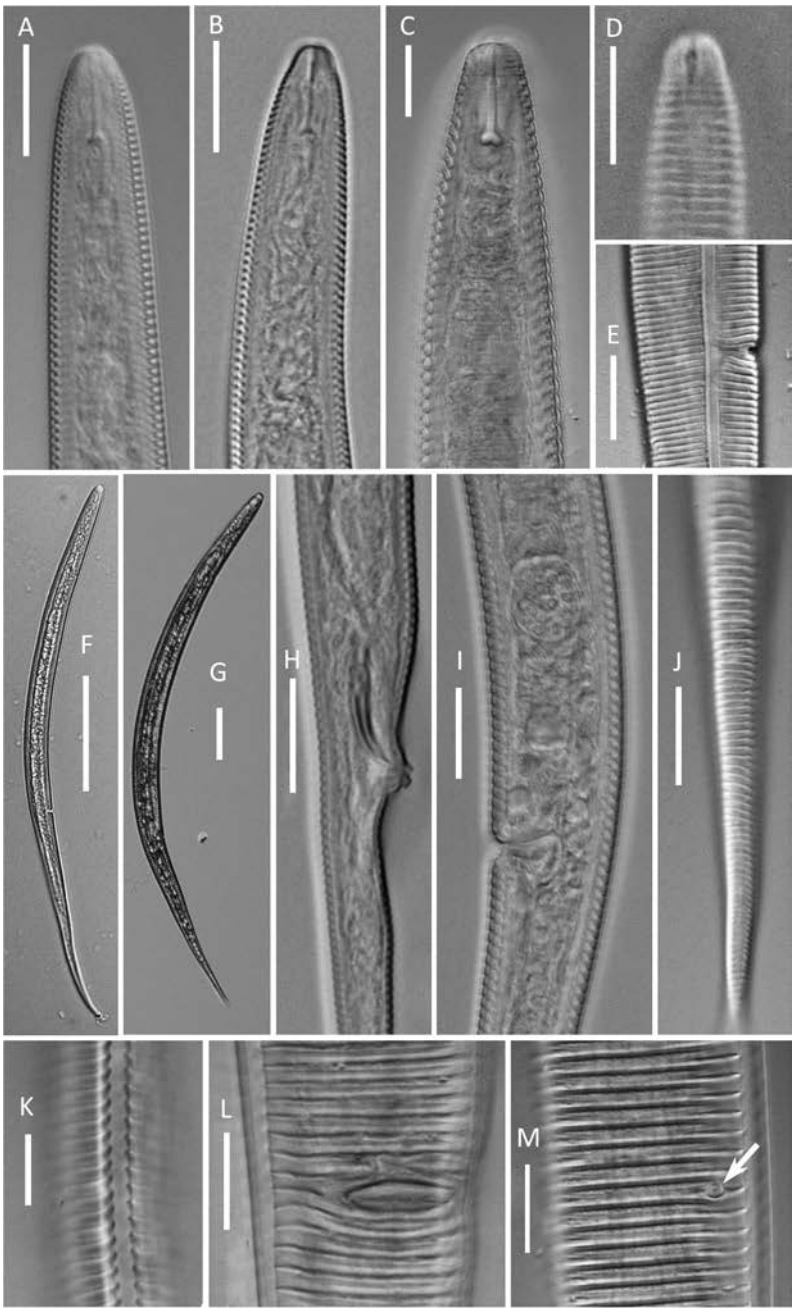

Fig. 1. LM picture of *Malenchus sexlineatus* n. sp. (A, E, F), *M. nanellus* (B, D, H) and *M. pachycephalus* (C, G, I-M). A-C: Female anterior end; D: Amphidial fovea; E: Lateral view of vulval region; F, G: Female habitus; H: Spicules and protruding cloacal lips; I: Vulva and spermatheca; J: Annules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

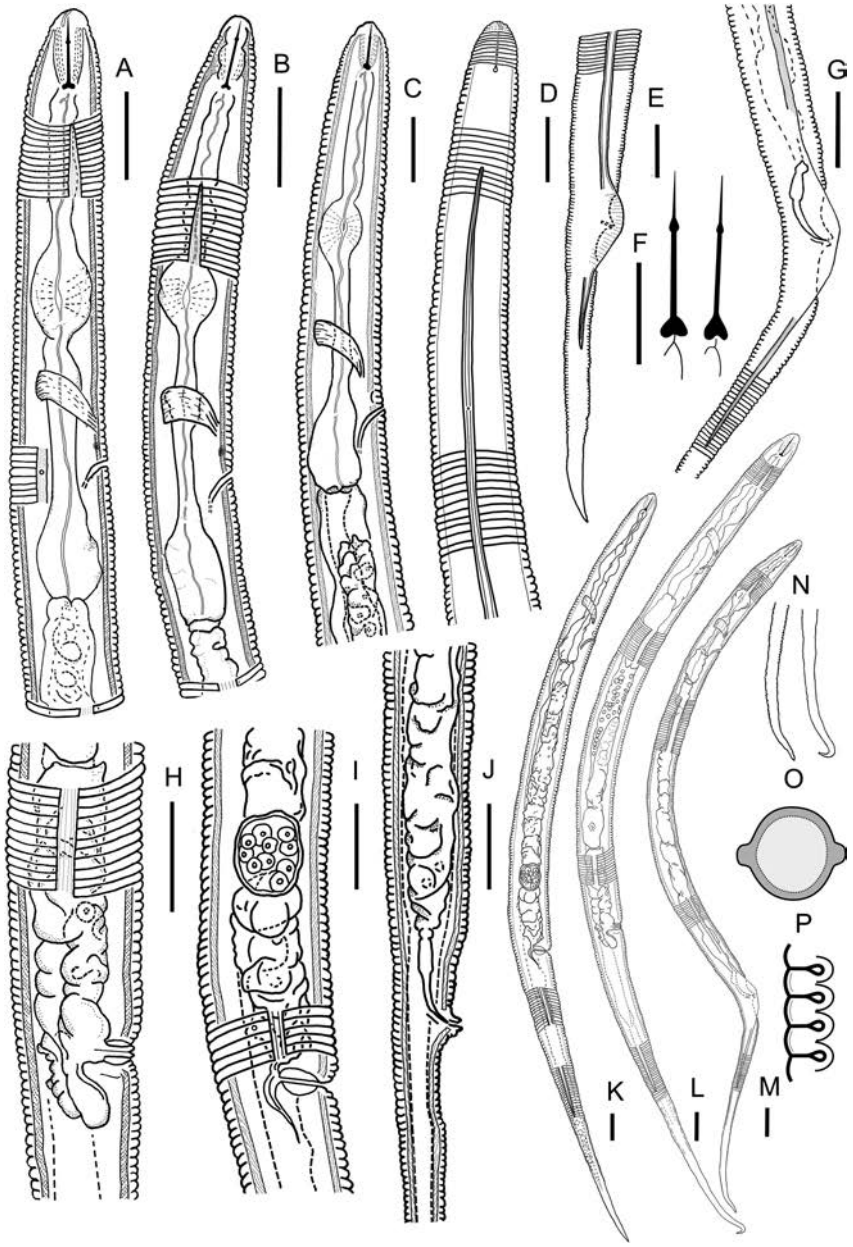

Fig. 2. *Malenchus sexlineatus* n. sp. from the Philippines, female holotype and male paratype (A, B, F-H, L-P) and Chinese population of *M. nanellus* Siddiqi, 1979 (C-E, I-K). A: Female anterior body; B: Male anterior body; C, D: Female anterior body; E: Male tail; F: Female stylet; G: Male tail showing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

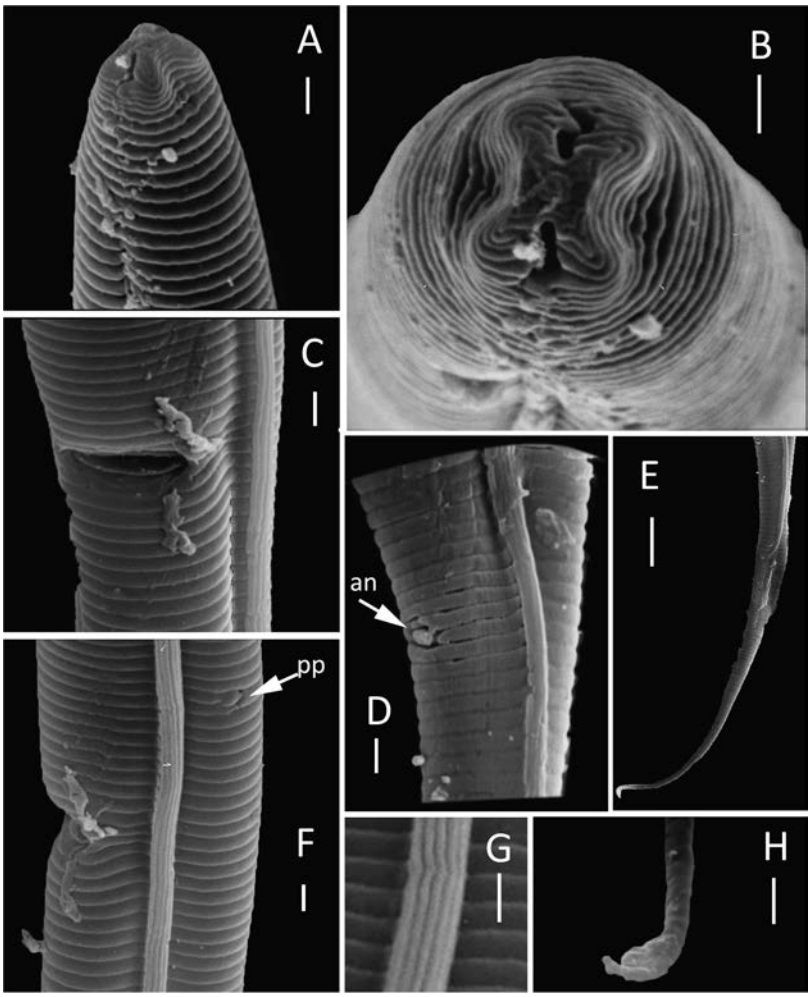

Fig. 3. SEM of female *Malenchus sexlineatus* n. sp. from the Philippines. A: Lip region; B: *En face* view; C: Ventral view of vulva showing epiptygmata; D: Anus (an = opening of anus); E: Tail; F: Lateral view of vulva (pp = prophasmid); G: Six incisures in lateral region; H: Hook-shaped tail tip. (Scale bars: A-D, F-H = 1 μm; E = 5 μm.)

Type material

Holotype female, four female paratypes and one male paratype deposited at the Museum Voor Dierkunde (Collection number UGMD104304), 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 species name has been registered in ZooBank (zoobank.org) under the identifier 6EE3BA51-E178-43C6-AD88-056083AA3D82.

Diagnosis and relationships

The new species is characterised by having six lines in the lateral fields, an exceptionally short body (270-288 μm), narrow annulations (0.7-0.8 μm) and a dorsally bent male tail after DESS relaxation. Although only 12 out of the 35 listed valid species by Geraert (2008) have an SEM image (7-12 lines have been detected), and whilst LM is unable to discern the exact number of lateral lines, the unique combination of features in *M. sexlineatus* n. sp. differentiates it from all other nominal *Malenchus* species.

Table 1. Morphometric data for *Malenchus exiguus*, *M. sexlineatus* n. sp., *M. nanellus*, *M. pachycephalus* and *Malenchus* sp. All measurements are in μm and in the form: mean ± s.d. (range).

Character	*M. exiguus*		*M. sexlineatus* n. sp.			*M. nanellus*		*M. pachycephalus*	*Malenchus* sp.
	Chinese population		Philippines			Chinese population		Chinese population	Chinese population
	Female	Male	Female		Male	Female	Male	Female	Female
			Holotype	Paratypes	Paratypes				
n	5	2	–	7	2	4	1	5	1
L	354 ± 5.0 (349-360)	360 ± 28 (333-387)	288	278 ± 6.2 (270-288)	289 ± 10 (281-296)	323 ± 17 (310-348)	312	476 ± 23 (456-502)	626
a	20.9 ± 1.2 (19.2-22.3)	26.3 ± 0.2 (26.2-26.5)	23.6	23.6 ± 1.7 (20.8-26.3)	30.1 ± 1.3 (29.2-31.0)	24.7 ± 2.45 (21.5-27.1)	26.2	18.9 ± 1.8 (16.9-20.8)	30.3
b	4.4 ± 0.3 (4.1-4.7)	4.4 ± 0.4 (4.1-4.7)	4.5	4.3 ± 0.2 (4.2-4.6)	4.1 ± 0.2 (4.0-4.3)	5.1 ± 0.4 (4.5-5.5)	4.5	5.5 ± 0.3 (5.2-6.0)	7.1
c	4.2 ± 0.2 (4.0-4.4)	3.7 ± 0.2 (3.5-3.8)	4.1	4.3 ± 0.1 (4.1-4.3)	3.8 ± 0.2 (3.7-3.9)	4.2 ± 0.6 (3.6-4.8)	4.0	6.3 ± 0.4 (5.8-6.7)	4.7
c′	10.7 ± 1.2 (9.3-12.2)	11.2 ± 1.4 (10.3-12.2)	11.8	11.6 ± 0.8 (10.8-12.7)	11.8 ± 1.1 (11.0-12.6)	11.8 ± 2.4 (9.1-13.3)	10.0	6.8 ± 1.0 (5.4-7.4)	11.4
V	62.4 ± 1.3 (61.4-64.2)	–	62.5	61.2 ± 1.0 (59.9-62.8)	–	60.2 ± 2.5 (58.3-63.8)	–	67.2 ± 1.4 (65.9-69.2)	64.3
V′	81.9 ± 0.8 (80.8-83.1)	–	82.6	79.7 ± 1.6 (78.9-82.7)	–	79.3 ± 3.1 (74.7-81.3)	–	80.0 ± 2.6 (77.6-83.5)	81.8
Tail length/vulva-anus distance	1.7 ± 0.1 (1.6-1.7)	–	1.9	1.5 ± 0.2 (1.4-1.8)	–	1.7 ± 0.3 (1.4-2.0)	–	1.0 ± 0.2 (0.8-1.2)	1.5
Start of lateral line to anterior end	24 ± 2.5 (21-26)	27 ± 1.5 (26-28)	12	12 ± 1.4 (11-15)	13 ± 0.3 (13-13)	19 ± 0.7 (18-20)	–	9.5 ± 0.1 (9.4-9.5)	36
Width of lateral line	2.0 ± 0.1 (2.0-2.1)	1.9 ± 0.03 (1.9-1.9)	2.0	2.0 ± 0.2 (1.6-2.3)	1.7 ± 0.4 (1.4-2.0)	2.4 ± 0.3 (1.2-2.7)	1.9	2.7 ± 0.2 (2.5-2.8)	2.3
Body diam.	17 ± 1.1 (16-17)	14 ± 1.3 (13-15)	12	12 ± 0.7 (11-13)	9.6 ± 0.1 (9.5-9.6)	13 ± 0. (12-15)	12	26 ± 2.8 (23-28)	21
Stylet	8.2 ± 0. 4 (7.5-8.7)	8.1 ± 0.6 (7.7-8.5)	6.2	7.0 ± 0.5 (6.2-7.5)	7.6 ± 0.2 (7.5-7.7)	8.3 ± 0.5 (7.8-9.0)	8.0	13 ± 0.6 (12-14)	15
MB	42 ± 2.1 (41-46)	43 ± 1.8 (42-44)	51	51 ± 1.9 (49-55)	51 ± 0.9 (50-51)	48 ± 3.2 (46-52)	52	50 ± 0.7 (49-50)	57
Excretory pore	64 ± 3.7 (60-67)	64 ± 7.4 (58-69)	49	47 ± 1.2 (45-49)	49 ± 1.2 (48-49)	57 ± 3.2 (54-60)	58	84 ± 3.2 (79-88)	62
Pharynx	82 ± 3.6 (77-85)	82 ± 0.5 (82-83)	64	64 ± 2.4 (60-65)	70 ± 0.8 (70-71)	64 ± 4.5 (58-70)	70	87 ± 2.6 (83-90)	88
Deirid	62 ± 2.9 (59-64)	63 ± 6.2 (59-68)	48	47 ± 0.9 (46-48)	49 ± 0.5 (49-50)	58 ± 1.6 (56-60)	–	82 ± 2.7 (79-85)	61
Nerve ring	59 ± 6.2 (52-65)	65 ± 4.5 (62-68)	42	41 ± 2.1 (39-44)	41 ± 2.2 (41-43)	43 ± 3.9 (40-48)	50	66 ± 2.6 (63-68)	70

Table 1. (Continued.)

Character	*M. exiguus*		*M. sexlineatus* n. sp.			*M. namellus*		*M. pachycephalus*	*Malenchus* sp.
	Chinese population		Philippines			Chinese population		Chinese population	Chinese population
	Female	Male	Female		Male	Female	Male	Female	Female
			Holotype	Paratypes	Paratypes				
Anus width or cloacal aperture width	8.0 ± 0.9	8.7 ± 0.4	5.9	5.8 ± 0.5	6.5 ± 0.6	6.3 ± 1.2	7.8	12 ± 1.9	12
	(7.1-9.1)	(8.5-9.0)		(5.1-6.4)	(6.1-7.0)	(5.1-7.4)		(10-14)	
Post-uterine sac or gubernaculum	7.6 ± 0.5	4.0 ± 0.6	7.6	7.2 ± 0.5	3.6 ± 0.2	7.2 ± 1.1	2.5	13 ± 2.9	13
	(7.2-8.3)	(3.6-4.4)		(6.6-7.4)	(3.5-3.8)	(6.5-8.0)		(9.4-15)	
Prophasmid to vulva or spicule	24 ± 3.2	14 ± 0.7	11	11 ± 2.0	11 ± 0.4	8.4 ± 2.1	14	26 ± 5.7	38
	(22-25)	(14-15)		(8.6-11)	(11-11)	(7.2-10)		(22-30)	
End of lateral line to tail tip	46 ± 1.8	51 ± 7.8	53	47 ± 2.8	53 ± 10	59 ± 17	45	41 ± 1.6	78
	(43-47)	(46-57)		(44-53)	(46-60)	(45-71)		(40-43)	
Tail	84 ± 2.7	98 ± 16	70	66 ± 2.7	77 ± 0.3	78 ± 13	78	76 ± 1.5	132
	(81-87)	(87-109)		(63-67)	(77-77)	(67-91)		(75-78)	
Annuli	1.1 ± 0.1	1.1 ± 0.1	0.8	0.8 ± 0.03	0.8 ± 0.1	1.2 ± 0.1	1.0	2.1 ± 0.1	1.7
	(1.0-1.3)	(1.0-1.1)		(0.7-0.8)	(0.7-0.9)	(1.1-1.3)		(2.0-2.2)	

Malenchus sexlineatus n. sp. is assigned to *Malenchus* based on the combination of the following morphological features: dorsoventrally compressed and anteriorly flattened lip region, very prominent cuticle annulations, protruding and conspicuous lateral field and body markedly narrowing posterior to vulva. At the subgenus level, the few lateral lines point to *Telomalenchus* Siddiqi, 2000, although this subgenus is characterised by straight amphidial apertures *vs* the S-shaped amphidial aperture typical for the subgenus *Malenchus* (Siddiqi, 2000). Furthermore, all *Telomalenchus* paratypes (*M. williamsi*, *M. leiodermis*, *M. parthenogeneticus*) examined by LM in the present study showed many differences with the proposed new species in morphological characters such as annulations (relatively weak annulations *vs* prominent cuticle annulations), vulval 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 species with available SEM in the subgenus *Malenchus*, these having numerous lateral lines. Nevertheless, *M. sexlineatus* n. sp. comes closer to the subgenus *Malenchus* because of the six incisures that are tightly arranged in one protruding ridge (two lines in LM) and the S-shaped amphidial apertures. Therefore, phylogenetic analyses are needed to verify/test the position of this species and other species in this subgenus.

Malenchus sexlineatus n. sp. is distinguished from *M. williamsi*, the only species in the genus with six lateral lines (based on currently available SEM data), by a shorter body of 278 (270-288) *vs* 452 (425-495) μm, shorter stylet (7.0 (6.2-7.5) *vs* 11-12 μm), narrower annulations (0.8 (0.7-0.8) *vs* 1.2-1.6 μm), lateral field comprising one elevated ridge in LM *vs* six well-separated incisures (resembling the lateral lines in *Cephalenchus* Goodey, 1962) in LM, the presence of S-shaped *vs* straight amphidial apertures, and vulval flaps absent or only one annuli long *vs* distinct. By having an exceptionally short body, *M. sexlineatus* 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 (see Table 2).

MOLECULAR CHARACTERISATION

Tree topologies inferred by ML and BI were largely congruent, except for several unresolved clades that were collapsed (original BI and ML tree available online at http://nematodes.myspecies.info). Bootstrap values and

Table 2. The taxonomically important features among related species. All measurements are in μm.

Species	Number of lateral lines in SEM	Stylet	Amphidial aperture	Vulva flap	Body length (♀)	Annuli width (♀)	Tail length (♀)	Ratios (♀)				
								a	c	c′	V	MB
M. acarayensis	⩾12	8.0-8.5	S-shaped	Absent/very small	280-410	1.00-1.70	70-76	19-26	3.8-5.5	7.5-10	61-66	47
M. bryanti	NA*	7.0-8.5	S-shaped	Absent	250-340	1.80-1.90	50-55	18-23	5.2-7.2	5.9-7.2	65-68	48
M. sexlineatus n. sp.	6	6.2-7.7	S-shaped	Absent/very small	270-288	0.69-0.79	63-67	21-26	4.1-4.3	11-13	60-63	49-55
M. parvus	>12	8.0-10	S-shaped	Absent	226-231	1.20-1.50	45-65	18-23	4.8-6.3	5.6-9.2	61-68	44-53
M. williamsi	6	11-12	Straight	Present	425-495	1.20-1.60	79-85	21-29	5.2-6.0	7.6-9.2	63-67	42-45

* NA = not available.

posterior probabilities are summarised on the Bayesian consensus tree (Fig. 4).

In all analyses, *M. sexlineatus* n. sp. was robustly supported (PP = 100, BS = 98) as sister taxon to *M. exiguus* (Massey, 1969) Andrássy, 1980. This clade together with *Malenchus* sp. P4 and sp. P9, *M. labiatus* Maqbool & Shahina, 1985 and *L. leptosome* form a fully supported clade (PP = 100, BS = 100) where P4 and P9 (unidentified due to only two juveniles recovered *ca* 500 m from the type locality) showed no genetic distance and only differed in sequence length. However, our phylogenetic analysis could not reveal any supported relationships of this clade with other taxa of Tylenchidae. Surprisingly our analyses did not prove the monophyly of the genus *Malenchus*, as *M. pressulus* (Kazachenko, 1975) Andrássy, 1981 was placed in a separate, unresolved position. The alternative topology showing the monophyly of the genus was tested and this morphologically based hypothesis was rejected based on SH and AU tests (SH test $p = 0.031$, AU test $p = 0.026$).

Malenchus pachycephalus Andrássy, 1981
(Fig. 1C, G, I-M)

Measurements

See Table 1.

Description

Female

General morphology typical of genus. Body relatively large, ventrally curved. Cuticular annulations coarse and wide. Lateral field consisting of two incisures as seen by light microscopy, deeply crenate, originating 3-4 annuli anterior to stylet base, ending at about mid-tail length. Lip region less dorsoventrally flatted than other species in genus. Stylet robust, cone *ca* one-third stylet length, one-fourth to one-fifth 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, *ca* one annulus wide. Vagina perpendicular to body axis, *ca* 10 μm long. Spermatheca elongated, simple/unilobed or bilobed (sperm present in proximal region of uterus), with round sperm cells, 27-49 μm long and 12-15 μm diam. Prophasmid *ca* 11 annuli anterior to vulva. Tail slightly ventrally curved, tip sharply pointed.

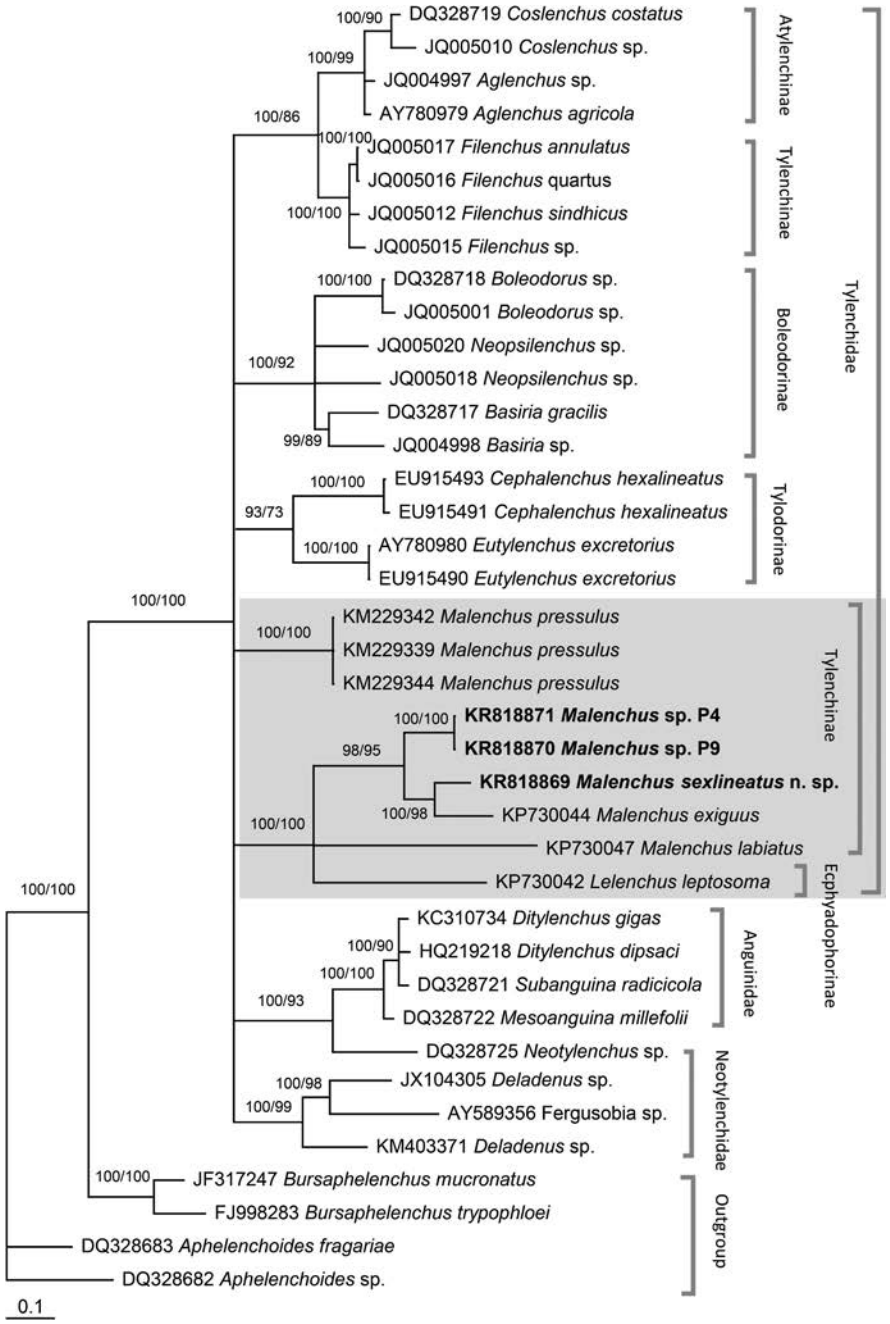

Fig. 4. Bayesian strict consensus phylogeny highlighting the phylogenetic position of *M. sexlineatus* n. sp. in relation with other relevant sequences from GenBank based on the D2-D3 domain of LSU rDNA sequences. Branch support is indicated in following order: PP value in BI analysis/BS value from ML analysis. New sequences generated in this study are highlighted in bold.

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

Malenchus 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 the original description (Andrássy, 1981), except for a slightly longer tail (76 (74-78) *vs* 65-72 μm) and the end point of the lateral field at 50 *vs* 25-33% of tail length. This Chinese population also resembles the Spanish population (Gómez-Barcina *et al.*, 1992), but has a longer tail (76 (74-78) *vs* 56-69 μm).

***Malenchus nanellus* Siddiqi, 1979**
(Figs 1B, D, H; 2C-E, I-K; 5)

MEASUREMENTS

See Table 1.

DESCRIPTION

Female

Body short. Lip region typical of genus, dorsoventrally flattened. Cuticle strongly annulated. SEM showing fine, longitudinal striae on annuli. Lateral field smooth, *ca* one-sixth body diam., starting at mid-point of procorpus or 16 annuli from anterior end (or *ca* one stylet length posterior to stylet base) and ending at 75% of tail length. SEM showing large amphidial apertures at lateral borders of labial plate, continuing as sinuous slits along lateral side of lip region. Stylet slender, cone *ca* one-third of total length, cone width one-third of anterior shaft and one-fourth of posterior shaft. Median bulb oval with distinct valve. Excretory pore located mid-way between nerve ring and basal bulb. Deirid at level of excretory pore. Prophasmid 9-10 annuli anterior to vulva. Reproductive system monodelphic, prodelphic, ovary outstretched with oocytes arranged in a single row. Crustaformeria 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, 10 μm long and 6.6 μm broad), and with oval sperm cells. Tail 67-91 μm long, tail tip fine, ventrally bent.

Male

Less common than female. Resembling female in most features except for genital system and narrower annulations. Bursa *ca* 28 μm long, starting at level of spicule capitulum.

HABITAT AND LOCALITY

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

REMARKS

Malenchus nanellus was originally described by Siddiqi (1979) from maize rhizosphere in 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 μm), shorter tail (67-91 *vs* 80-90 μm) and some variation of MB (46-52 *vs* 42-45).

Study of the amphidial aperture shows that the lateral slit is not visible using LM in early juvenile stages, only the presence of oval holes being indicated in the labial plate (Fig. 5E1). In late juvenile stages, very narrow sinuous slits are visible both in SEM (Fig. 5D) and LM, indicating a gradually laterally expansion of the slit (Fig. 5E2, 3). In the adult stage, the width of this S-shape slit increases (Fig. 5E4).

Notably, although the starting point of the lateral field was used as a 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) varying from stylet knob level, mid-region

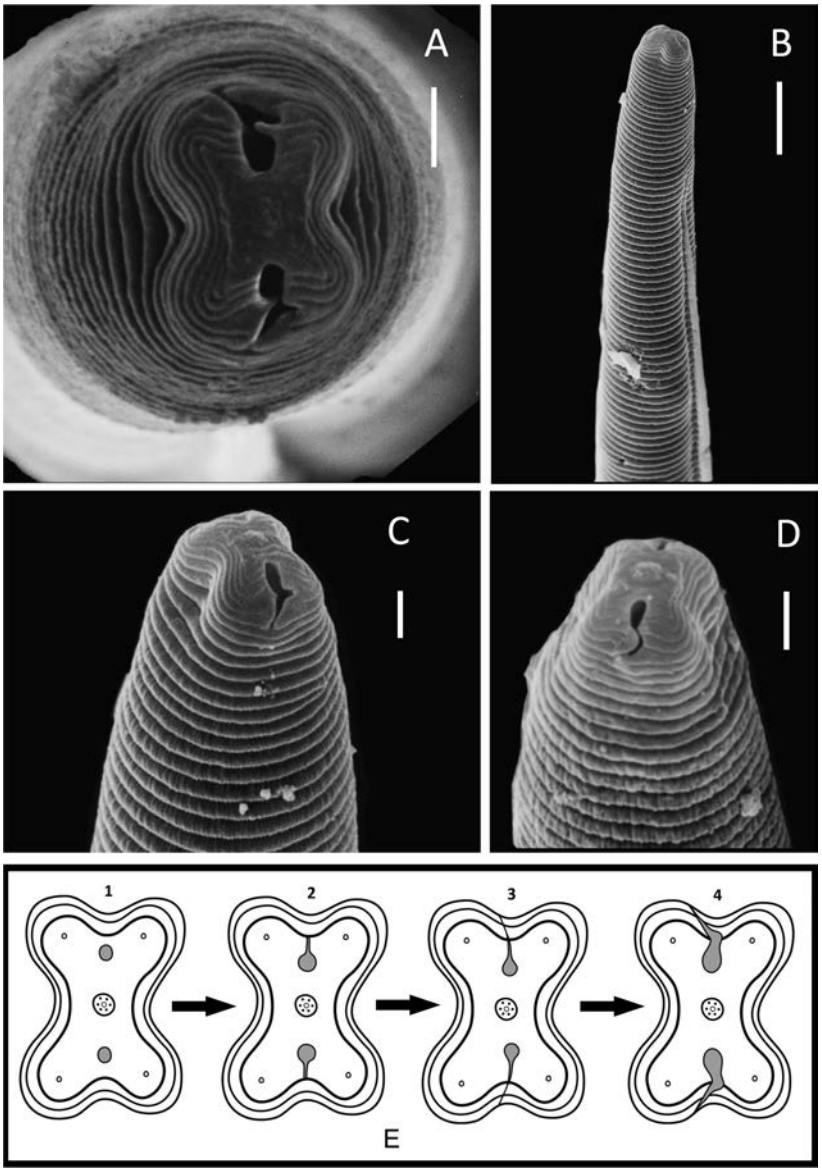

Fig. 5. SEM of female and juvenile of *Malenchus nanellus* from China, and the possible development process of amphidial aperture. A: *En face* view of female showing oval hole in anterior part of amphidial aperture; B: Anterior part of female; C: Lateral view of female lip region; D: Lip region of juvenile; E: Possible development process of amphidial aperture. (Scale bars: A, C, D = 10 μm, B = 50 μm.)

of the procorpus to the 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 question. However, in spite of some variation in the starting point of the lateral field, it is always located at more or less the mid-region of the

procorpus in the present Chinese population, indicating that this feature is stable within the studied population herein.

The spermatheca shape in *Malenchus* 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 the Chinese population is high, *i.e.*, from rounded to elongate; sperm constrained in the spermatheca to present both in the spermatheca and in the proximal part of the uterus (in the latter case spermatheca appearing bilobed). 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 exiguus* (Massey, 1969) Andrássy, 1980**

= *Aglenchus exiguus* Massey, 1969
= *Ottolenchus sulcus* Wu, 1970
= *M. sulcus* (Wu, 1970) Siddiqi, 1979
(Figs 6A, B, G, H, J; 7A, B, D, E-G, I, J, M-O, R)

MEASUREMENTS

See Table 1.

DESCRIPTION

Female

Body small to middle-sized. Lip region typical of genus, dorsoventrally flattened. Lateral lines consisting of one ridge, slightly crenate, starting close to median bulb (21-26 annuli from posterior to end of lip region) and ending at mid-point of tail. Amphidial aperture sinuous-shaped. Stylet slender, cone *ca* one-third of stylet length long. Median bulb oval, valvular apparatus round, conspicuous. Prophasmid inconspicuous, 9-13 annuli anterior to vulva. Reproductive system monodelphic-prodelphic, ovary outstretched, crustaformeria with five cells in each row. Vulva sunken into body, vagina thickened, lateral flap distinct, 2-3 annuli long. Spermatheca rounded, simple/unilobed, offset, filled with sperm. Tail ventrally bent, filiform with pointed terminus.

Male

Less common than female. 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-shaped, sperm cells round. Bursa *ca* 30 μm long, starting at level of spicule 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. on Mount 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 and morphometrics of *M. exiguus*, except for a slightly shorter stylet (8.1 (7.7-8.5) *vs* 9-10 μm). Although, the key of Geraert (2008) brought us initially to *M. acarayensis*, clear differences with the type material of *M. acarayensis* include a higher tail/vulva-anus ratio (1.7 (1.6-1.7) *vs* 1.3-1.4 μm), narrower annuli (1.1 (1.0-1.1) *vs* 1.5-1.7 μm) and broader lip region (relatively round *vs* more compressed and flattened).

***Malenchus* sp.**

(Figs 6C-F, I; 7C, H, K, L, P, Q)

MEASUREMENTS

See Table 1.

DESCRIPTION

Female

Only a single specimen of this species was collected. Body large. Cuticle coarsely annulated and folded between annuli. Lateral field not crenate, consisting of two incisures, starting five annuli posterior to stylet knobs and ending at mid-tail. Lip region continuous, not elevated, slightly flattened or not, 9.1 μm wide at base. Amphidial aperture S-shaped. Stylet prominent, cone 5.9 μm long, cone base width 25% of anterior and 20% of posterior shaft width. Median bulb relatively robust for 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 end with a pointed terminus.

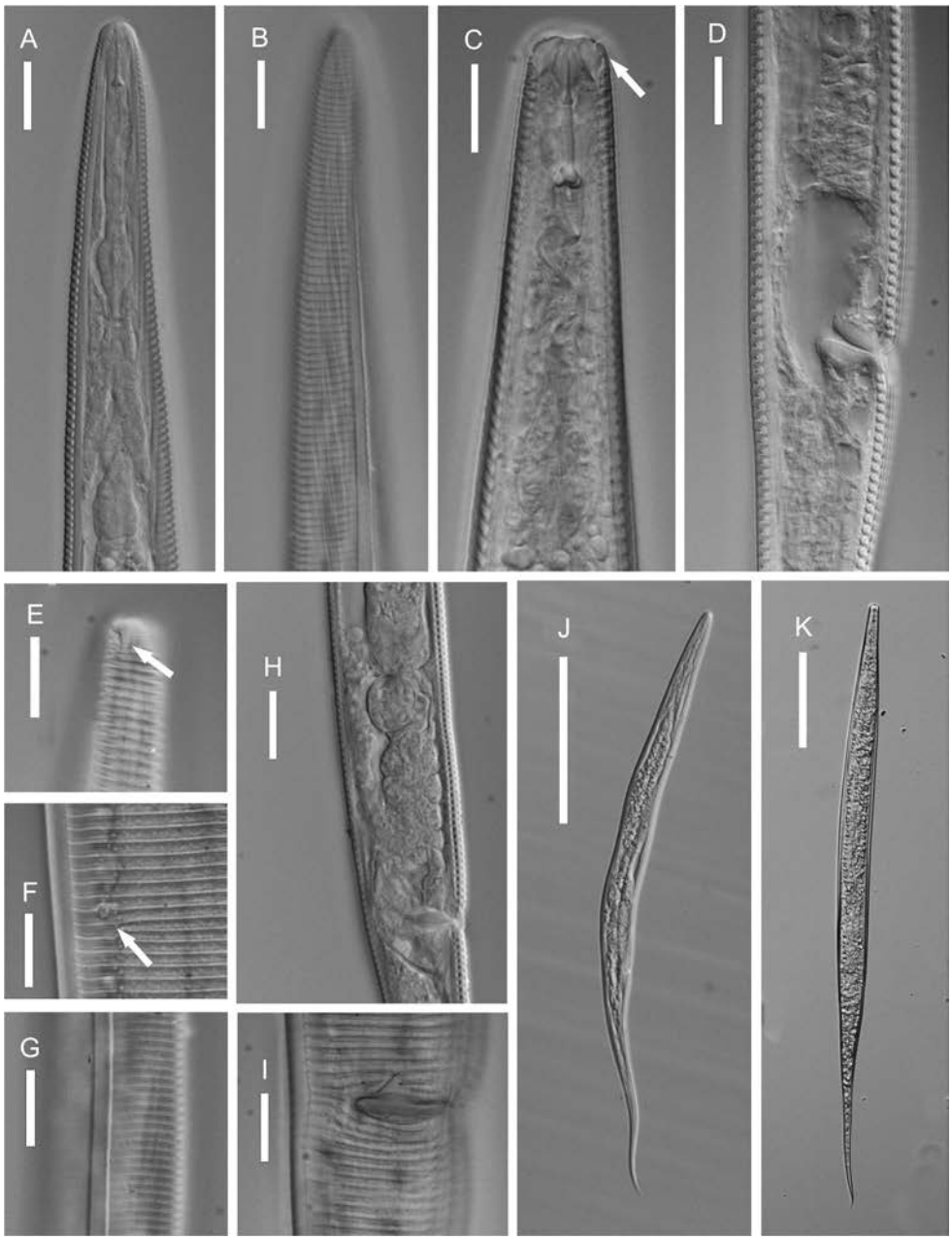

Fig. 6. LM picture of *Malenchus exiguus* (A, B, G, H, J, K) and *Malenchus* sp. (C-F, I). A, B: Female anterior body; C: Ventral view of female anterior body (arrow showing amphidial fovea); D: Female reproductive system showing sunken vulva, thicken vaginal wall; E: Female lip region (arrow showing amphidial aperture); F: Prophasmid (arrow); G: Lateral region with offset ridge; H: Female reproductive system showing part of ovary, spermatheca, uterus, vagina and sunken vulva; I: Ventral view of vulva; J, K: Female body habitus. (Scale bars: A-G, I = 10 μm; H, J, K = 100 μm.)

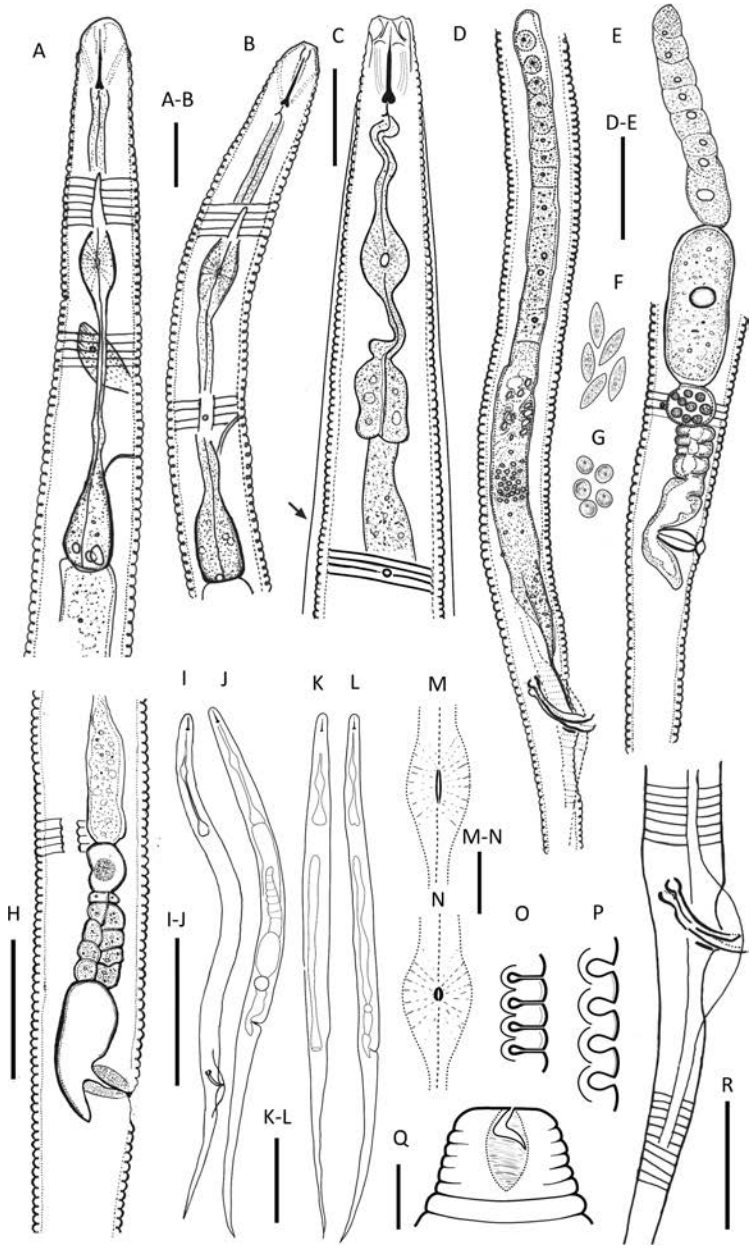

Fig. 7. *Malenchus exiguus* (A, B, D, E-G, I, J, M-O, R) and *Malenchus* sp. (C, H, K, L, P, Q). A: Female anterior body; B: Male anterior body; C: Ventral view of female anterior body, arrow showing elevated lateral ridge; D: Male reproductive system; E: Female reproductive system; F: Spermatids from *vesicula seminalis*; G: Sperm cells from *vesicula seminalis*; H: Female reproductive system; I: Male habitus; J-L: Female habitus; M: Male median bulb showing elongated valvular apparatus; N: Female median bulb showing round valvular apparatus; O, P: Folded cuticle; Q: Lateral view of lip region showing amphidial aperture and fovea; R: Male tail. (Scale bars: A, B = 10 μm; C, D, E, H, R = 20 μm; I-L = 100 μm; M, N, Q = 5 μm; F, G, O, P = diagrammatic.)

Male

Not seen.

Habitat and locality

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

Remarks

The single specimen recovered has an exceptionally large body, which makes it close to *M. novus*. This rare species is known only from the type description from eastern Russia. The general morphology of the single female fits well with the original description of that species 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 specimen.

Discussion

Molecular characterisation and phylogeny

Recent studies (van Megen *et al.*, 2009; Bert *et al.*, 2010; Atighi *et al.*, 2013) based on 18S rDNA 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 or geographic location was provided (Holterman *et al.*, 2006). Recently, a 28S rDNA-based phylogeny indicated a robustly (PP = 69, BS = 99) supported clade harbouring all *Malenchus* spp. species and *Lelenchus* (Yaghoubi *et al.*, 2015). However, we have not been able to reproduce this result (especially the high BS value), even with the same data and using the 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. sexlineatus* n. sp., *M. exiguus* and an unidentified *Malenchus* species, but the relationship of *M. labiatus* and *L. leptosoma*, 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 character of the lateral field being represented by a single elevated ridge. However, *M. presulus* also has a single ridge and appears within the 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 dorsoventrally 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* 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 characterisation and position of *Malenchus* within Tylenchidae is still unsettled. Moreover, morpho-

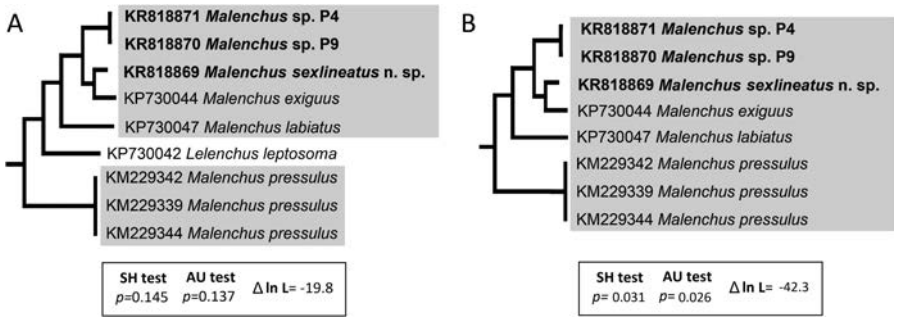

Fig. 8. Comparing alternative hypotheses using AU and SH test. The topological schemas (hypotheses) are compared with the originally obtained topology (Fig. 4). Clades containing *Malenchus* species are highlighted in grey. A: The hypothesis of paraphyly of *Malenchus* as robustly supported (BS = 99) in the analysis of Yaghoubi *et al.* (2015); B: The hypothesis of monophyly of *Malenchus*. Δ ln 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.

logical 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. sexlineatus* n. sp. as a new species within *Malenchus*. Nevertheless, a wider and more comprehensive analysis using additional genetic markers is needed, not only for this genus but for Tylenchidae in general, in order to define molecularly based clades and associated morphological apomorphies.

Remarks on amphidial aperture development

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

Generally, the present observations agree with the studies of Andrássy (1981) and Gómez-Barcina *et al.* (1992) in that 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, inspection of a Chinese population of *M. nanellus* showed that the morphology of the amphidial aperture changes according to the life stage of the species (Fig. 5A, C-E). However, a straight aperture, as known for the subgenus *Telomalenchus*, was never observed and the oval hole in the labial plate

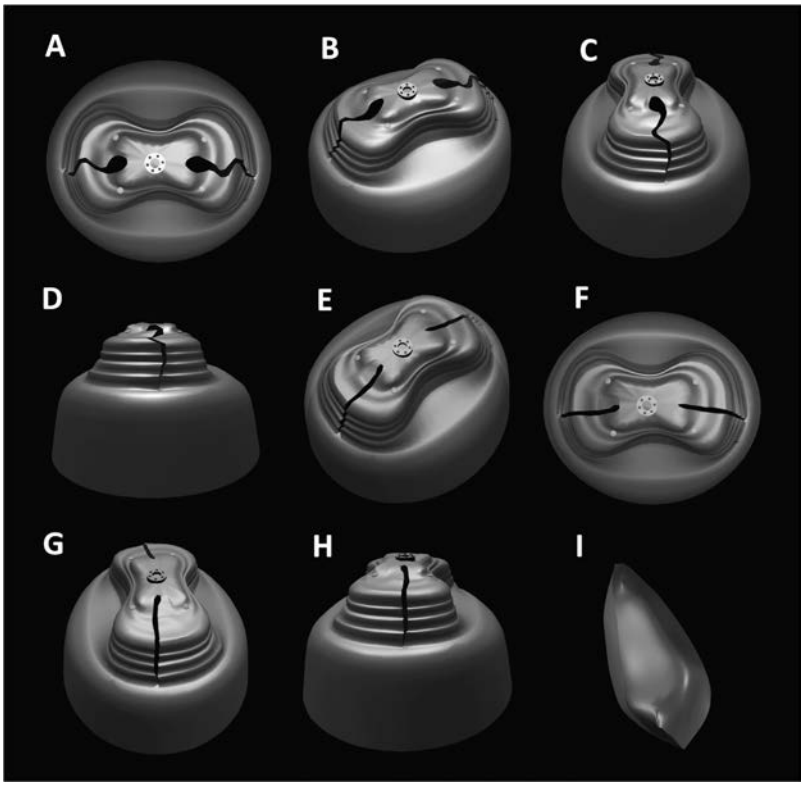

Fig. 9. 3D models of the lip region of the two subgenera of *Malenchus*. A-D: S-shaped amphidial aperture, subgenus *Malenchus*; E-H: Straight amphidial aperture, subgenus *Telomalenchus*; I: Lateral view of amphidial fovea.

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 is an intrinsic character of the subgenus *Malenchus*. The change during development may be explained as an adaptation to its multiple functions, *e.g.*, feeding habit, mating, moving, sensing chemicals or moisture (Bumbarger *et al.*, 2009) within the different life stages, or simply as structural changes in different developmental stages but without any functional link.

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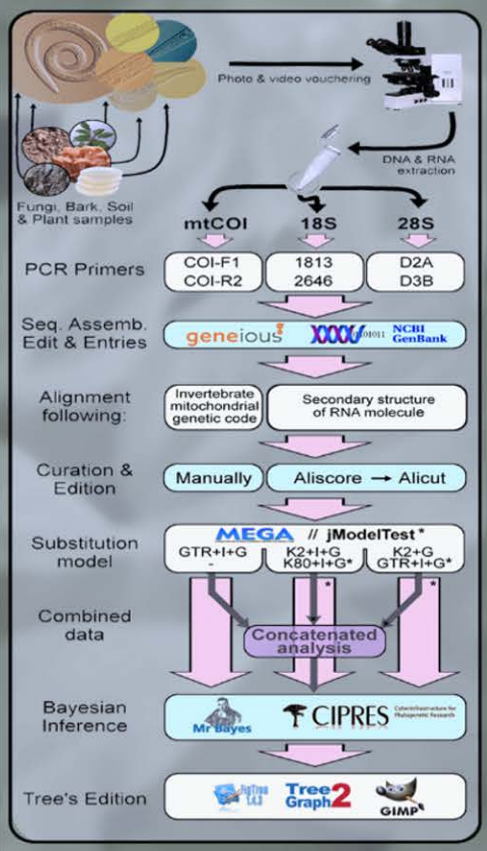
The genus *Aphelenchoides* belongs to one of the four plant-parasitic lineages within Nematoda. Although it is composed of mostly mycophagous species, 14 are plant-parasites, including the foliar nematodes, *i.e. A. besseyi*. *A. ritzemabosi* and *A. fragariae*. This genus is complex in many ways and is an excellent example of the challenges associated with Nematology: it has an overwhelming diversity, it is highly abundant in diverse substrates and its possible associations with microorganisms and invertebrates are understudied. Yet, most described species lack molecular data and comprehensive morphological data.

In this thesis we updated the knowledge on the range of plants associated with the plant-parasitic species, we compared the phylogenetic resolution of three molecular markers and their usefulness for diagnosis (see figure at the right side for methodology overview) and we deepened our understanding of the phylogenetic relationships and taxonomy of the genus by combining molecular and morphological data. An amendment to the genus diagnosis and a new grouping system based on tail-shape morphology are proposed, and the mitochondrial COI gene is put forth as an appropriate barcoding region for *Aphelenchoides*.



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