Phylogenetic relationships within major nematode clades based on multiple molecular markers

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CONTENTS

CHAPTER 1
INTRODUCTION

I. Molecular phylogenetics, DNA barcoding and their implication for nematode detection

Molecular phylogenetics tries to infer the process of organismal evolution from patterns of DNA/protein variation. In the age of rapid accumulation of this macromolecular data, comparisons of homologous gene and protein sequences have become more advanced and can reveal compelling information about relationships between different organisms. This can be very helpful; especially in situations where there is lack of fossil data or when morphological features are scarce. However, not every genomic sequence serves as a good molecular marker. This depends on the nature of the organismal group under investigation as well as on the taxonomic level of interest. Only the ones evolving at a proper rate and thus harboring an informative amount of mutations for a given phylogenetic question can be used. Otherwise a phylogenetic signal will be either too ambiguous, when sequence evolves at a very fast rate, or too limited, when it evolves slowly. Additionally, due to different ranges of intra and interspecific genetic variation, it is impossible to employ a universal molecular marker that could be used for all known organismal kingdoms. Consequently, it may happen that a gene can give a very good resolution in one group of organisms, being at the same time uninformative in another. For instance, mitochondrial genes like cytochrome c oxidase subunit I (COI) are extensively used in animals and fungi but they show little variation in plants. For deep phylogenetic studies in plants other mitochondrial genes such as *atp1*, *matR* and *nad5* are combined with phylogenetic signal generated by chloroplast and nuclear genes [1]. In bacteria and also many invertebrates, small subunit ribosomal DNA (SSU rDNA), a relatively conserved nuclear gene, is frequently used to study deep to moderately shallow phylogenetic relationships. It is *e.g.* widely used in phylogenetic studies of nematodes, and it is currently the only gene that is used to deduce phylum-wide evolutionary relationships [2] [3] [4]. To obtain more detailed resolution and while striving to convert current gene trees into an approximation of 'the true organismal trees', additional genes need to be engaged. Most molecular markers derive from genes associated with critical physiological functions. They are positively selected in the process of evolution and therefore they can be easily found in almost all organisms. Preferentially, genes used in phylogenetics should be single copy genes or multiple copy genes with the same function subject to efficient intrachromosal and/or extrachromosomal homogenization mechanisms. After all, orthologous genes are separated by the process of speciation. All this conditions are not inconsequential for DNA barcoding, a method that exploits the presence of short characteristic genomic sequences, also called DNA sequence signatures, for species identification. Detection of organisms on the basis of unique DNA motifs finds a broad application in nature conservation studies, biodiversity surveys or organismal detection where flagging of species that are

extinct, cryptic, yet unclassified or newly found is crucial. Thus, it is not surprising that DNA barcoding is especially important in such organismal groups as nematodes. Nematodes are a ubiquitous and speciose group of predominantly vermiform organisms that are mainly found in soil and sediments. The lack of informative morphological characters in combination with their high societal impact - numerous plant and animal pathogenic species reside amongst them – prompted researchers to explore possibilities to exploit taxon-specific DNA motifs for their qualitative or quantitative detection in complex DNA backgrounds.

Large molecular frameworks offer opportunities for the development of DNA-based methods for nematode detection. Within such a framework, unique DNA sequence signatures can be identified and these motifs can be exploited for the (quantitative) detection of nematode families, genera and even species. Probes/primers designed base on those sequences allow for taxon detection and identification. Currently proposed detection methods are based on techniques such as direct sequencing [5], PCR DGGE [6], T-RFLP [7] or quantitative PCR [3] [8] [9] [10].

II. Biology and economic relevance of cyst, root-knot, and lesion nematodes

Root-knot, lesion and cyst nematodes, members of the genera *Meloidogyne, Pratylenchus* and *Heterodera / Globodera,* pose a serious threat to main agricultural crop such as potato, sugar beet, and soybean. As such these distal representatives of the order Tylenchida constitute the most economically detrimental group of plant parasitic nematodes. Among the three genera mentioned above, root-knot nematodes such as *Meloidogyne incognita, M. hapla,* and *M. chitwoodi*, are most polyphagous, being able to infect almost all domesticated plants worldwide [11]. The invasion of plant roots by root-knot and cyst nematodes leads to the formation of nematode feeding sites. In case of sedentary endoparasites, establishment of so called 'giant cells' or 'syncytia' (root-knot and cyst nematodes respectively) is considered as one of the most sophisticated adaptations of plant parasitism. These feeding sites are used till the end of the nematode life cycle and serve as sink tissues to which nutrients are imported in a symplastical or apoplastical way [12]. On the other hand, migratory endoparasites, such as *Pratylenchus* species, exhibit feeding strategies that may be considered as less refined but no less successful keeping their proliferation and host range in mind. Various life stages of lesion nematodes move freely through the root to feed and reproduce, creating numerous local tissue lesions, which are used as an entrance for the secondary pathogens such as bacteria or fungi. The feeding takes place mostly in the root cortex but the uptake of nutrients, especially by the younger stages unable to perforate thicker epidermis, may also derive from the root hair area [13]. The parasitic success of the mentioned groups of nematodes is undoubtedly a result of their unusual ability to overcome the barrier of the plant cell wall. This biological obstacle is mechanically and chemically weakened by the stylet-driven puncturing of the cell wall in combination with the local release of cell wall-degrading and modifying proteins.

III. Horizontal gene transfer and its possible implications in nematodes

Any movement of genetic information, other than its vertical transmission from parents to their offspring via process of reproduction, is defined as horizontal or lateral gene transfer (HGT or LGT). Although well described in Archaea and Bacteria, knowledge about HGT in higher eukaryotes is mostly limited to the mobile genetic elements and endosymbiotic theory concerning the ancient acquisition of plastids and mitochondria. Relocation of genes from those organelles to the nucleus and transfer of genetic material between unrelated species represent two ways of HGT among eukaryotes. Phylogenetic studies on simple eukaryotes show that intra-domain gene transfers are not so uncommon as previously thought. Insufficient samplings as well as lack of systematic investigations of completely sequenced genomes may even have caused a significant underestimation of those events [14] [15]. On the other hand, genetic transfers from prokaryotes to simple eukaryotes are relatively frequent. So far, lots of genes of probable bacterial origin have been found in most of the investigated protist genomes while genes of symbiotic *Wolbachia* bacteria have been found in nuclear genomes of many eukaryotes such as fruit flies or nematodes [16] [17]. Strikingly, sequencing project of a filarial nematode *Brugia malayi* revealed more than 200 contigs containing probably non-functional *Wolbachia*-like sequences [17].

The biological association between *Wolbachia* and *B. malayi* is one of many examples of various interactions involving bacteria and nematodes. Being extremely abundant and speciose in soil and sediments, some nematodes unavoidably co-exist with bacteria in these environments. Members of the subfamily *Stilbonematinae* live in symbiosis with sulfate-reducing bacteria in sulfide-rich marine sediments [18], while entomopathogenic nematodes, members of the genera *Heterorhabditis* and *Steinernema*, form intimate relationships with the γ-proteobacteria *Xenorabdus* or *Photorhabdus* spp. [19]. In a few occasions bacteria can be pathogenic for nematodes causing series of diseases or even their death [20], but the simplest interaction is the one in which bacteria serve as a food source for nematodes [21] [22]. This constant exposure and, in some cases, strongly dependent relationships fulfill main HGT conditions including habitat overlap, physical contact, and symbiotic or trophic relationships which support the "you are what you eat" theory [23].

In fact, trophic preferences within phylum Nematoda are versatile, ranging from grazing on bacteria through feeding on fungal hyphae, algae, plants or other nematodes [22]. Analysis of neutral genes (not associated with feeding) suggests that most (if not all) trophic ecologies have arisen multiple times [2] [3]. According to Holterman's clade division of the phylum Nematoda, representatives of plant parasites can be found within at least three groups: Clade 1 - Triplonchida, Clade 2 - Dorylaimida and Clade 12 -Tylenchida. Phylum-wide phylogenetic data apparently support the evolution from bacterivores via fungivores to plant parasites for these three groups. At the very base of Clade 12, a sister relationship is observed between the superfamily

Aphelenchoidea [24], the predominantly fungivorous genera *Aphelenchus* and *Paraphelenchus,* and all other, mainly plant parasitic, members of Tylenchida. Another example is found in Clade 1: ribosomal DNA data suggest a sister relationship between the fungivorous genus *Diphtherophora* and the ectoparasitic Trichodoridae. The remaining major lineage of plant parasitic nematodes, the Longidoridae, resides in Clade 2. At least two fungivorous genera are represented in Clade 2, *Tylencholaimus* and *Tylencholaimellus,* but their relationship with the Longidoridae is so far unresolved [25].

Focusing on the Tylenchida, all plant parasitic nematode taxa investigated so far harbor genes encoding cell wall-modifying or cell wall-degrading enzymes (CWDE). This also holds true for *Aphelenchus avenae*, a predominantly fungivorous member of the order Aphelenchida (superfamily Aphelenchoidea). At least eight different cellulases have been found amongst CWDEs identified in a genome of this nematode and they all belong to the GHF5 [26]. This becomes quite surprising when considering a fact that the cell wall of true fungi does not contain cellulose. Interestingly, although *A. avenae* is generally described as a fungivore, there are several reports showing that members of this genus can feed on plants as well [27] [28]. Hence, at least some *Aphelenchus* species are facultative plant parasites. It is additionally noted that members of the genus *Paraphelenchus*, the other genus in this superfamily, are apparently without exception truly fungivorous [24].

It is hypothesized that the evolution of plant parasitism among nematodes is related to the acquisition of CWDEs from different microbial donors via multiple independent HGT events [29]. If this is true for Clade 12, we hypothesize that at least one HGT event - the one that provided the members of this clade with GHF5 cellulases - took place in the common ancestor of the orders Tylenchida and Aphelenchida (superfamily Aphelenchoidea). A repertoire of those proteins, including β-1,3 - and β-1,4 -endoglucanases, pectate lyases, polygalacturonases, expansins or cellulose-binding proteins, have also been found in some plant parasitic nematode species outside of Clade 12, namely in the superfamily Aphelenchoidoidea (*e.g. Bursaphelenchus xylophilus*) and Dorylaimida (*e.g. Xiphinema index*).

IV. Cellulases in plant parasitic nematodes

β-1,4-endonucleases were the first discovered in two cyst nematode species *Globodera rostochiensis* and *Heterodera glycines* [30], and are relatively well studied cell-wall degrading enzymes among nematodes. They are encoded by a multi-copy gene family, which is expressed in the subventral esophageal glands of the infective second stage juveniles (J_2). A biochemical activity of these enzymes is determined as a hydrolysis of β-1,4 glycosidic bonds of cellulose microfibrils. According to their biochemical characteristics, cellulases are represented in various glycoside hydrolase (GH) families. All members of the order Tylenchida investigated so far (even the insect parasite *Delandenus siridicola*) harbor cellulases belonging to the

family 5 (GHF5; glutamic acid (Glu) residues essential for catalysis). A facultative plant parasite belonging to a non-Tylenchida lineage, the pinewood nematode *Bursaphelenchus xylophilus*, was shown to produce cellulases from glycoside hydrolase family 45 (GHF 45; aspartic acid (Asp) residues essential for catalysis) [31]. More recently, GHF5 cellulases were characterized from another facultative plant parasite, *Aphelenchoides fragariae* [32]. Notably, this finding was the result of a directed search as the authors used degenerated GHF5 primers to screen for the presence of cellulases in *A. fragariae.* Remarkably, the expression levels of *Afr-eng*-1 (and even the bare presence), one of the cellulases of *A. fragariae* depended on the food source. After changing the diet from plants to fungus, the cellulase expression levels decreased, and after 100 generations feeding on the fungus *Cylindrocladium*, the *Afr-eng*-1 transcript was no longer detectable [32]. It is worthwhile mentioning that the families Aphelenchoididae (*A. fragariae*) and Parasitaphelenchidae (*B. xylophilus*) are relatively related: each family is residing in one of the branches of a single major subclade in Clade 10 [4].

The GHF5 genes in nematodes comprise at least a catalytic domain, and occasionally this is connected to a linker and/or a type II cellulose binding domain (CBDII). Due to the slight differences in intron-exon composition and noticeable phylogenetic distance, those cellulases are thought to belong to at least two distinct lineages [33] [34]. These lineages are referred to as *CelI* and *CelII* by Rehman *et al.* [35], and in a more recent study these are similar to catalytic domains type B and type C respectively [36]. Phylogenertic analysis of nematode GHF5 cellulases suggests for an early acquisition of this category of plant cell wall degrading enzymes, maybe even by the common ancestor of the order Tylenchida and the family Aphelenchidae (subfamilies Aphelenchinae and Paraphelenchinae) [36].

Although recent nematode genome sequencing projects for *Meloidogyne incognita* [37]; *Meloidogyne hapla* [38], *Globodera palida*; (UK consortium 2011; http://www.sanger.ac.uk/resources/downloads/helminths/globodera-pallida.html) resulted in discoveries of numerous new cellulases (so far all belonging to GHF5), this approach is evidently laborious and too undirected to discover the phylogenetic history of a single gene family.

V. Scope of this thesis

In the first part of this thesis, we investigate and exploit the phylogenetic signal present in the small and large subunit (SSU and LSU) of the ribosomal DNA to elucidate the evolutionary relationships within the ecologically relevant subclass Dorylaimia (members are highly sensitive to environmental disturbances; **Chapter 2**) and within the genus *Aphelenchoides*, a taxonomically highly unstable group harboring a number of plant parasites (usually referred to as foliar nematodes) that are morphologically barely distinguishable from their harmless fungivorous relatives (**Chapter 3**). Next to the phylogenetic relationships, it is

presented how characteristic SSU rDNA motifs can be used in quantitative PCR (qPCR) assays for the detection and the quantification of the ecologically (Dorylaimia) and economically (*Aphelenchoides*) significant groups of nematodes.

In **Chapter 2**, the SSU-rDNA-based analysis for the subclass Dorylaimia provided ample phylogenetic resolution with the order Mononchida (mainly carnivores) whereas the resolution within the sister order Dorylaimida was relatively poor. In order to solve this, a 1,000 bp fragment of the 5′ region of the more variable LSU rDNA was sequenced, and indeed this resulted in a clearer and better-supported topology within this order. These results raised doubts about the tenability of number of Dorylaimid families such as the Qudsianematidae, the Nordiidae and the Dorylaimidae. Fortunately, this did not come as a surprise for taxonomic experts on this particular group of nematodes. Moreover, the data set implied that plant parasitism had arisen at least twice in the order Dorylaimida, but the relationship with two fungivorous genera within this order, *Tylencholaimus* and *Tylencholaimellus*, could not be established unequivocally.

In **Chapter 3** it is shown how problem of the distinction between plant parasitic foliar nematodes and fungal feeding *Aphelenchoides* can be overcome by using the molecular identification method. The detection assays have been designed for four *Aphelenchoides* species: *A. besseyi, A. fragariae, A. ritzemabosi* and *A. subtenuis*. The SSU rDNA-based phylogeny that included all currently available Aphelenchoididae showed a well-supported split among the foliar nematodes into two subclades. Together with a number of exclusively fungivorous species *A. fragariae* and *A. subtenuis* were placed in branch distinct from the one in which two notorious plant parasites, *A. besseyi* and *A. ritzemabosi*, are residing.

In the second part of my thesis, a wide range of PCR primers and (q)PCR approach were used in order to search for new β-1,4-endonucleases belonging to GHF 5 (**Chapter 4**) and to amplify the largest subunit (RPB1) of RNA polymerase II (**Chapter 5**) among members of Tylenchida. We focused mainly on *Meloidogyne* and *Pratylenchus* genera, in which the phylogenetic relationship – studied based on small subunit ribosomal DNA (SSU rDNA) – remained unresolved [39].

In **Chapter 4** we not only report new cellulase sequences in *Pratylenchus spp.* but also for the first time in genus *Hirschmaniella*, nematodes *Meloidogyne ichinochei, M. artiellia, M. ardenensis* and *Globodera pallida*. Joined phylogenetic analysis of the currently known GHF5 cellulases from Tylenchida revealed three types of catalytic domains (A, B and C). Moreover, the overall topology of the, numerically dominant, catalytic domain type B remarkably resemble the one of the SSU rDNA-based tree.

In **Chapter 5** the rDNA SSU and the largest subunit (RPB1) of RNA polymerase II genes are employed in order to identify a living representative of the genus *Pratylenchus* that could be the closest to the Meloidogynidae clade.

References

1. Soltis DE, Smith SA, Cellinese N, Wurdack KJ, Tank DC, Brockington SF, Refulio-Rodriguez NF, Walker JB, Moore MJ, Carlsward BS *et al*. (2011) **Angiosperm phylogeny: 17 genes, 640 taxa**. *American Journal of Botany* **98**(4):704-730.

2. Blaxter ML, De Ley P, Garey JR, Liu LX, Scheldeman P, Vierstraete A, Vanfleteren JR, Mackey LY, Dorris M, Frisse LM *et al*. (1998) **A molecular evolutionary framework for the phylum Nematoda**. *Nature* **392**(6671):71-75.

3. Holterman M, van der Wurff A, van den Elsen S, van Megen H, Bongers T, Holovachov O, Bakker J, Helder J (2006) **Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown clades**. *Molecular Biology and Evolution* **23**(9):1792-1800.

4. Van Megen H, Van Den Elsen S, Holterman M, Karssen G, Mooyman P, Bongers T, Holovachov O, Bakker J, Helder J (2009) **A phylogenetic tree of nematodes based on about 1200 full-length small subunit ribosomal DNA sequences**. *Nematology* **11**(6):927-950.

5. Floyd R, Abebe E, Papert A, Blaxter M (2002) **Molecular barcodes for soil nematode identification**. *Molecular Ecology*, **11**(4):839-850.

6. Okada H, Oba H (2008) **Comparison of nematode community similarities assessed by polymerase chain reaction-denaturing gradient gel electrophoresis (DGGE) and by morphological identification**. *Nematology* **10**(5):689-700.

7. Donn S, Neilson R, Griffiths BS, Daniell TJ (2012) **A novel molecular approach for rapid assessment of soil nematode assemblages – variation, validation and potential applications.** *Methods in Ecology and Evolution* **3**:12-23.

8. Yan G, Smiley RW, Okubara PA (2012) **Detection and quantification of *Pratylenchus thornei* in DNA extracted from soil using real-time PCR**. *Phytopathology* **102**(1):14-22.

9. Vervoort MTW, Vonk JA, Mooijman PJW, van den Elsen SJJ, van Megen HHB, Veenhuizen P, Landeweert R, Bakker J, Mulder C, Helder J (2012) **SSU Ribosomal DNA-Based Monitoring of Nematode Assemblages Reveals Distinct Seasonal Fluctuations within Evolutionary Heterogeneous Feeding Guilds**. *PLoS ONE* **7**(10):e47555.

10. Rybarczyk-Mydłowska K, Mooyman P, Van Megen H, Van den Elsen S, Vervoort MTW, Veenhuizen P, Van Doorn J, Dees R, Karssen G, Bakker J, Helder J (2012) **Small Subunit Ribosomal DNA-Based Phylogenetic Analysis of Foliar Nematodes (*Aphelenchoides* spp.) and Their Quantitative Detection in Complex DNA Backgrounds**. *Phytopathology*, **102**(12):1153-1160.

11. Trudgill DL, Blok VC (2001) **Apomictic, polyphagous root-knot nematodes: Exceptionally successful and damaging biotrophic root pathogens**. *Annual Review of Phytopathology* **39**:53-77.

12. Hoth S, Stadler R, Sauer N, Hammes UZ (2008) **Differential vascularization of nematode-induced feeding sites**. *Proceedings of the National Academy of Sciences of the United States of America* **105**(34):12617-12622.

13. Zunke U (1990) **Ectoparasitic feeding behaviour of the root lesion nematode, *Pratylenchus penetrans*, on root hairs of different host plants**. *Revue Nématol* **13**(3):331-337.

14. Andersson JO (2005) **Lateral gene transfer in eukaryotes**. *Cellular and Molecular Life Sciences*, **62**(11):1182-1197.

15. Keeling PJ, Palmer JD (2008) **Horizontal gene transfer in eukaryotic evolution**. *Nature Reviews Genetics* **9**(8):605-618.

16. Salzberg SL, Hotopp JC, Delcher AL, Pop M, Smith DR, Eisen MB, Nelson WC (2005) **Serendipitous discovery of *Wolbachia* genomes in multiple Drosophila species**. *Genome biology* **6**(3).

17. Dunning Hotopp JC, Clark ME, Oliveira DCSG, Foster JM, Fischer P, Muñoz Torres MC, Giebel JD, Kumar N, Ishmael N, Wang S *et al.* (2007) **Widespread lateral gene transfer from intracellular bacteria to multicellular eukaryotes**. *Science* **317**(5845):1753-1756.

18. Hentschel U, Berger EC, Bright M, Felbeck H, Ott JA (1999) **Metabolism of nitrogen and sulfur in ectosymbiotic bacteria of marine nematodes (Nematoda, Stilbonematinae)**. *Marine Ecology Progress Series* **183**:149-158.

19. Forst S, Dowds B, Boemare N, Stackebrandt E (1997) ***Xenorhabdus* and *Photorhabdus* spp.: Bugs that kill bugs**. *Annual Review of Microbiology* 51: 47-72.

20. Darby C (2005) **Interactions with microbial pathogens**. *WormBook : the online review of C. elegans biology* 1-15.

21. Blanc C, Sy M, Djigal D, Brauman A, Normand P, Villenave C (2006) **Nutrition on bacteria by bacterial-feeding nematodes and consequences on the structure of soil bacterial community**. *European Journal of Soil Biology* **42**(SUPPL. 1):S70-S78.

22. Yeates GW, Bongers T, De Goede RGM, Freckman DW, Georgieva SS (1993) **Feeding-Habits in Soil Nematode Families and Genera - an Outline for Soil Ecologists**. *Journal of Nematology* **25**(3):315-331.

23. Ford Doolittle W (1998) **You are what you eat: A gene transfer ratchet could account for bacterial genes in eukaryotic nuclear genomes**. *Trends in Genetics* **14**(8):307-311.

24. Hunt DJ (1993) **Aphelenchida, Longidoridae and Trichodoridae: their systematics and bionomics**. Wallingford, UK CAB International

25. Holterman M, Rybarczyk K, Van den Elsen S, Van Megen H, Mooyman P, Pena-Santiago R, Bongers T, Bakker J, Helder J (2008) **A ribosomal DNA-based framework for the detection and quantification of stress-sensitive nematode families in terrestrial habitats**. *Molecular Ecology Resources* **8**(1):23-34.

26. Karim N, Jones JT, Okada H, Kikuchi T (2009) **Analysis of expressed sequence tags and identification of genes encoding cell-wall-degrading enzymes from the fungivorous nematode Aphelenchus avenae.** BMC Genomics. Nov 16;10:525

27. Barker KR (1963) **Parasitism of tobacco callus and Kentucky bluegrass by *Aphelenchus avenae***. *Phytopathology* **53**:870.

28. Chin DA, Estey RH (1966) **Studies on the pathogenicity of *Aphelenchus avenae* Bastian, 1865** *Phytoprotection* **47**:66-72.

29. Danchin EGJ, Rosso MN, Vieira P, De Almeida-Engler J, Coutinho PM, Henrissat B, Abad P (2010) **Multiple lateral gene transfers and duplications have promoted plant parasitism ability in nematodes**. *Proceedings of the National Academy of Sciences of the United States of America* **107**(41):17651-17656.

30. Smant G, Stokkermans J, Yan YT, de Boer JM, Baum TJ, Wang XH, Hussey RS, Gommers FJ, Henrissat B, Davis EL *et al.* (1998) **Endogenous cellulases in animals: Isolation of beta-1,4-endoglucanase genes from two species of plant-parasitic cyst nematodes**. *Proceedings of the National Academy of Sciences of the United States of America* **95**(9):4906-4911.

31. Kikuchi T, Jones JT, Aikawa T, Kosaka H, Ogura N (2004) **A family of glycosyl hydrolase family 45 cellulases from the pine wood nematode *Bursaphelenchus xylophilus***. *FEBS Letters* **572**(1-3):201-205.

32. Fu Z, Agudelo P, Wells CE (2012) **Differential expression of a beta-1,4-endoglucanase induced by diet change in the foliar nematode *Aphelenchoides fragariae***. *Phytopathology* **102**(8):804-811.

33. Ledger TN, Jaubert S, Bosselut N, Abad P, Rosso MN (2006) **Characterization of a new beta-1,4-endoglucanase gene from the root-knot nematode *Meloidogyne incognita* and evolutionary scheme for phytonematode family 5 glycosyl hydrolases**. *Gene* **382**:121-128.

34. Kyndt T, Haegeman A, Gheysen G (2008) **Evolution of GHF5 endoglucanase gene structure in plant-parasitic nematodes: No evidence for an early domain shuffling event**. *BMC Evolutionary Biology* **8**(1).

35. Rehman S, Butterbach P, Popeijus H, Overmars H, Davis EL, Jones JT, Goverse A, Bakker J, Smant G (2009) **Identification and characterization of the most abundant cellulases in stylet secretions from *Globodera rostochiensis***. *Phytopathology* **99**(2):194-202.

36. Rybarczyk-Mydłowska K, Ruvimbo Maboreke H, Van Megen H, Van den Elsen S, Mooyman P, Smant G, Bakker J, Helder J (2012) **Rather than by direct acquisition via lateral gene transfer, GHF5 cellulases were passed on from early Pratylenchidae to root-knot and cyst nematodes**. *BMC Evolutionary Biology* **12**.

37. Abad P, Gouzy J, Aury JM, Castagnone-Sereno P, Danchin EGJ, Deleury E, Perfus-Barbeoch L, Anthouard V, Artiguenave F, Blok VC *et al.* (2008) **Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita***. *Nature Biotechnology* **26**(8):909-915.

38. Opperman CH, Bird DM, Williamson VM, Rokhsar DS, Burke M, Cohn J, Cromer J, Diener S, Gajan J, Graham S *et al.* (2008) **Sequence and genetic map of *Meloidogyne hapla*: A compact nematode genome for plant parasitism**. *Proceedings of the National Academy of Sciences of the United States of America* **105**(39):14802-14807.

39. Holterman M, Karssen G, Van den Elsen S, Van Megen H, Bakker J, Helder J (2009) **Small Subunit rDNA-Based Phylogeny of the Tylenchida Sheds Light on Relationships Among Some High-Impact Plant-Parasitic Nematodes & the Evolution of Plant Feeding**. *Phytopathology* **99**(3):227-235.

CHAPTER 2

A RIBOSOMAL DNA-BASED FRAMEWORK FOR THE DETECTION AND QUANTIFICATION OF STRESS-SENSITIVE NEMATODE FAMILIES IN TERRESTRIAL HABITATS

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Abstract

Indigenous communities of soil-resident nematodes have a high potential for soil health assessment as nematodes are diverse, abundant, trophically heterogeneous and easily extractable from soil. The conserved morphology of nematodes is the main operational reason for their under-exploitation as soil health indicators, and a user-friendly bio-sensor system should preferably be based on non-morphological traits. More than 80% of the most environmental stress-sensitive nematode families belong to the orders Mononchida and Dorylaimida. The phylogenetic resolution offered by full length small subunit ribosomal DNA sequences (SSU rDNA) within these two orders is highly different. Notwithstanding several discrepancies between morphology and SSU rDNA-based systematics, Mononchida families (indicated here as M1-M5) are relatively well-supported and, consequently, family-specific DNA sequences signatures could be defined. Apart from Nygolaimidae and Longidoridae, the resolution among Dorylaimida families was poor. Therefore, a part of the more variable Large Subunit (LSU) rDNA (≈ 1,000 bp from the 5′-end) was sequenced for 72 Dorylaimida species. Sequence analysis revealed a subclade division among Dorylaimida (here defined as D1-D9, PP1-PP3) that shows only distant similarity with "classical" Dorylaimid systematics. Most subclades were trophically homogeneous, and - in most cases - specific morphological characteristics could be pinpointed that support the proposed division. To illustrate the practicability of the proposed molecular framework, we designed primers for the detection of individual subclades within the order Mononchida in a complex DNA background (*viz.* in terrestrial or freshwater nematode communities) and tested them in quantitative assays (real time PCR). Our results constitute proof-of-principle for the concept of DNA sequence signatures-based monitoring of stress sensitive nematode families in environmental samples.

Introduction

Nematodes are among the most widespread and abundant invertebrates in soils and (freshwater and marine) sediments. These relatively small, vermiform organisms reside in water (films) in densities up to several millions of individuals and up to 100 species per square meter [1]. The high density and the variety of trophic ecologies represented within this phylum – nematodes may feed on bacteria, fungi, algae, other nematodes, plants, (in)vertebrates or a combination of these [2] – render them a key position in the soil food web [3][4]. Furthermore, nematodes themselves serve as a food source for a wide range of soil inhabitants [3]. Nematodes show a broad range of sensitivities towards disturbances such as subtle temperature changes, changing moisture conditions, exposure to pollutants, and changes in the nutritional status of their environment. Taking into consideration that nematodes – unlike bacteria and fungi – can easily be extracted from soil, they have a great potential as indicators for soil health (recently reviewed by Dmowska & Ilieva-Makulec [5]).

In terrestrial habitats, the great majority (> 80%) of the environmental stress-sensitive nematode families - as indicated by the *c-p* values 4 and 5 (*c,* colonizer; *p,* persister [6]) - belongs to two orders, namely the Dorylaimida and the Mononchida (16 and 6 families, respectively). Despite their common overall sensitivity to environmental stresses, their responsiveness towards different kinds of physical, chemical or biological disturbances is diverse. Therefore, the monitoring of shifts in Dorylaimida and Mononchida communities in terrestrial and fresh water habitats at family or even genus level is ecologically relevant [6] [7] [8] [9]. As compared to Mononchida, Dorylaimida are highly speciose; Jairajpuri & Ahmad [10] estimated that more than 10% of all currently known nematode species belong to this order. The range of trophic ecologies represented by these two orders is just marginally smaller than the diversity within the phylum Nematoda as a whole. Its members can be found in all soil types as well as in freshwater environments, while - remarkably - they are absent in marine habitats. In comparison to the well-known free-living nematode *Caenorhabditis elegans* (∈1 mm in length), Dorylaimida and Mononchida are relatively large (typically 1-5 mm), have long generation times (months instead of 3-4 days for *C. elegans*), and produce a relatively low number of eggs. The low reproduction rates imply that Dorylaimida and Mononchida populations will only slowly recover from disturbances. The high sensitivity to pollution could be partially explained by the permeability of their cuticle. Generally spoken these nematodes have relatively permeable cuticles [11] [12]. All these characteristics combined make members of the Dorylaimida and Mononchida sensitive indicators for the impact of environmental stress on soil life.

Dorylaimida display a mosaic of morphological characters and are notoriously difficult to identify, even for experts. As it is unclear which characters are relevant for the establishment of phylogenetic relationships within this order and which characters suffer from homoplasy, there have been

numerous rearrangements of genera, families and superfamilies within this order (for a historical overview see [10]). Apart from the scarcity of informative morphological characteristics, the potential of Mononchida and Dorylaimida as sensitive bio-indicators is underexploited because routine analysis of soil samples is very time consuming (a single mass-slide takes on average 2 hours), and because only adults are taken into consideration (juveniles can not always be identified). It is concluded that a nematode-based bio-sensor should be based on non-morphological traits.

Molecular analysis has become a powerful tool to clarify evolutionary relationships, and, generally spoken, well-resolved relationships are a strong basis for DNA sequence signature-based taxon identification. The small subunit ribosomal DNA (SSU rDNA) gene has proven to be useful for the reconstruction of phylogenetic relationships among nematode taxa [13] [14] [15] [16], and recently a subdivision of the phylum into twelve clades has been proposed [15]. Members of the orders Dorylaimida and Mononchida were shown to reside within a single major clade (Clade 2), and the representatives were distributed over two well-resolved order-specific branches. In contrast to the families within the order Mononchida, the phylogenetic relationships within the family-rich order Dorylaimida were fully unclear [15]. The low diversity of the SSU rDNA within the order Dorylaimida prompted us to sequence a part of the more variable large subunit (LSU) ribosomal DNA gene (≈ 1,000 bp from the 5'-end). In many (45) cases both the SSU (full length) and LSU (fragment) rDNAs were sequenced from the same individual nematode. In this paper we present SSU and LSU rDNA-based phylogenetic analysis of Mononchida and Dorylaimida, and show the possibilities of using SSU and LSU rDNA-based sequence signatures for the quantitative detection of individual stress-sensitive nematode families in environmental samples.

Materials and Methods

Taxon sampling

Nematodes were collected from various habitats throughout The Netherlands, and extracted from the soil using standard techniques. Prior to DNA extraction, individual nematodes were identified using a light microscope (Zeiss Axioscope) equipped with DIC optics. A CCD camera (CoolSnap, RS Photometrics) was used to take a series of digital images from each nematode.

SSU rDNA sequences

Part of the SSU rDNA sequences used in this study came from an earlier study on the phylogeny of the Nematoda [15], and new sequences were acquired as described in this paper. SSU rDNA sequences were deposited at GenBank under the accession numbers EF207244 to EF207254. For a full list of sequences used for this study see Supplementary Tables S1 and S2.

DNA extraction, LSU rDNA amplification and sequencing

Single nematodes were transferred to a 0.2 ml PCR tube containing 25µl sterile water. An equal volume of lysis buffer containing 0.2 M NaCl, 0.2 M Tris-HCl (pH 8.0), 1% (v/v) β-mercaptoethanol and 800µg/ml proteinase-K was added. Lysis took place in a Thermomixer (Eppendorf, Hamburg, Germany) at 65 °C and 750 rpm for 2 hrs followed by 5 min incubation at 100°C. Lysate was used immediately or stored at -20°C. LSU rDNA was amplified using either primer 28-61for or 28-81for (28-61for, 5'-gtcgtgattacccgctgaactta-3'; 28-81for, 5'-ttaagcatatcatttagcggaggaa-3') in combination with either primer 28-1006rev or 28-1032rev (28-1006rev, 5'-gttcgattagtctttcgcccct-3'; 28-1032rev, 5'-tcggaaggaaccagctacta-3'). PCR was performed in a 25-µl final volume containing 3 µl of 100X diluted crude DNA extract, 0.1 µM of each PCR primer and a 'Ready-To-Go PCR bead' (Amersham). The following PCR protocol was used: 94ºC, 5 min; 5X (94ºC, 30 s; 45ºC, 30 s; 72ºC, 70 s) followed by 35X (94ºC, 30 s; 54ºC, 30 s; 72ºC, 70 s) and 72ºC, 5 min. Gel-purified (Marligen) amplification products were cloned into a TOPO TA vector (Invitrogen) and sequenced using standard procedures. Newly generated LSU rDNA sequences were deposited at GenBank under the following accession numbers: AY592994 - AY593065 and EF207234 - EF20743 (see also Supplementary Table S2).

Sequence alignment

SSU rDNA sequences were supplemented with publicly available sequences (Supplementary Table S1) and aligned using the ClustalW algorithm as implemented in BioEdit 5.0.9 [17] and manually improved using secondary structure information from arthropods (*http://www.psb.ugent.be/rRNA/secmodel/index.html)*, in accordance with Wuyts *et al.* [18].

Newly generated nematode LSU rDNA sequences were supplemented with one publicly available sequence (*Xiphinema rivesi* AY210845). The outgroup consisted of Mononchida; *viz*. *Mononchus tunbridgensis*, *Mononchus truncatus* and *Anatonchus tridentatus*. The LSU rDNA sequences were aligned using the same methods as for the SSU rDNA sequences, and further improved with secondary structure information from yeast (*http://www.psb.ugent.be/rRNA/varmaps/Scer_lsu.html*; see also Ben Ali *et al.* [19]). The final alignment consisted of 74 partial LSU rDNA sequences (each sequence spans about 1,000 bp from the 5'-end onwards) and contained 1,309 aligned positions (including gaps).

Phylogenetic analyses

The SSU rDNA trees were constructed using Bayesian inference. Modeltest selected the GTR model with invariable sites and gamma distribution as the best fitting models for both SSU datasets. In essence the data set was analyzed as described in Holterman *et al.* [15], except for now the gamma parameter was included. The Dorylaimia SSU rDNA dataset was run for 2,000,000 generations and the burnin was 100,000 generations (Fig. 1). The Dorylaimida SSU rDNA dataset was run for 1,800,000 generations and the burnin was 500,000 generations (Fig. 2).

Three different methods were used to construct a phylogenetic tree from the LSU rDNA; neighbor-joining (NJ), maximum parsimony (MP) and Bayesian inference (BI). Modeltest 3.06 [20] was used to determine the most appropriate nucleotide substitution model. Both the likelihood ratio test and the Akaike information criterion selected the general time reversible (GTR) model with invariable sites (I) and a γ-shaped distribution of substitution rates (Γ) as the best fitting substitution model. The neighbor-joining tree was constructed using PAUP* 4.0b10 [21] using the model parameters calculated by Modeltest. The resulting tree was bootstrapped 1,000 times. The maximum parsimony tree was also constructed using PAUP*, the default parameters were used. This tree was bootstrapped 1,000 times as well. The Bayesian tree was constructed using the program MrBayes 3.1.2 [22]. The alignment was divided into a stem and a loop partition according to the secondary structure. For both partitions, GTR + I + Γ was used. The stem regions were analyzed under the Doublet model. It is noted that the Doublet + GTR model explained our data only marginally better that the GTR only approach (data not shown). The default flat priors were used for the parameters and the parameters were unlinked between the partitions. Four independent runs with different random starting trees were performed. Each run was made with four Markov chains and run for 3,000,000 generations with a sample frequency of 200 generations. The first 200,000 generations were discarded as burn-in. The sampled trees from the four runs were combined in a single 50% majority-rule tree. The program Tracer v.1.2.1 [23] was used to check if all parameters had converged. The program MacClade v.4.0 [24] was used to infer the ancestral character states of several traits along the Bayesian LSU rDNA tree.

Detection and quantification of individual subclades

To detect and quantify clusters as defined in Fig. 1 (M1-M5), subclade-specific SSU rDNA-based primers were designed using the software package ARB [25]. An alignment of about 1,200 full length SSU rDNA sequences covering a substantial part of the nematode biodiversity in terrestrial and freshwater habitats was used as input file to identify subclade-specific sequence motives. Potential close non targets were selected by changing the ARB mismatch setting to - at most - 4 nucleotides. One or two mismatches were always considered as close non targets unless they were positioned very close to the 3'-end of the foreseen PCR primer. Three and four mismatches were only included if they were clustered and positioned in the 5'-end region.

Subclades are defined in Fig. 1, and for each of the subclades the closest non-targets are shown in Table 1. Primer combinations as presented in Table 1 were tested using cloned SSU rDNA fragments. Bacterial clones harboring a TOPO TA vector with a SSU rDNA fragment of interest were grown in 2ml of LB medium supplemented with 100 µg/ml of ampicillin at 37°C. Plasmid extraction was performed using the Wizard Plus Minipreps DNA Purification System (Promega). DNA concentrations were measured with a NanoDrop spectrophotometer (NanoDrop Technologies) and adjusted to 10 ng/µl. For

Q-PCR application 3 µl of 1000X diluted template was mixed with a subclade-specific primers (end concentrations for both primers 200 nM) and 12.5 µl iQ SYBR Green Supermix (Bio-Rad) in a total reaction volume of 25 µl. In order to increase the specificity occasionally locked nucleid acids (LNAs) were incorporated (Tab. 1). Thermal cycling was performed on a Bio-Rad MyiQ thermal cycler (Bio-Rad) and consisted of 98°C for 3 min; followed by 60 cycles of 98°C for 30 sec, subclade-specific annealing temperature (Tab. 1) for 1 min and 72°C for 30 sec.

Table 1. Primer combinations used for the detection and quantification of Mononchida subclades as defined in Fig. 1. Real time PCR results are presented in Figure 4. Close non-targets species belonging to the subclass Dorylaimia are given in italics.

Subclade Identifier	Primer combination (F-forward; R-reverse)	Close non-targets	Annealing temperature [°C]
M1	F:5′-cgatccgtcggtgttaaatat*t R:5′-ctcg*agctgatgactcgaa*	*Prionchulus punctatus* 1 *Mononchus truncatus* 1 Haliplectus sp. 1 Prismatolaimus dolichurus 2 Pratylenchus pratensis	63
M2	F:5′-cgcatttattagaccaaaaccag* R:5′-tagaagacccagttaaactcctt*	*Mermfamil Mermitide* Mesocriconema xenoplax 1 Malenchus andrassyi 1 Ditylenchus dipsaci 9	64
M3	F:5′-cgagcttcttagagggacag* R:5′-ccaattcttaccagaaaaggttttaa	*Mylonchulus rotundicaudatus 2*** *Opisthodorylaimus sylphoides* ** *Granonchulus sp1* Diphtherophora obesa 1 Trischistoma sp. 1 ** Eumonhystera filiformis 1 ** Steinernema glaseri1 ** Prochromadora sp.1 **	65
M4	F:5′- cgatccgtcggtgttaag* R:5′- ccaattcttaccagaaaaggttttaa	*Mylonchulus sp. 1* *Mylonchulus sigmaturus 3* *Mylonchulus brachyuris 2* *Mylonchulus rotundicaudatus 2* *Mononchus truncatus 1* *Clarkus papillatus 1* *Coomansus parvus 1* *Granonchulus sp. 1* *Bathyodontus mirus 1* *Cryptonchus tristis1* Prionchulus punctatus 2 Prismatolaimus dolichurus 2 Euteratocephalus sp. 1	62
M5	F:5′-gacgaagaattttatatgttttttgtg* R:5′-ggttgtaaagcacactgctattc*	*Anatonchus tridentatus* 1 *Granonchulus sp* 1 *Coomansus parvus* 1	63

*- LNA ** - starts giving a positive signal in later cycles

Results and Discussion

Currently nematode community analysis for ecological soil classification invariably includes time-consuming light microscopy-based identification (mostly till family level) and counting of relatively small samples (typically 75 up to 200 individuals). In most cases nematode families are defined on the basis of a series of morphological characters that are not always visible in juvenile life stages. For a transformation of prevalent nematode community analysis tools into DNA sequence signature-based methods it is relevant to know whether DNA sequence data do or do not confirm the existence of these nematode families as they are currently defined. Here, this is investigated for members of the subclass Dorylaimia, with a focus on Mononchida and Dorylaimida; two orders whose members live exclusively in terrestrial and freshwater habitats and share a high sensitivity to environmental stress. Subsequently, we show how subclade-specific DNA sequence signatures can be used for life-stage independent, large-scale analysis of terrestrial and freshwater nematode communities.

Phylogeny of the subclass Dorylaimia

Six orders of the Dorylaimia are represented in our analysis (Figs.1 and 2): Dorylaimida, Mononchida, Mermithida (insect parasites), Trichinellida (animal parasites), Dioctophymatida (animal parasites) and Isolaimida (order that comprises only a single family with one genus; *Isolaimium*). SSU rDNA sequence data offer a remarkably good resolution within the Mononchida, Mermithida and Trichinellida (Fig.1), whereas the phylogenetic resolution among representatives of the Dorylaimida was poor (Figs. 2 and S1). Addition of sequences from representatives of the Cryptonchidae and the Soboliphymatidae - both positioned basally in two major branches (see Fig. 1) - resulted in intra-clade relationships that differ from the relationships presented by Mullin *et al*. [26] and Holterman *et al*. [15]. The current data set suggests a basal node that defines the Dorylaimida on the one hand, and the Mononchida, Mermithida, Trichinellida, and Dioctophymatida on the other. Within the second group, a sister relationship is observed between the Mononchida and Mermithida, and the Trichinellida and Dioctophymatida. As animal parasites are not taken into consideration in ecological soil assessment, the within-order relationships of Mermithida, Trichinellida, and Dioctophymatida will not be discussed here. The order Isolaimida was placed outside the Dorylaimia, a confirmation of a result that was recently published by Mullin *et al*. [26]. We will focus on the Mononchida and Dorylaimida as the numerous representatives of these orders are stress-sensitive as reflected by their high *c-p* values (4 or 5 on a 1-5 scale as defined at family level [27]).

Mononchida - phylogenetic relationships

SSU rDNA-based phylogenetic relationships among Mononchida are to some extent similar to the current classification of the order. Most striking is the positioning of the Bathyodontidae and the Cryptonchidae, two families harboring

exclusively bacterial feeding nematodes [2], at the base of the Mononchida subclade. This would suggest that predatory (see below) Mononchida and insect parasitic Mermithida arose from bacterivorous ancestors. In this respect it is noteworthy that the food preference of nematodes may change during their life cycle; contrary to adults (and J3/J4), initial juvenile stages of several members of the Mononchida feed on bacteria (*e.g.* Yeates [28] 1987). The remarkable and firm placement of the Mermithidae (insect parasites) within the Mononchida confirms previous findings by Blaxter *et al* [14], Mullin *et al* [26] and Holterman *et al*. [15]. Apparently, there are no morphological characters that support this positioning.

The family Mylonchulidae was shown to be polyphyletic as *Granonchulus* did not cluster with representatives of the genus *Mylonchulus* (M1 in Fig. 1). The characters considered to be diagnostic for the Mylonchulidae are the strong tapering of the stoma (mouth-like opening) at its base, and the arrangement of the denticles in transverse rows [29]. However, the stoma of *Granonchulus* is ovoid, not tapering strongly at the base, and members of this genus have only one transverse row of denticles (instead of multiple); the remaining denticles are ordered in longitudinal rows. Hence, morphological data are available that seem to support our SSU rDNA–based results.

The Mononchidae also turned out to be paraphyletic, with *Mononchus* being a sistergroup of *Mylonchulus*, separate from *Coomansus*, *Clarkus* and *Prionchulus* (M2 in Fig 1). There is morphological support for this separation. Both *Mononchus* and *Mylonchulus* have well developed tail glands and a (reduced) spinneret (a cuticular cone connected to the caudal glands). On the contrary, *Coomansus*, *Clarkus* and *Prionchulus* (M3 in Fig. 1) have only rudimentary tail glands and no spinneret [29]. In addition *Mononchus* has a transverse rib on each ventrosublateral wall of the stoma and *Mylonchulus* has denticles on the ventrosublateral walls arranged in transverse rows. In contrast *Coomansus*, *Clarkus* and *Prionchulus* have longitudinal ridges on their ventrosublateral stoma walls [29]. These characteristics support the results from our phylogenetic analysis.

The Anatonchidae appeared to be paraphyletic, too. Representatives of the genus *Anatonchus* (M4 in Fig. 1) are placed at the base of a subclade that includes most Mononchidae and Mylonchulidae. Our analysis suggests that it is probably not possible to define Anatonchidae-specific DNA sequence signatures that would cover all members of the genera *Anatonchus* and *Miconchus*.

Bathyodontidae (M5 in Fig. 1) are represented by two species only. So far this family seems to be monophyletic.

Dorylaimida – SSU rDNA-based phylogenetic relationships

Within the Dorylaimida two suborders are distinguished, the Dorylaimina and the Nygolaimina [30]. These are characterized by the nature of their stoma [31]. The Dorylaimina are equipped with an axial odontostyle, whereas the Nygolaimina have a mural tooth. In the SSU rDNA-derived tree (Fig. 2), the Nygolaimina are placed in a single, well-supported subclade (D9). SSU rDNA sequence data do not allow for the deduction of family relationships among Dorylaimina. The only family for which all members are present in a single well-supported cluster is the plant parasitic family Longidoridae. However, because it is part of a large polytomy, the relationship between Longidoridae and other Dorylaimida families could not be established.

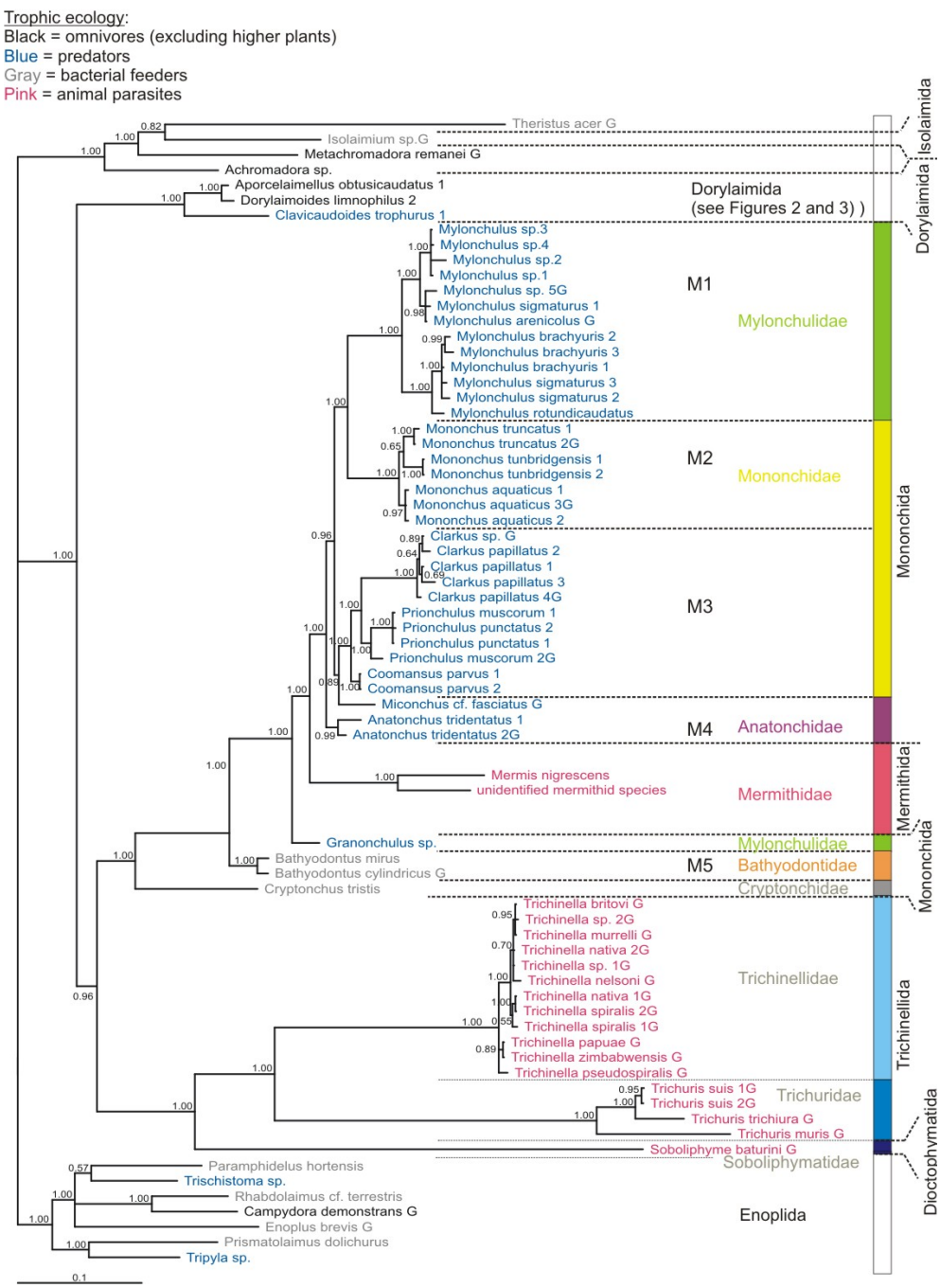

Figure 1. SSU rDNA-based Bayesian phylogeny of the subclass Dorylaimia (Clade 2 in Holterman *et al.* [15]). Numbers near nodes represent posterior probabilities. The colored bar indicates to which order, family, and subclade (M1-M5) a species belongs. The color of the species name indicates the feeding type (according to Yeates *et al.* [2]).

Dorylaimida – LSU rDNA-based phylogenetic relationships

The conserved nature of the SSU rDNA among representatives of the Dorylaimida prompted us to sequence a part of the LSU rDNA in addition to the SSU rDNA (≈ 1,000 bp from 5′ end). In many cases (45 of 72 sequences) both SSU and LSU rDNA were amplified from the same individual nematode. The LSU rDNA-based phylogram is constructed on the basis of 75 sequences: 72 representatives of the Dorylaimida and 3 members of the Mononchida. The LSU trees show a better resolution within the Dorylaimida, although a large basal polytomy still remains. The Bayesian tree (Fig. 3), parsimony tree (Fig. S2, electronic supplement) and neighbor-joining tree (Fig. S3, electronic supplement) are nearly identical and therefore only the Bayesian tree is depicted here. All parameters of the evolutionary model for the Bayesian tree had converged after a burn-in of 200,000 generations.

As there is limited congruence between the current family subdivision of the Dorylaimina and the subclades suggested by LSU rDNA sequence data, it was decided to define 12 subclades, D1-D9 and PP1-PP3, that are well-supported by molecular data. Dorylaimina systematics has been subject to numerous revisions, and we investigated whether morphological support could be found for the currently proposed subclade division:

D1. Being quite heterogeneous from a morphological point of view, D1 shows several general (although not totally common) patterns (Fig. S4). (i) It groups several long-tailed taxa which currently are dispersed in separate families (and even superfamilies): Dorylaimidae (Dorylaimoidea), Mydonomidae (Tyencholaimoidea) and Belondiridae (Belondiroidea). (ii) It groups several taxa (*Paractinolaimus*, *Dorylaimus*, *Opisthodorylaimus* and *Mesodorylaimus*) which display sexual dimorphism in the tail shape (females have an elongated or cone-shaped tail and males a rounded tail), a feature not found elsewhere in the studied taxa. (iii) It assembles several taxa which share the feature of being opisthodelphic (= uterus directed posteriorly) in total or in part (*Opisthodorylaimus*, *Ecumenicus, Dorylaimoides, Oxydirus*), an infrequent feature in Dorylaimidae and its relatives; remaining subclades are dominated by didelphic (= two uteri) or predominantly didelphic taxa, with the exception of PP2 (*Pungentus* species) and D5 (*Tylencholaimus* species).

D2. This cluster consists of *Aporcelaimellus* and *Allodorylaimus* species that belong to the Aporcelaimidae and Qudsianematidae respectively. To the best of our knowledge there are no morphological characters supporting this clade.

D3. This cluster includes several Qudsianematidae (*Epidorylaimus*, *Eudorylaimus* and *Thonus*), *Enchodelus* (Nordiidae - Pungentinae) and *Prodorylaimus* (Dorylaimidae) and it was also distinguished on the basis of SSU rDNA data (Fig. 2). Although these taxa share several characters, none of these characters are unique for this subclade (Fig. S4). These shared characteristics are: (i) guiding ring simple (double in *Enchodelus* and *Prodorylaimus*), (ii) pharynx widening near or slightly behind the middle, (iii) vagina sclerotized and (iv) tail shape equal in both sexes. Although none of these characteristics is D3-specific by itself, their combination is fairly unique. The only other genus which combines all these 6 characteristics is *Allodorylaimus*, which is placed in clade D2.

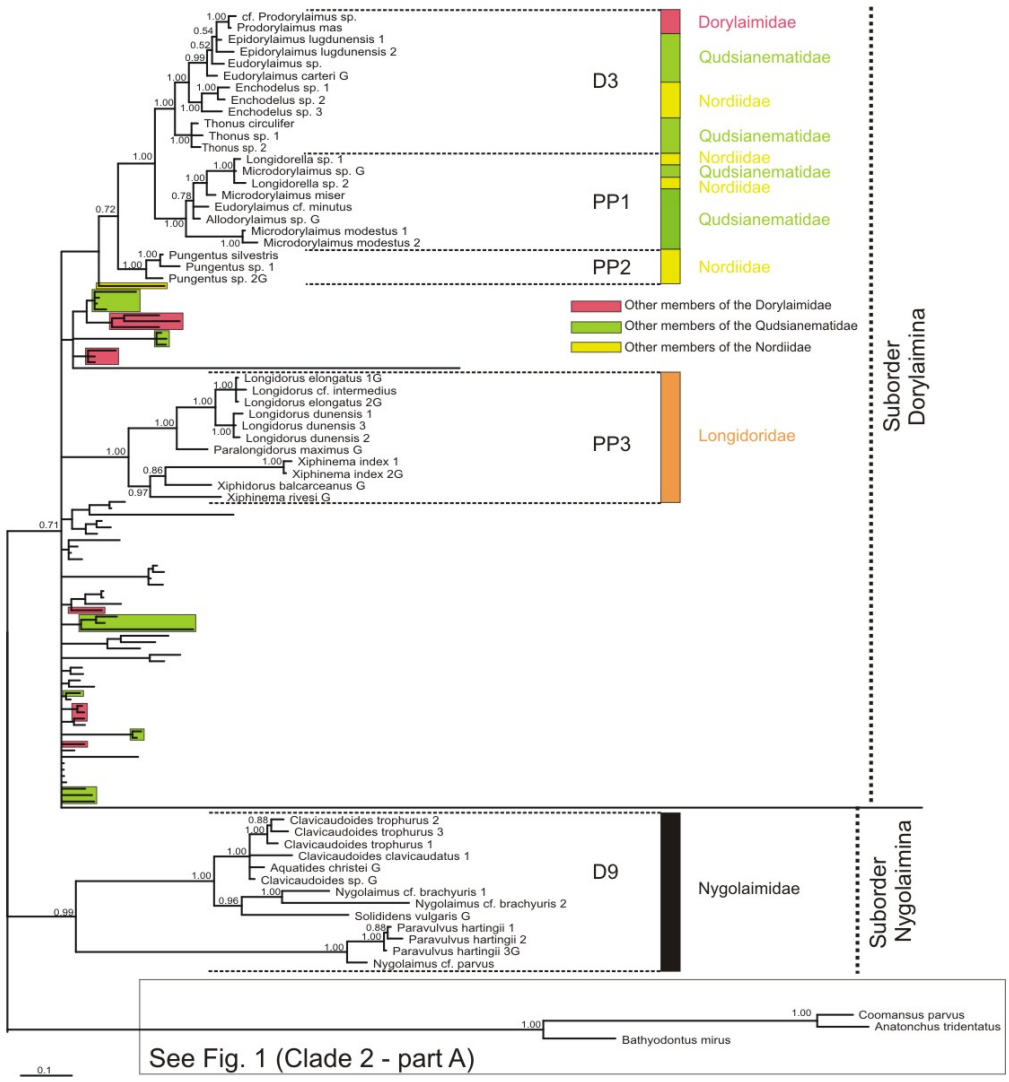

Figure 2. SSU rDNA-based Bayesian phylogeny of the order Dorylaimida. Numbers near nodes represent posterior probabilities. The colored bars indicate to which suborder, family and subclade a species belongs. Only species names belonging to well-resolved subclades are given (for detailed phylogenetic tree see Fig. S1).

PP1. This subclade consists of *Microdorylaimus, Longidorella* and *Eudorylaimus cf. minutus*. This subclade is characterized by having a pharynx that spans about a third of their total body length (possibly related to their relatively small size - 0.4-0.7 mm).

PP2 consist solely of the genus *Pungentus*.

D4 consists of members of the Qudsianematidae subfamilies Carcharolaiminae and Discolaiminae. These subfamilies were previously regarded

as a separate family - the Discolaimidae [32], see also De Ley *et al.* [33] - and based on these results it seems reasonable to reinstate this family.

D5, PP3 and **D6** are monophyletic subclades that so far appear to correspond with the families Tylencholaimidae, Longidoridae and Tylencholaimellidae, respectively. **D7** includes representatives of the genus *Sectonema*, the only genus within the subfamily Sectonematinae. Hence, this cluster corresponds to a subfamily as currently defined within the Qudsianematidae. **D8**, defined here by a single LSU rDNA sequence only, corresponds to the Qudsianematidae subfamily Chrysonematinae. **D9** covers Nygolaimidae (Nygolaimina) and this subclade could be clearly distinguished on the basis of SSU rDNA sequence data as well (Fig. 2).

The origin of plant parasitism within the order Dorylaimida

Plant parasitism arose at least three times independently during the evolution of the phylum Nematoda, one time within the order Dorylaimida (Longidoridae), and two times in the (infra)orders Triplonchida and Tylenchomorpha [14]. For the latter case, Holterman *et al.* [15] provided molecular support for a long-standing hypothesis stating that plant parasitic nematodes arose from fungivorous ancestors [34]. We investigated the positioning of the Longidoridae (PP3) *vis-à-vis* the two fungivorous subclades D5 and D6. However, the current data set provided insufficient resolution to make a statement about the origin of the Longidoridae.

Remarkably, two more groups of plant feeders within the order Dorylaimida, members of the genera *Longidorella* and *Pungentus* (ectoparasites of higher plants [2]; but also see Trudgill [35]), evolved independently from the Longidoridae. From our LSU rDNA data we conclude that *Longidorella* (subclade PP1) presumably arose from an omnivorous ancestor. The current LSU rDNA tree provides no insight into the possible feeding type of the ancestor of *Pungentus*.

The use of DNA sequence signatures for quantitative detection of Mononchida subclades.

Unique DNA sequence signatures (unique among 1,200 full length SSU rDNA sequences from all over the phylum Nematoda) were determined for the five subclades (M1- M5) within the order Mononchida as defined in Fig. 1. On the basis of these signatures primers were designed that would work under similar annealing temperatures, and these were tested for their specificity (Fig. 4). It is noted that close non-targets as given in Tab. 1 do not necessarily belong to the Dorylaimia. On the contrary, except for M5 all primer combinations tested were shown to have close non-targets that are phylogenetically completely unrelated to the target sequence. In four cases (M1, M2, M4 and M5) primers were shown to be highly specific as hardly any signal could be detected even after 60 PCR cycles. Primers designed for M3 were slightly less specific, but the ΔC_T (C_T - cycle number at which the fluorescent signal exceeds a given threshold value) was still around 20. It is concluded that nematode subclades as defined here provide a firm basis for the development of assays for the detection and quantification of stress sensitive nematode families in soils.

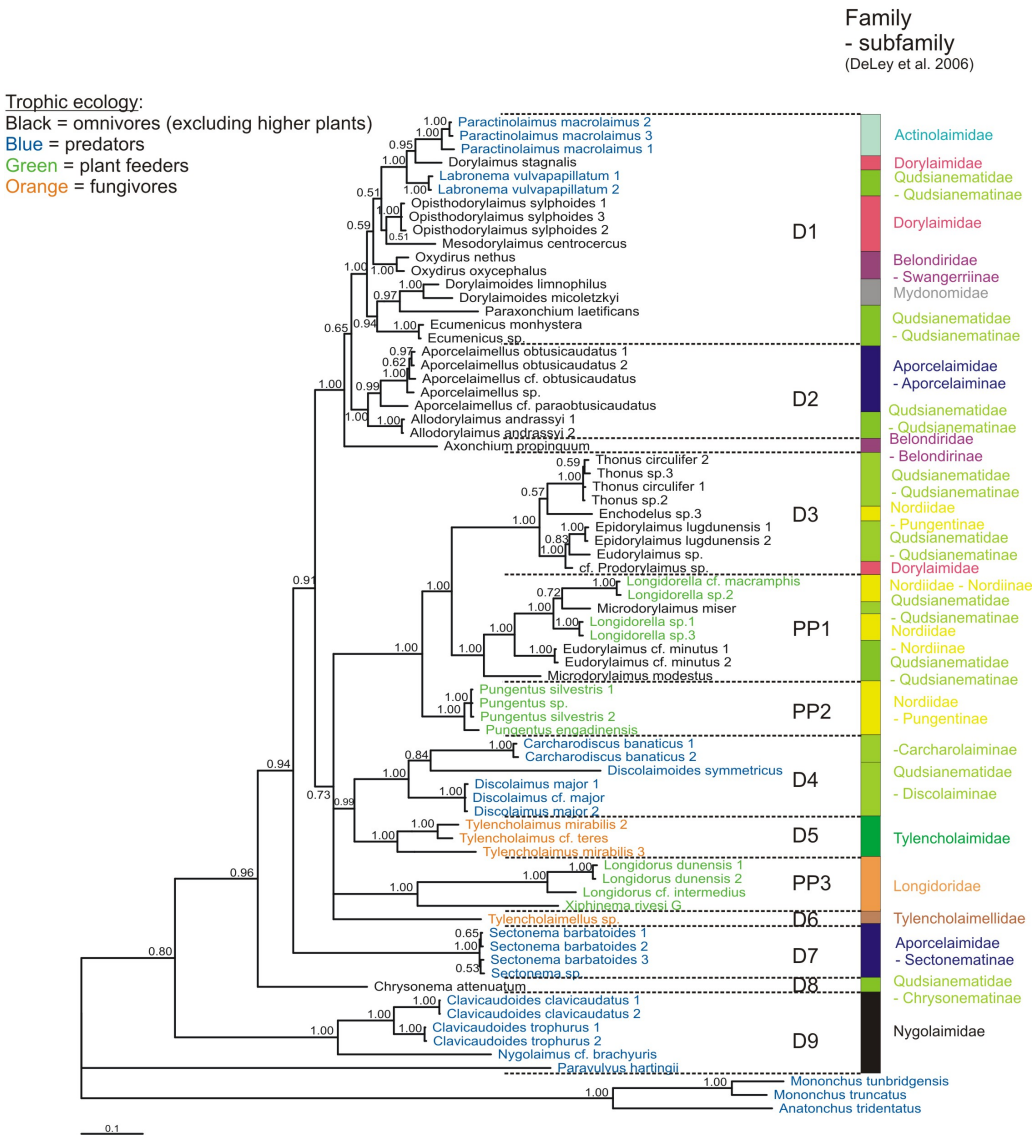

Figure 3. LSU rDNA-based Bayesian phylogeny of the order Dorylaimida. Numbers near nodes represent posterior probabilities. The colored bar indicates to which (sub-) family a species belongs. The Dorylaimida are divided into 12 subclades (D1-D9, PP1-PP3). The color of the species name indicates the feeding type (according to Yeates *et al.* [2]).

DNA sequence signature-based identification of Dorylaimida subclades

LSU rDNA sequence analysis resulted in a subdivision of the Dorylaimida into nine free-living subclades (D1-D9), and three clusters that include multiple parasites of higher plants (PP1- PP3). Contrary to LSU, SSU rDNA data are available from a considerable number of taxa well spread over the phylum

Nematode. Hence, subclades are preferably defined on the basis of specific, shared SSU rDNA sequence motives. As can be seen in Fig. 2, this is achievable only for D3, D9, and PP1-PP3. The remaining subclades will be defined by shared LSU rDNA motives. Currently, the LSU rDNA database is dominated by representatives of the Dorylaimida and the Tylenchomorpha, and consequently subclade specific primers could potentially have cross reactivity outside Clade 2 and 12. It should be noted that - as compared to SSU rDNA - the relatively high degree of variability of the LSU rDNA genes among nematodes reduces the chances of unwanted cross reactivity considerably. Nevertheless, we are planning to alleviate possibly uncertainties about specificity by the development of SSU-rDNA-based suborder Dorylaimina specific primers. In absence of (major) cross reactivity the total Dorylaimina signal minus the D3, D9, and PP1-PP3 signals should be similar to the D1-D2 plus D4-D8 signals.

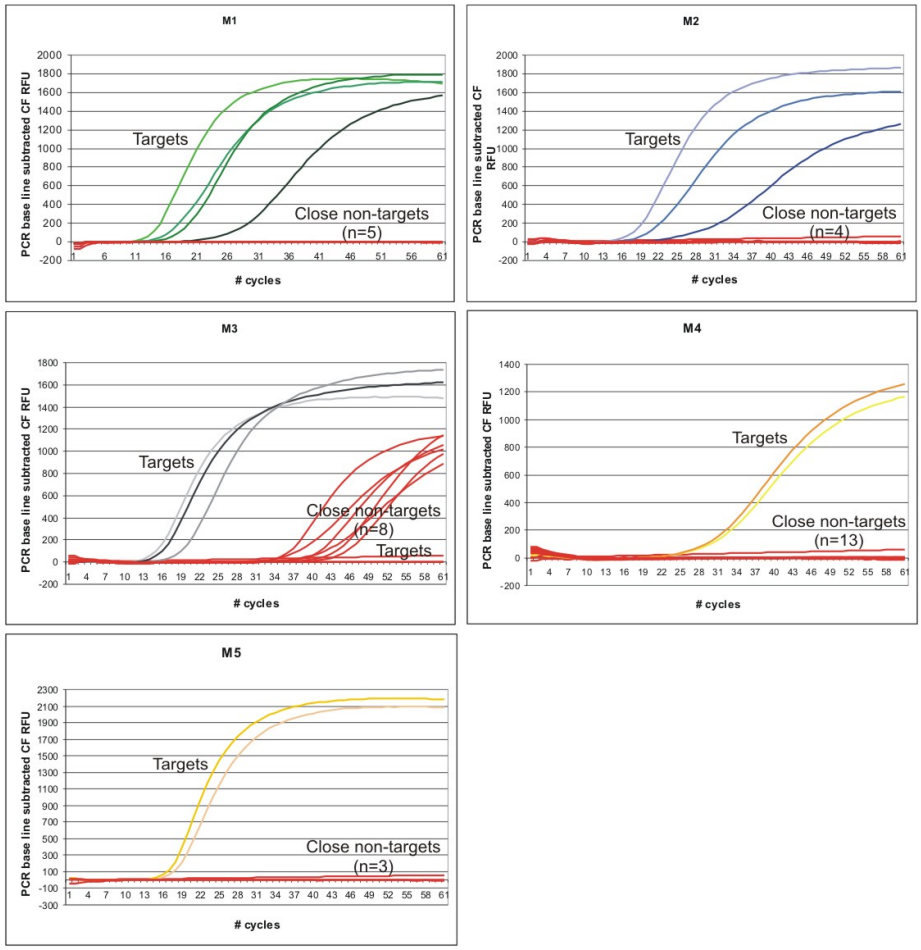

Figure 4. Real time PCR amplification curves showing the detection of trophically homogeneous subclades within the order Mononchida as defined in Fig. 1 on the basis of full length SSU rDNA sequences (M1-M5). Target species, closest non-targets, primers and annealing temperatures are given in Table 1.

Further development of this detection system will include the determination of the average quantitative PCR signal yield per family or subclade. For this, we are currently generating series of 1, 5, 10, 50 and 100 microscopically-determined individuals from single genera. By determining the PCR signal yield per genus, we will get insight in the within-family variation. As nematodes with individual families or subclades tend to have similar body sizes (*e.g.* Bongers [36]), we expect a moderate variation, and this would enable us to define factors that translate the quantitative PCR signal into a reasonable estimate of the number of individuals to which this signal is corresponding. Finally, in parallel analyses of field samples on the basis of morphological and molecular characteristics will be needed to further validate this entirely novel approach for the analyses of soil and fresh water nematode communities.

Conclusion

Although the potential of nematodes as indicators for the ecological condition of soil and freshwater sediments is widely recognized (*e.g.* Bongers & Ferris [3]), the large scale exploitation of this group so far has been hampered mainly by their conserved morphology. The molecular framework for the detection of two major, trophically heterogenous groups of stress sensitive nematodes combined with the relatively simple quantitative PCR-based analysis tool as presented here offers great perspectives for the exploitation of this group as it lifts - at least in part - the need for specialist taxonomic expertise, detects all developmental stages (instead of - mainly - adults), facilitates the analysis of relatively large and numerous soil and/or sediment samples, and greatly reduces the sample handling time.

Acknowledgements

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References

1. Anderson DT (2001) **Invertebrate Zoology**, 2 edn. Oxford University Press.

2 Yeates GW, Bongers T, De Goede RGM, Freckman DW, Georgieva SS (1993) **Feeding-Habits in Soil Nematode Families and Genera - an Outline for Soil Ecologists.** Journal of Nematology **25**, 315-331.

3. Bongers T, Ferris H (1999) **Nematode community structure as a bioindicator in environmental monitoring.** Trends in Ecology & Evolution **14**, 224-228.

4. De Ruiter PC, Neutel AM, Moore JC (1998) **Biodiversity in soil ecosystems: the role of energy flow and community stability.** Applied Soil Ecology **10**, 217-228.

5. Dmowska E, Ilieva-Makulec K (2004) **Past and present status of nematode community indicators.** In: Proceedings of the fourth international congress of nematology, 8-13 june 2002, Tenerife, Spain (eds. Cook R, Hunt DJ), pp. 487-501. Brill, Leiden.

6. Bongers T (1999) **The Maturity Index, the evolution of nematode life history traits, adaptive radiation and cp-scaling.** Plant and Soil **212**, 13-22.

7. Bongers T, Ilieva-Makulec K, Ekschmitt K (2001) **Acute sensitivity of nematode taxa to CuSO4 and relationships with feeding-type and life-history classification.** Environmental Toxicology and Chemistry **20**, 1511-1516.

8. Ettema CH, Bongers T (1993) **Characterization of nematode colonization and succession in disturbed soil using the maturity index.** Biology And Fertility Of Soils **16**, 79-85.

9. Georgieva SS, McGrath SP, Hooper DJ, Chambers BS (2002) **Nematode communities under stress: the long-term effects of heavy metals in soil treated with sewage sludge.** Applied Soil Ecology **20**, 27-42.

10. Jairajpuri MS, Ahmad W (1992) **Dorylaimida** E.J. Brill, Leiden.

11. Hollis JP (1961) **Nematode reactions to coal-tar dyes.** Nematologica **6**, 315-325.

12. Premachandran D, Vonmende N, Hussey RS, McClure MA (1988) **A Method for Staining Nematode Secretions and Structures.** Journal of Nematology **20**, 70-78.

13. Aleshin VV, Kedrova OS, Milyutina IA, Vladychenskaya NS, Petrov NB (1998) **Relationships among nematodes based on the analysis of 18S rRNA gene sequences: molecular evidence for monophyly of chromadorian and secernentian nematodes.** Russian Journal of Nematology **6**, 175-184.

14. Blaxter ML, De Ley P, Garey JR, et al. (1998) **A molecular evolutionary framework for the phylum Nematoda.** Nature **392**, 71-75.

15. Holterman M, van der Wurff A, van den Elsen S, et al. (2006) **Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown clades.** Molecular Biology and Evolution **23**, 1792-1800.

16. Rusin LY, Aleshin VV, Tchesunov AV, Atrashkevich GI (2003) **The 18S ribosomal RNA gene of Soboliphyme baturini Petrow, 1930** (Nematoda : Dioctophymida) and its implications for phylogenetic relationships within Dorylaimia. Nematology **5**, 615-628.

17. Hall TA (1999) **BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT.** Nucleic Acids Symposium Series **41**, 95-98.

18. Wuyts J, De Rijk P, Van de Peer Y, et al. (2000) **Comparative analysis of more than 3000 sequences reveals the existence of two pseudoknots in area V4 of eukaryotic small subunit ribosomal RNA.** Nucleic Acids Research **28**, 4698-4708.

19. Ben Ali A, Wuyts J, De Wachter R, Meyer A, Van de Peer Y (1999) **Construction of a variability map for eukaryotic large subunit ribosomal RNA.** Nucleic Acids Research **27**, 2825-2831.

20. Posada D, Crandall KA (1998) **MODELTEST: testing the model of DNA substitution.** Bioinformatics **14**, 817-818.

21. Swofford DL (1998) **PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods).** Version 4. Sinauer Associates, Sunderland, Massachusetts.

22. Ronquist F, Huelsenbeck JP (2003) **MrBayes 3: Bayesian phylogenetic inference under mixed models.** Bioinformatics **19**, 1572-1574.

23. Rambaut A, Drummond A (2005) Tracer v1.2.1. available at: http://evolve.zoo.ox.ac.uk/.

24. Maddison DR, Maddison WP (2000) **MacClade 4: Analysis of phylogeny and character evaluation.** Sinauer Associates, Sunderland, Massachusets.

25. Ludwig W, Strunk O, Westram R, et al. (2004) ARB: a software environment for sequence data. Nucleic Acids Research **32**, 1363-1371.

26. Mullin PG, Harris TS, Powers TO (2005) **Phylogenetic relationships of Nygolaimina and Dorylaimina (Nematoda : Dorylaimida) inferred from small subunit ribosomal DNA sequences.** Nematology **7**, 59-79.

27. Bongers T (1990) **The maturity index - an ecological measure of environmental disturbance based on nematode species composition.** Oecologia **83**, 14-19.

28. Yeates GW (1987) **Nematode Feeding and Activity - the Importance of Development Stages.** Biology and Fertility of Soils **3**, 143-146.

29. Zullini A, Peneva V (2006) **Order Mononchida. In: Freshwater nematodes: ecology and taxonomy** (eds. Abebe E, Traunspurger W, Andrassy I), pp. 468-496. CABI Publishing, Wallingford.

30. De Ley P, Decraemer W, Abebe E (2006) **Introduction: Summary of present knowledge and research addressing the ecology and taxonomy of freshwater nematodes. In: Freshwater Nematodes, Ecology and Taxonomy** (eds. Abebe E, Andrassy I, Traunspurger W), pp. 3-30. CABI Publishing, Wallingford.

31. Coomans A (1964) **Stoma structure in members of the Dorylaimina.** Nematologica **9**, 587-601.

32. Siddiqi MR (1969) **Crateronema N Gen (Crateronematidae N Fam) Poronemella N Gen (Lordellonematinae N Sub-Fam) and Chrysonemoides N Gen (Chrysonematidae N Fam) with a Revised Classification of Dorylaimoidea (Nematoda).** Nematologica **15**, 81-&.

33. De Ley P, Van Driessche R, Coomans A (2005) **Terrestrial nematodes from the Galapagos Archipelago. 10. Morphological and taxonomical analysis of Carcharolaimus ramirezi Thorne, 1967** (Dorylaimida : Qudsianematidae). Nematology **7**, 133-147.

34. Maggenti AR (1971) **Nemic relationships and the origin of plant parasitic nematodes. In: Plant parasitic nematodes** (eds. Zuckerman BM, Mai WF, Rohde RA), pp. 65-81. Academic Press Inc., New York.

35. Trudgill DL (1976) **Observations on Feeding of Pungentus-Thornei on Roots of Petunia-Hybrida.** Plant Pathology **25**, 117-&.

36. Bongers T (1994) **De nematoden van Nederland**, 2edn. Koninklijke Nederlandse Natuurhistorische Vereniging

Supplementary information

Table S1: GenBank accession numbers of SSU rDNA sequences (full length)

CLADE 2 (according to Holterman *et al.* 2006) – DORYLAIMIDA			
Species	**accession**	**Species**	**accession**
Allodorylaimus andrassyi	AY284801	*Longidorus dunensis* 3	AY284819
Allodorylaimus sp. G	AJ966472	*Longidorus elongatus* 1G	AF036594
Aporcelaimellus cf. paraobtusicaudatus	AY284812	*Longidorus elongatus* 2G	AY687992
Aporcelaimellus obtusicaudatus 1	AY284811	*Mesodorylaimus avernas*	AY593947
Aporcelaimellus obtusicaudatus 2G	DQ141212	*Mesodorylaimus bastiani* G	AJ966488
Aporcelaimellus sp. 1	AY284813	*Mesodorylaimus centrocercus* 2	EF207248
Aporcelaimellus sp. 2G	AJ875153	*Mesodorylaimus cf. Nigritulus* G	AJ966490
Aporcelaimellus sp. 3G	AJ875155	*Mesodorylaimus japonicus* G	AJ966489
Aporcelaimellus sp. 4G	AJ875154	*Mesodorylaimus* sp.	AY284780
Aquatides christei G	AY552963	*Mesorylaimus centrocercus* 1	AY284799
Axonchium propinquum	AY284820	*Metaporcelaimus simplex*	AY593948
Californidorus sp. G	AY283155	*Microdorylaimus miser*	AY284804
Carcharodiscus banaticus	AY284827	Microdorylaimus modestus 1	AY284805
Chrysonema attenuatum 1	AY593945	*Microdorylaimus modestus* 2	AY284806
Chrysonema attenuatum 2	AY284779	*Microdorylaimus sp.* G	AJ966492
Chrysonema attenuatum 3	EF207245	*Nygolaimus cf. brachyuris* 1	AY284770
Clavicaudoides clavicaudatus	AY593944	*Nygolaimus cf. brachyuris* 2	AY284771
Clavicaudoides sp. G	AY552967	*Nygolaimus cf. parvus* G	AY552974
Clavicaudoides trophurus 1	AY284772	*Opisthodorylaimus sylphoides*	AY284785
Clavicaudoides trophurus 2	AY284773	*Oxydirus nethus*	EF207251
Clavicaudoides trophurus 3	AY593943	*Oxydirus oxycephaloides*	AY284823
Discolaimus cf. Major	EF207252	*Oxydirus oxycephalus* 1	AY284824
Discolaimus major	AY284828	*Oxydirus oxycephalus* 2	AY284825
Dorylaimellus montenegricus	AY284821	*Paractinolaimus macrolaimus* 1	AY284826
Dorylaimellus virginianus G	AY552969	*Paractinolaimus macrolaimus* 2G	AY993978
Dorylaimoides limnophilus 2	AY593950	*Paractinolaimus sp.* G	AY552975
Dorylaimoides micoletskyi	AY284830	*Paralongidorus maximus* G	AJ875152
Dorylaimoides sp.	AY593951	*Paravulvus hartingii* 1	AY284774
Dorylaimus stagnalis 1	AY284777	*Paravulvus hartingii* 2	AY284775
Dorylaimus stagnalis 2	AY284776	*Paravulvus hartingii* 3G	AY552976
Ecumenicus monohystera 1	AY284783	*Paraxonchium laetificans* 1	AY284808
Ecumenicus monohystera 2	AY284784	*Paraxonchium laetificans* 2	AY284809
Ecumenicus sp. 1	AY284781	*Paraxonchium laetificans* 3	AY284810
Ecumenicus sp. 2	AY284782	*Prodorylaimus mas*	AY593946
Enchodelus sp. 1	AY284792	*Prodorylaimus* sp. 1	EF207246
Enchodelus sp. 2	AY284793	cf. *Prodorylaimus* sp.2	AY284778
Enchodelus sp. 3	EF207247	*Pungentes silvestris*	AY284788
Epidorylaimus lugdunensis 1	AY284802	*Pungentes sp.* 1	AY284791
Epidorylaimus lugdunensis 2	AY284803	*Pungentes sp.* 2G	AJ966501
Eudorylaimus carteri G	AJ966484	*Sectonema barbatoides*	AY284814
Eudorylaimus sp. 1	AY284800	*Sectonema sp.*	AY284815
Eudorylaimus sp. 2	AY284794	*Solididens vulgaris* G	AY552977
Labronema ferox G	AY552972	*Thonus circulifer*	AY284795
Labronema vulvapapillatum	AY284807	*Thonus sp.* 1	AY284796
Leptonchus granulosus	AY284831	*Thonus sp.* 2	AY284797
Longidorella sp. 1	AY284789	*Thonus sp.* 3	AY284798
Longidorella sp. 2	AY284790	*Thornia steatopyga*	AY284787
Longidorus cf. Intermedius	AY284816	*Tylencholaimellus affinis* G	AY552978
Longidorus dunensis 1	AY284817	*Tylencholaimus cf. teres*	EF207254
Longidorus dunensis 2	AY284818	*Tylencholaimus sp.* 1G	AJ966510

Tylencholaimus sp. 3	AY284834	*Xiphinema index* 1	EF207249
cf. Tylencholaimus sp. 1	AY284832	*Xiphinema index* 2G	AY687997
cf. Tylencholaimus sp. 2	AY284833	*Xiphinema rivesi* G	AF036610
Xiphidorus balcarceanus G	AY297839	*Xiphinema* sp. 1	EF207250
CLADE 2 - OTHER ORDERS			
Species	**accession**	***Species***	**accession**
Anatonchus tridentatus 1	AY284768	*Mylonchulus sigmaturus 2*	AY284756
Anatonchus tridentatus 2G	AJ966474	*Mylonchulus sigmaturus 3*	AY284757
Bathyodontus cylindricus G	AY552964	*Mylonchulus sp. 1*	AY284758
Bathyodontus mirus	AY284744	*Mylonchulus sp. 2*	AY284759
Clarkus papillatus 1	AY284748	*Mylonchulus sp. 3*	AY284760
Clarkus papillatus 2	AY284750	*Mylonchulus sp. 4*	AY284761
Clarkus papillatus 3	AY284749	*Mylonchulus sp. 5G*	AJ875156
Clarkus papillatus 4G	AY552966	*Prionchulus muscorum 1*	AY284745
Clarkus sp. G	AJ966479	*Prionchulus muscorum 2G*	AJ966500
Coomansus parvus 1	AY284766	*Prionchulus punctatus 1*	AY284746
Coomansus parvus 2	AY284767	*Prionchulus punctatus 2*	AY284747
Cryptonchus tristis	EF207244	*Soboliphyme baturini G*	AY277895
Granonchulus sp.	AY593953	*Trichinella britovi G*	AY851257
Mermis nigrescens G	AF036641	*Trichinella murrelli G*	AY851259
Mermithidae sp.	AY284743	*Trichinella nativa 1G*	AY487254
Miconchus cf. fasciatus G	AY552973	*Trichinella nativa 2G*	AY851256
Mononchus aquaticus 1	AY284764	*Trichinella nelsoni G*	AY851261
Mononchus aquaticus 2	AY284765	*Trichinella papuae G*	AY851263
Mononchus aquaticus 3G	AY297821	*Trichinella pseudospiralis G*	AY851258
Mononchus truncatus 1	AY284762	*Trichinella sp. 1*	AY851260
Mononchus truncatus 2G	AJ966493	*Trichinella sp. 2*	AY851262
Mononchus tunbridgensis 1	AY284763	*Trichinella spiralis 1G*	U60231
Mononchus tunbridgensis 2	AY593954	*Trichinella spiralis 2G*	AY497012
Mylonchulus arenicolus G	AF036596	*Trichinella zimbabwensis G*	AY851264
Mylonchulus brachyuris 1	AY284754	*Trichuris muris G*	AF036637
Mylonchulus brachyuris 2	AY284752	*Trichuris suis 1G*	AY851265
Mylonchulus brachyuris 3	AY284753	*Trichuris suis 2G*	AY856093
Mylonchulus rotundicaudatus	AY284751	*Trichuris trichiura G*	DQ118536
Mylonchulus sigmaturus 1	AY284755		
CLADE 3 – 5			
Species	**accession**	***species***	**accession**
Achromodora sp.	AY284717	*Metachromadora remanei*	AY854216
Isolaimium sp. G	AY552971	*Theristus acer G*	AJ966505
CLADE 1			
Species	**accession**	***species***	**accession**
Campydora demonstrans G	AY552965	*Rhabdolaimus cf. terrestris*	AY284712
Enoplus brevis G	U88336	*Tripyla sp.*	AY284737
Paramphidelus hortensis	AY284739	*Trischistoma sp.*	AY284735
Prismatolaimus dolichurus	AY593957		

Table S2: GenBank accession numbers of LSU rDNA sequences (≈ 1,000 bp from 5′-end)

CLADE 2 (according to Holterman *et al.* 2006) – DORYLAIMIDA			
Species	**accession**	**Species**	**accession**
Allodorylaimus andrassyi 1	AY593015	*Longidorus dunensis* 1	AY593056
Allodorylaimus andrassyi 2	AY593016	*Longidorus dunensis* 2	AY593057
Aporcelaimellus cf. Obtusicaudatus	AY593017	*Mesodorylaimus sp.*	AY593005
Aporcelaimellus cf. *Paraobtusicaudatus*	AY593020	*Mesorylaimus centrocercus*	AY593007
		Microdorylaimus miser	AY593046
Aporcelaimellus obtusicaudatus 1	AY593018	*Microdorylaimus modestus*	AY593049
Aporcelaimellus obtusicaudatus 2	AY593019	*Nygolaimus cf. brachyuris*	AY593061
Aporcelaimellus sp.	AY593021	*Opisthodorylaimus sylphoides* 1	AY593008
Axonchium propinquum	AY593022	*Opisthodorylaimus sylphoides* 2	AY593009
Carcharodiscus banaticus 1	AY593023	*Opisthodorylaimus sylphoides* 3	AY593010
Carcharodiscus banaticus 2	AY593024	*Oxydirus nethus*	AY593011
Chrysonema attenuatum	AY593029	*Oxydirus oxycephalus*	AY593012
Clavicaudoides clavicaudatus 1	EF207234	*Paractinolaimus macrolaimus* 1	AY592998
Clavicaudoides clavicaudatus 2	EF207235	*Paractinolaimus macrolaimus* 2	AY592999
Clavicaudoides trophurus 1	EF207236	*Paractinolaimus macrolaimus* 3	AY593000
Clavicaudoides trophurus 2	EF207237	*Paravulvus hartingii*	AY593062
Discolaimoides symmetricus	EF207238	*Paraxonchium laetificans*	AY593001
Discolaimus cf. Major	EF207239	*Prodorylaimus* sp.	EF207241
Discolaimus major 1	AY593025	cf. *Prodorylaimus* sp.	AY593034
Discolaimus major 2	AY593026	*Pungentus*	AY593054
Dorylaimoides limnophilus	AY593003	*Pungentus engadinensis*	AY593050
Dorylaimoides micoletzkyi	AY593004	Pungentus silvestris 1	AY593052
Dorylaimus stagnalis	AY592994	*Pungentus silvestris* 2	AY593053
Ecumenicus monohystera	AY593013	*Sectonema barbatoides* 1	AY593030
Ecumenicus sp.	AY593014	*Sectonema barbatoides* 2	AY593031
Enchodelus sp.	EF207240	*Sectonema barbatoides* 3	AY593032
Epidorylaimus lugdunensis 1	AY593035	*Sectonema* sp.	AY593033
Epidorylaimus lugdunensis 2	AY593036	*Thonus circulifer* 1	AY593038
Eudorylaimus cf. minutus 1	AY593047	*Thonus circulifer* 2	AY593039
Eudorylaimus cf. minutus 2	AY593048	*Thonus* sp. 2	AY593040
Eudorylaimus sp.	AY593037	*Thonus* sp. 3	AY593041
Labronema vulvapapillatum 1	AY592996	*Tylencholaimellus* sp.	AY593055
Labronema vulvapapillatum 2	AY592997	*Tylencholaimus cf. teres*	EF207243
Longidorella cf. Macramphis	AY593042	*Tylencholaimus mirabilis* 2	AY593027
Longidorella sp. 1	AY593045	*Tylencholaimus mirabilis* 3	EF207242
Longidorella sp. 2	AY593043	cf. *Tylencholaimus* sp.	AY593028
Longidorella sp. 3	AY593044	*Xiphinema rivesi* G	AY210845
Longidorus cf. Intermedius	AY593058		
MONONCHIDA			
Species	**accession**	**species**	**accession**
Anatonchus tridentatus	AY593065	*Mononchus tunbridgensis*	AY593063
Mononchus truncatus	AY593065		

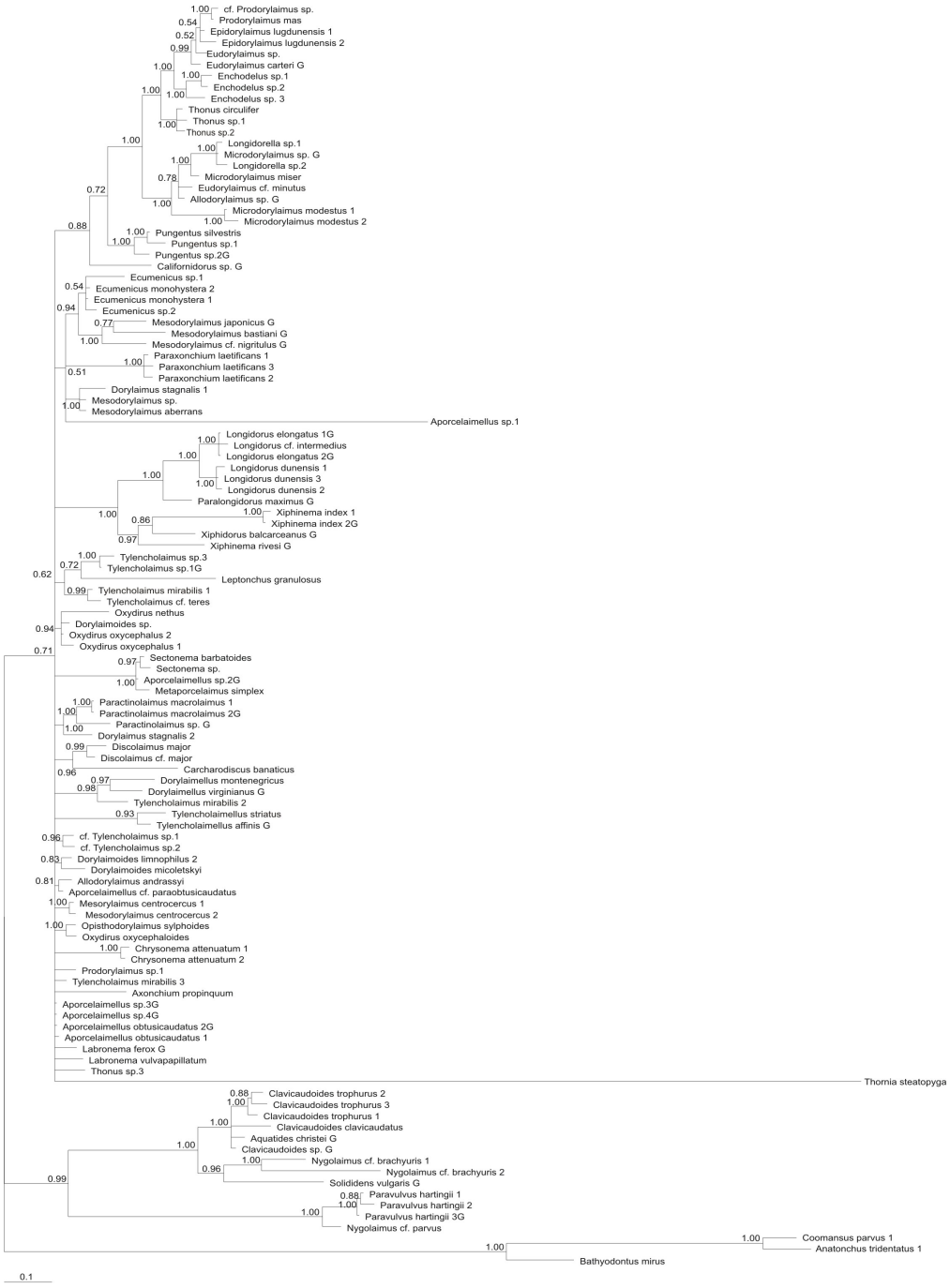

Figure S1. Complete Bayesian SSU rDNA tree of the Dorylaimida (depicted in compressed form in Fig. 2). Numbers near nodes indicate posterior probabilities. A 'G' behind the name indicates that the sequence was acguired from GenBank.

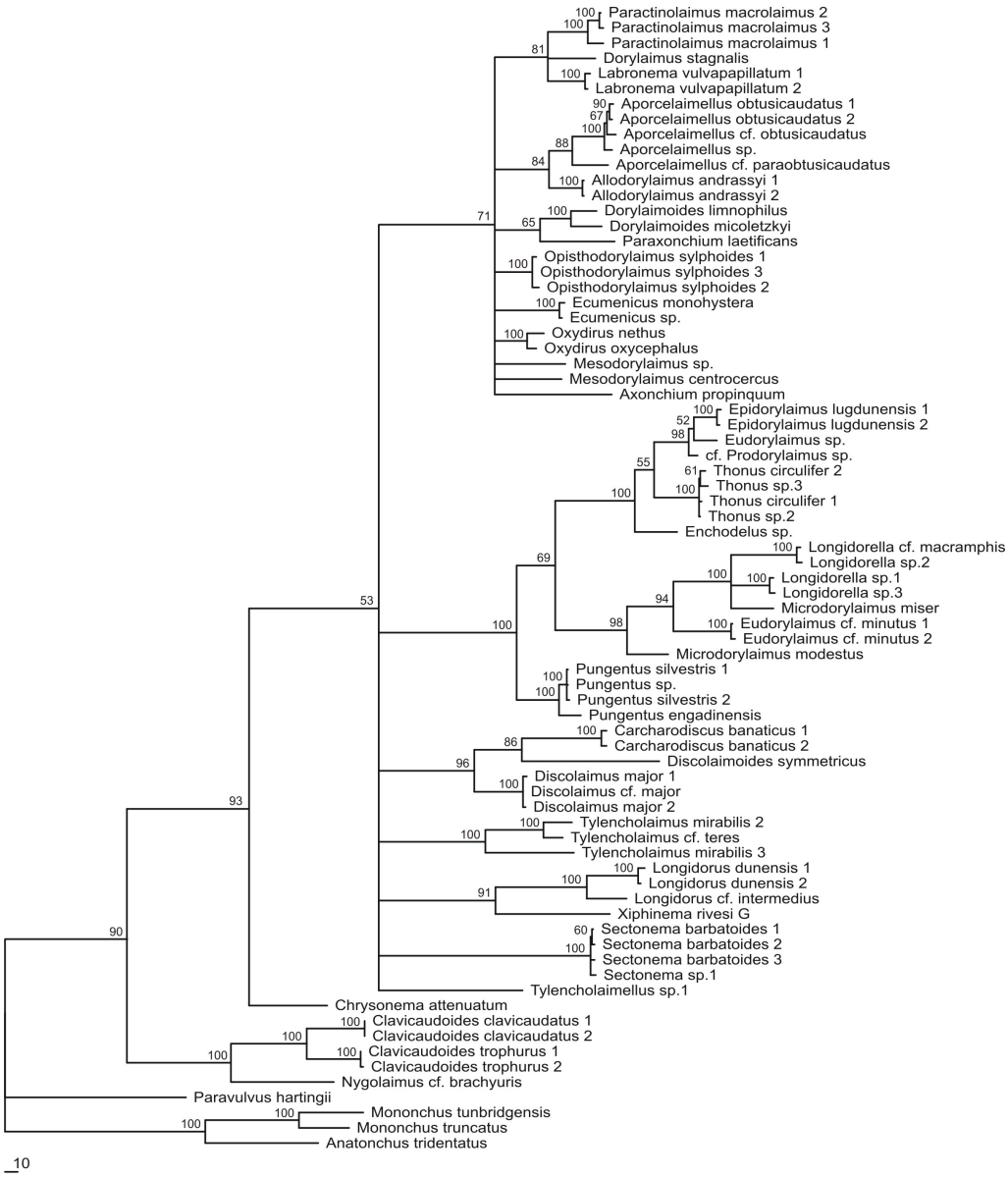

Figure S2. LSU rDNA (5′-end)-based maximum parsimony tree of the Dorylaimida. Numbers near nodes indicate bootstrap values. A ′G′ behind the name indicates that the sequence was acguired from GenBank.

Figure S3. LSU rDNA (5′-end)-based neighbor-joining tree of the Dorylaimida. Numbers near nodes indicate bootstrap values. A 'G' behind the name indicates that the sequence was acguired from GenBank.

Figure S4. Simplified LSU rDNA (5′-end)-based Bayesian tree of the Dorylaimida. Letters indicate a change in the character with the new character state given.

CHAPTER 3

SSU rDNA-BASED PHYLOGENETIC ANALYSIS OF FOLIAR NEMATODES (*APHELENCHOIDES* SPP.), AND THEIR QUANTITATIVE DETECTION IN COMPLEX DNA BACKGROUNDS

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Abstract

Foliar nematodes, plant-parasitic representatives of the genus *Aphelenchoides*, constitute a minority in a group dominated by fungivorous species. Distinction between (mostly harmless) fungal feeding *Aphelenchoides* species and high impact plant parasites such as *A. besseyi*, *A. fragariae, A. ritzemabosi,* and *A. subtenuis* is severely hampered by the scarcity of informative morphological characters, some of which are only observable in specific developmental stages. Poor description of a number of non plant-parasitic *Aphelenchoides* species further complicates identification. Based on (nearly) full-length small subunit ribosomal DNA (SSU rDNA) sequences (≈ 1,700 bp), a phylogenetic tree was generated, and the four target species appeared as distinct, well-supported groups. Notably, this genus does not constitute a monophyletic group: *A. besseyi* and *A. ritzemabosi* cluster together, and they are phylogenetically isolated from *A. fragariae, A. subtenuis* and other, most fungivorous species. A phylum-wide SSU rDNA framework was used to identify species-specific DNA motifs. For the molecular detection of four plant-parasitic *Aphelenchoides* species, PCR primers were developed with high, identical annealing temperatures (63°C). Within the molecular framework presented here, these primers can be used for the rapid screening of plant material and soil for the presence of one or multiple foliar nematode species.

Introduction

Foliar nematodes are the common name of plant-parasitic members of the genus *Aphelenchoides.* Almost all foliar nematodes feed as ecto- or endoparasites on aboveground plant parts, and some species may cause substantial economical losses in both mono- and dicotyledonous crop species. *Aphelenchoides fragariae* is the causal agent of strawberry crimp, and - apart from strawberry - this nematode is reported to attack over 100 plant species belonging to various families. "White-tip disease" refers to a characteristic whitening of leaf tips of rice as a result of parasitism by *A. besseyi.* However, rice is not its only host; it also reproduces on strawberry ("summer dwarf") and other plant species such as *Polianthes tuberosa* and *Capsicum annuum* [1]. Another economically important member of the genus *Aphelenchoides,* the chrysanthemum foliar nematode *A. ritzemabosi,* causes a typical brown discoloration of angular sections between large leaf veins, and chrysanthemum and various other members of the Asteraceae are susceptible hosts. An atypical example of a 'foliar nematode' is the root parasite *A. subtenuis* as it has been shown to feed on roots of narcissus [2]. In interaction with other bulbous hosts such as gladiolus this nematode was found in corms and pseudostems [3]. These foliar nematode species all feed on fungi as well [4]. Apart from their possible survival on fungi in absence of a plant host, the management of some of the foliar nematodes is hindered by their desiccation tolerance.

Besides foliar nematodes, the genus *Aphelenchoides* harbors over 100 mainly mycetophagous species. Species such as *A. bicaudatus, A. composticola* and *A. saprophilus* are frequently found in habitats that also harbour plant parasites. Only taxonomic experts are able to distinguish between (often) harmless mycetophagous species and true foliar nematodes on the basis of morphological characteristics. It is noted that the systematics of this genus is unstable; Hunt [4] indicated that "many nominal species are inadequately characterized for reliable recognition and the genus is in urgent need of a major revision".

Many taxonomic classifications suggest a common ancestry between the families Aphelenchoididae (*Aphelenchoides* being the most speciose genus of the family) and Aphelenchidae (for most recent overview see De Ley *et al.* [5]). However, molecular phylogenetics based on full-length small subunit ribosomal DNA (SSU rDNA) sequences suggested a sister relationship between the Aphelenchoididae (*Aphelenchoides, Laimaphelenchus, Schistonchus*) and the Parasitaphelenchidae (*Bursaphelenchus* spp.) [6] [7], and both families appeared to be only distantly related to the Aphelenchidae [8]. The genus *Aphelenchoides* was shown to be polyphyletic as the foliar nematode species *A. besseyi* and *A. ritzemabosi* together with most *Laimaphelenchus* species reside in a subclade robustly separately from the other members of genus *Aphelenchoides* [8]. So far the relatively conserved SSU rDNA gene seemed to offer sufficient phylogenetic signal for the distinction between *Aphelenchoides* species [8].

Possibilities to explore the ribosomal DNA cistron for *Aphelenchoides* detection purposes have been investigated before. Ibrahim *et al.* [9] amplified the

internal transcribed spacers (ITS) and the entire 5.8S gene from eight *Aphelenchoides* species, and looked for RFLPs in these ≈ 1,000 bp fragments. The restriction enzyme *Alu* I was most successful for the differentiation of *Aphelenchoides* species, but failed in case of *A. besseyi.* More recently, McCuiston *et al.* [10] concentrated on the ITS1 region (≈ 170 bp), and on the basis of a framework of twenty ITS-1 sequences (including five *Aphelenchoides* species) *A. fragariae*-specific PCR primers were developed. ITS regions are relatively variable as they are non-coding. This can be advantageous as these regions show high inter-species variation, but among nematodes the intra-species variation can be considerable as well (see *e.g.* McCuiston *et al.* [10], Fig. 1). Hence, for detection purposes thorough insight in ITS sequence variation among populations is a prerequisite. To avoid this, and to broaden the applicability of assays, tests for the detection of plant-parasitic nematode species are more and more based on coding regions within the nuclear or mitochondrial genome.

Previous results [7] [8] [11] suggested that SSU rDNA contains sufficient phylogenetic signal for the identification of *Aphelenchoides* species. Here an alignment of 48 *Aphelenchoides* SSU rDNA sequences was used in combination with a larger phylum-wide framework comprising ≈ 2,500 nematode taxa. Apart from a phylogenetic analysis, quantitative PCR-based assays are presented for the detection of four foliar nematode species, namely *A. besseyi, A. fragariae, A. ritzemabosi,* and *A. subtenuis.* A single PCR programme can be used for all assays, and by using phylum-wide unique DNA sequence signatures, these four assays are suitable for detection in both simple (plant material) and complex (soil or substrate) DNA backgrounds. It is noted that by far not all (> 100) *Aphelenchoides* species described in literature were included in this paper. In part this is due to the unstable systematic status of this genus and to the very poor description of many species. Hence, specificity of the tests presented here can only be guaranteed within the molecular framework used in this study.

Materials and Methods

Taxon sampling and acquisition of SSU rDNA sequences

Nematode collection and identification, followed by lysis, amplification and sequencing of two overlapping SSU rDNA fragments were performed as described by Holterman *et al.* [6]. Apart from newly generated sequences, all publicly available (nearly) full length SSU rDNA data from Aphelenchoididae were included. A full list of rDNA sequences and the corresponding GenBank numbers are given in Figure 1.

Phylogenetic analyses

The SSU rDNA trees were constructed using Bayesian inference and the maximum likelihood method. For the Bayesian analysis, the alignment was divided into a stem and a loop partition according to the secondary structure. For both partitions, GTR + γ + I was used. Four independent runs were made with 4 Markov chains per run. The program was run for 2 million generations

with a sample frequency of 200 generations. The first 60,000 generations were discarded as burn-in. The program Tracer v1.4 [12] was used to check the stabilization of likelihood and parameters.

For the Maximum Likelihood (ML) analyses, the RAxML-HPC BlackBox program available at the CIPRES Science Gateway V. 3.1 (http://www.phylo.org/sub_sections/portal/) was used [13]. The following parameters were chosen: estimated proportion of invariable sites (GTRGAMMA + I), finding best tree using maximum likelihood search, bootstrapping halted automatically and printed branch lengths.

Primer design and specificity testing

For the development of SSU rDNA-based detection assays for *A. besseyi, A. fragariae, A. ritzemabosi,* and *A. subtenuis*, we essentially followed the procedure as described in Neilson *et al.* [14]. As a starting point an alignment of approximately 2,500 full-length SSU rDNA sequences was used. The corresponding nematode taxa cover a substantial part of the nematode biodiversity in terrestrial and freshwater habitats in moderate climate zones till genus level. The Linux-based software package ARB [15] was used to identify unique rDNA sequence signatures for each of the four foliar nematode species. PCR primers (Table 1) were developed with a predicted optimal annealing temperature of 63°C. For each of the targeted *Aphelenchoides* species, the closest non-targets (Table 1) were identified by changing the mismatch settings in ARB as described in Neilson *et al.* [14].

To test the specificity of the primers, bacterial clones harboring a TOPO TA vector with an SSU rDNA fragment of the closest non-target species were grown at 37°C in 2 mL of LB medium supplemented with 100 µg/mL ampicillin. Plasmid extraction was performed using the Wizard Plus Minipreps DNA Purification System (Promega). DNA concentrations were measured with a NanoDrop spectrophotometer (Thermo Scientific) and adjusted to 10 ng/µL. For the qualitative nematode detection testing, 3 µl of 10^3× diluted sample was used.

For each Q-PCR reaction 3 µl of diluted template was mixed with species-specific primers (end concentrations for both primers 200 nM) and 12.5 µL Absolute SYBR Green fluorescein mix (Thermo Fisher) in a total reaction volume of 25 µL. Thermal cycling was performed on iQ5 (Bio-Rad) and the following PCR profile was used: 95 °C for 15 min; followed by 60 cycles of 95 °C for 30 s, 63 °C (or gradient from 61°C to 66°C) for 1 min and 72 °C for 30 s. For negative controls, template was replaced by an equal volume of Milli-Q water. Quantitative PCR output is expressed in C_t values, the cycle number (C_t) at which the reporter dye emission intensity crosses a predetermined threshold. Here, the C_t threshold was set at 80.

Table 1. Primer combinations and close non-target sequences used in detection assays for four *Aphelenchoides* species: *A. besseyi*, *A. fragariae*, *A. ritzemabosi* and *A. subtenuis*. All primer combinations were tested in the annealing temperature of 63°C. For the use of LNAs we essentially adhered to Nakitandwe *et al.* [28].

Target species	Primer combinations, positions and product lengths	Closest non targets
A. subtenuis	1454: 5'-gtagttggattgtctcgcc 1458: 5'-atgacttgtcttgagcacg Primer 1454 based on SNP at position 589 Primer 1458 based on SNP at position 697 Product length: 144 bp.	*Anomyctus xenurus* 1 *Aphelenchoides ritzemabosi* 1 *Aphelenchoides ritzemabosi* 2 *Aphelenchoides sp.* 10 *Alaimus parvus* 1 (AY284738) *Diplopeltula sp.*1 (EF591329) *Granonchulus sp.* 1 (AY593953) *Heterodera goettingiana* 1 (EU669915) *Plectonchus sp.* 1 (AY593920) *Pratylenchus neglectus* 1 (EU669923) *Tylolaimophorus typicus* 2 (JQ957901)
A. besseyi	1770: 5'-gcgggattcgtggttc*t 1772: 5'-cgacatgccgaaacatgag Primer 1454 based on SNP at position 1317 Primer 1458 based on SNP at position 1608 Product length: 325 bp.	*Acrobeloides cf thornei* 1 (JQ957903) *Ascolaimus cf elongates* 2 (EF591330) *Aphelenchoides ritzemabosi 2* *Clavicaudoides trophurus* 1 (AY284772) *Deladenus durus* 1 (JQ957898) *Domorganus macronephritices* 2 (FJ969122) *Ethmolaimus pratensis* 1 (AY593942) *Panagrobelus stammeri* 1 (AF202153) *Paracyatholaimus intermedius* 3 (JQ957906) *Rotylenchus uniformis* (AY593882) *Tripyla cf filicaudata* 1 (AY284730)
A. fragariae	1469: 5′-cttatcgcacgactttacg 1472: 5'-tcaaagtaatccgcatccaat Without LNA: 1844: 5′-ttatcgcacgactttacg 1847: 5′-caaagtaatccgcatccaat Primer 1454 based on SNP at position 223 Primer 1458 based on SNP at position 655 Product length: 470 bp.	*Anomyctus xenurus* 1 *Anoplostoma sp.* 2 (FJ040492) *Aphelenchoides sp.* 8 *Aphelenchoides cf bicaudatus* 1 *Aphelenchoides saprophilus* 1 *Aphelenchoides sp.* 1 *Aphelenchoides sp.* 5 *Pratylenchus penetrans* 1 (EU669925) *Neopsilenchus magnidens* 1 (AY284585) *Symplocostoma sp.* 1 (FJ040502)
A. ritzemabosi	1496: 5'-cgctggtgggtttcga 1499: 5'-cccgctaagaaatgatcac*c Primer 1454 based on SNP at position 986 Primer 1458 based on SNP at position 1299 Product length: 347 bp.	*Anomyctus xenurus* 1 *Aphelenchoides composticola* 1 *Aphelenchoides sp.* 8 *Aphelenchoides sp.* 10 *Geomonhystera villosa* 1 (EF591334) *Globodera sp.* 4 (JQ957897) *Laimaphelenchus penardi* 1 *Ogma menzeli* 1 (EU669919) *Oncholaimidae sp.* 1 (FJ040493) *Paratylenchus straeleni* 2 (AY284630) *Rotylenchus sp.* 1 (AY284608) *Synonchiella sp.* 1 (FJ040468) *Steinernema glaseri* 2 (FJ040422)

Quantitative detection of Aphelenchoides species

In order to produce standards to relate C_t to number of target nematodes, quantitative series of microscopically-identified nematodes were prepared: 1, 5, 10, 50 and 100 hand-picked individuals of each species were collected in 0,2 ml tube containing 25µl of Milli-Q water. For lysis an equal volume of lysis buffer

as described by Holterman *et al.* [6] was added. Lysis took place in a Thermomixer (Eppendorf) at 65 ˚C and 750 rpm for 1 h, followed by 5 min incubation at 100˚C. Three µl of 50x diluted sample from each range was used for Q-PCR. As a positive control relevant plasmid DNA was used. See previous section for Q-PCR conditions. Resulting data were used to define the slope and the Y-intercept of the regression line describing the linear relationship between log (# nematodes) and the corresponding C_t values.

Aphelenchoides-free nematode suspensions used as non-target backgrounds were extracted from freshly harvested narcissus bulbs with adhering soil (Flower Bulbs and Nursery Stock - PPO Lisse, The Netherlands). Suspensions were collected after incubation for two days in a mist chamber. Subsamples from suspension were checked microscopically for the absence of target *Aphelenchoides* species. Non-target backgrounds (≈ 1,000 individuals for each of the background samples) included plant-parasitic and free-living nematodes.

Results

SSU rDNA-based phylogenetic relationships among Aphelenchoididae

Phylogenetic analysis of 60 full-length SSU rDNA sequences from members of the family Aphelenchoididae (54) and its close relatives (6) gave rise to two major subclades (labeled A and B in Fig. 1). Notably, foliar nematodes do not constitute a monophyletic group as *Aphelenchoides besseyi* and *A. ritzemabosi,* two major impact plant parasites, reside in a subclade (B in Fig. 1) distinct from most other foliar nematode species (A in Fig. 1). This division is supported by two independent phylogenetic methods, namely Bayesian Inference (BI) and Maximum Likelihood (ML). Subclade B comprises a split between *A. besseyi*, *A. ritzemabosi*, and the recently described *A. paradalianensis* [16] on the one hand, and *Laimaphelenchus preissii* and *L. penardi* on the other. Subclade A is characterized by a sister relationship between *A. subtenuis*, and representatives of other *Aphelenchoides* species (and *L. heidelbergi*). Other members of the superfamily Aphelenchoidoidea, namely Parasitaphelenchidae, Ektaphelenchidae and Seinuridae, were used as outgroup. Noteworthy is the predatory nature of the *Seinura* spp., an unusual feeding type among the Aphelenchoidoidea; with rapid stylet thrusts it punctures the cuticle of its prey, after injection of pharyngeal gland secretions, the body content is ingested.

A remarkable characteristic of this *Aphelenchoides* phylogenetic tree is the inclusion of a relatively large number of sequences (# = 21) for which the corresponding nematode could not be identified to species level. The group consists of 11 accessions from GenBank ("G" behind the name), and 10 accessions generated in this research (for example "*Aphelenchoides* sp. 2"). This illustrates the paucity of informative characters for many species within this genus. In a few cases "*Aphelenchoides* sp." sequences reside within a well-supported monophyletic group with limited intra-species variation. *Aphelenchoides* sp. 2, 3 and 4

are positioned within a cluster of *A. fragariae* sequences, and we assume that *Aphelenchoides* sp. 2, 3 and 4 in fact are representatives of this species. The same holds for *Aphelenchoides* sp. 9G that is positioned in a small cluster of *A. besseyi* sequences. We assume that *Aphelenchoides* sp. 9G belongs to this species. A number of other "*Aphelenchoides* sp." sequences such as *Aphelenchoides* sp. 1G, 5G, 12G (etc.) could not be assigned to any characterized *Aphelenchoides* species.

Although it is realized that a part of the *Aphelenchoides* biodiversity is not represented in this tree - and even this statement can not be substantiated further due to poor description of many nominal species [4] - some foliar nematode species appeared as well-supported distinct groups with limited intra-species variation in this SSU rDNA-based phylogenetic analysis. This prompted us to investigate whether this gene could be used for the molecular identification of four plant-parasitic *Aphelenchoides* species.

The use of SSU rDNA sequence motifs for the (quantitative) detection of four plant-parasitic Aphelenchoides species

A phylum-wide database comprising approximately 2,500 (nearly) full length SSU rDNA sequences was used as a starting point to develop detection assays for four plant-parasitic *Aphelenchoides* species, namely *A. besseyi, A. fragariae, A. ritzemabosi*, and *A. subtenuis*. The software package ARB [15] was used to identify species-characteristic sequence motifs, and close non-target taxa for these particular fragments within the SSU rDNA. It is worth mentioning that these close non-targets are not necessarily systematically related to the target species. For the detection of *A. besseyi,* the most optimal primer combination (1770 / 1772, see Table 1) was designed on the basis of two typical SSU rDNA motifs. As shown in Table 1, a series of nematode species was identified within the SSU rDNA database with similar motifs. Apart from *A. ritzemabosi,* this list includes taxa that are taxonomically unrelated to *A. ritzemabosi* such an *Acrobeloides* (Cephalobidae), an *Ethmolaimus* (Ethmolaimidae), a *Panagrobelus* (Panagrolaimidae), and a *Rotylenchus* (Hoplolaimidae) species (three bacterivores and one plant parasite, respectively).

Similarly, PCR primer combinations were developed for the other three selected *Aphelenchoides* species (Table 1). To optimize specificity, PCR primers were designed to work optimally at a high annealing temperature (63^{o}C). Moreover, all PCR primers have the same annealing temperature. As a consequence of this uniformity, these *Aphelenchoides* tests can be run in parallel in any combination.

In Fig. 2A, the procedure for the testing of candidate *Aphelenchoides* species-specific primer combinations is illustrated. For detection of *A. subtenuis*, the annealing temperature characteristics were tested for primer combination 1454 / 1458 (Table 1) on plasmids DNA harboring the relevant SSU rDNA fragments. As can be seen in Fig. 2A, the optimal temperature (= the temperature giving the lowest C_t value) is indeed 63^{o}C. Subsequently, the specificity of primer combination 1454 / 1458 was tested with all relevant target (cloned SSU rDNA fragments from three *A. subtenuis* populations), and 11 close non-target species.

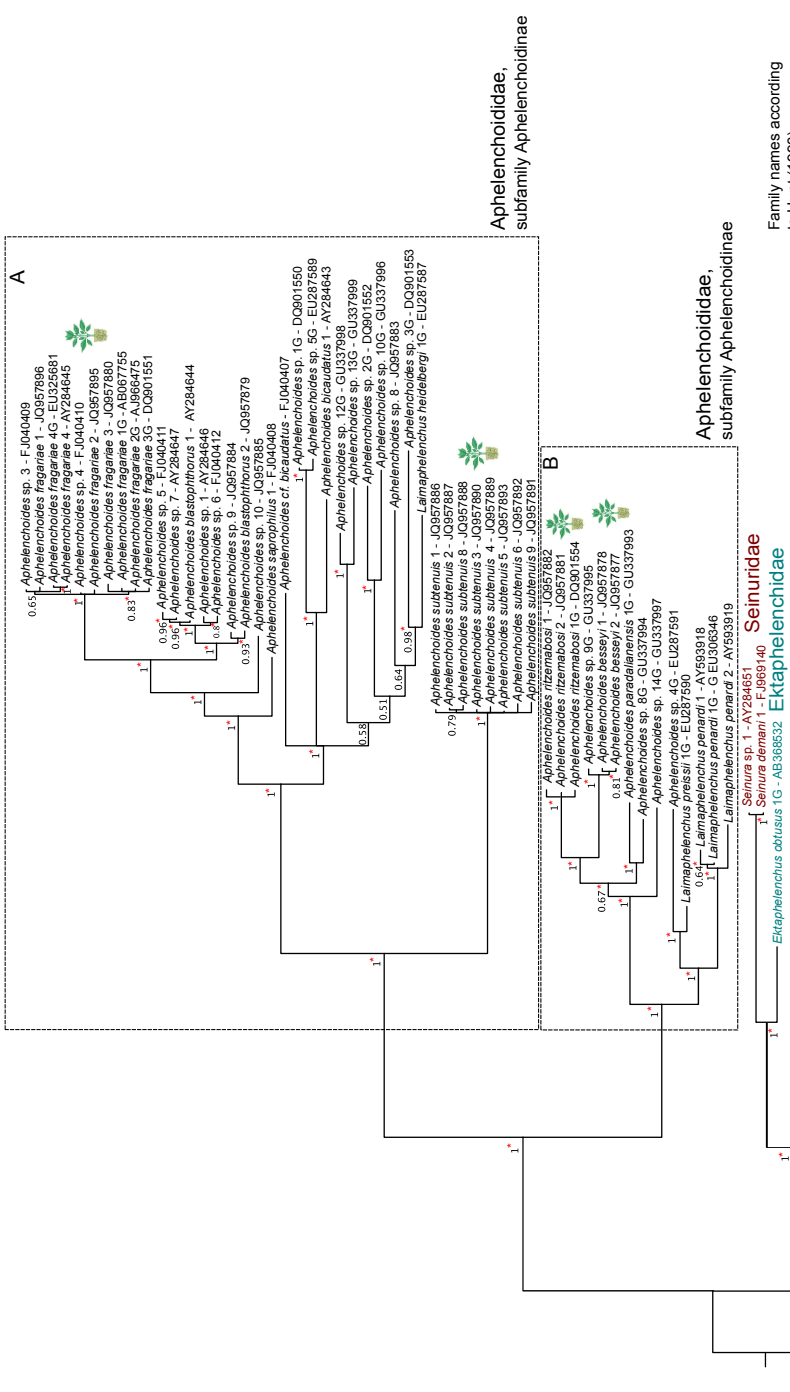

Figure 1. A small subunit ribosomal DNA-based Bayesian tree of the Aphelenchoididae (Nematoda). Support values (posterior probabilities) are given next to the nodes; nodes marked with an asterisk (*) are highly supported by ML (bootstrap > 90%). A 'G' behind a nematode name indicates that the sequence was acquired from GenBank. For the nomenclature of taxonomic groups we essentially conformed to Hunt [4].

As shown in Fig. 2B1, none of the close non-target species (marked in red) gave a significant PCR signal after 60 cycles. In Fig. 2B2, the negative first derivative of the melting curve (= temperature-dependent dissociation of the amplicon) is shown. It is noted that this particular amplicon gives rise to an asymmetric melting pattern.

Table 2. To verify the specificity of the SSU rDNA-based primers, target nematodes were added to twenty independent *Aphelenchoides*-free nematode backgrounds (each ~ 1,000 nematodes). The background suspensions were extracted from narcissus bulbs with adhering soil. Individual nematode suspensions were checked microscopically for the absence of *Aphelenchoides* spp.

***Aphelenchoides* species**			**SSU rDNA-based detection**	
	Background Sample ID	**# target nematodes**	**C_t (*A. fragariae*)**	**C_t (*A. ritzemabosi*)**
A. fragariae	6	5	34,00	N/A
	7		33,25	N/A
	8		33,09	N/A
	9		33,25	N/A
	10		32,57	N/A
	35		32,60	N/A
	36		32,72	N/A
	37		32,34	N/A
	38A		32,31	N/A
	38B		32,91	N/A
A. ritzemabosi	11	5	37,67	34,33
	12		N/A	36,53
	13		36,92	36,26
	14		37,22	35,78
	15		N/A	35,40
	16		N/A	34,44
	17		N/A	35,39
	18		N/A	34,75
	19		N/A	34,24
	20		N/A	34,62

To translate C_t values into numbers of nematodes, a calibration curve was generated. To this end, a series of 1, 5, 10, 50 and 100 hand-picked *A. subtenuis* individuals was analyzed twice (technical replication). The results of the analyses with primer combination 1454/1458 is shown in Fig. 2C. A robust linear relationship (R^2=0,99) was found between C_t values the log [number of target nematodes]. The relationship is characterized by a slope of -5,437 (slope is negative as more target DNA results in lower C_t values), and an intercept (= C_t value corresponding with a

single target nematode) of 30,65 cycles. Values on the x-axis are negative as only a small fraction of the nematode lysate was used for analysis.

In essence the same procedure was followed for *A. besseyi*, *A. fragariae*, and *A. ritzemabosi*. The selected primer combinations 1770/1772, 1469/1472 (1844/1847), and 1496/1499 (for primers see Table 1) were tested against 11, 10 and 13 close non-targets. The results are shown in Fig. 3 A1, B1 and C1. It was not always possible to design primers that give no detectable signal at all for all close non-targets. In case of the specificity test for *A. fragariae*, the gap between the latest target and the earliest non-target signal is 29.3 cycles, which will be sufficient for most practical applications. In addition, the negative first derivatives of the melting curves are shown in Fig. 3 A2, B2 and C2.

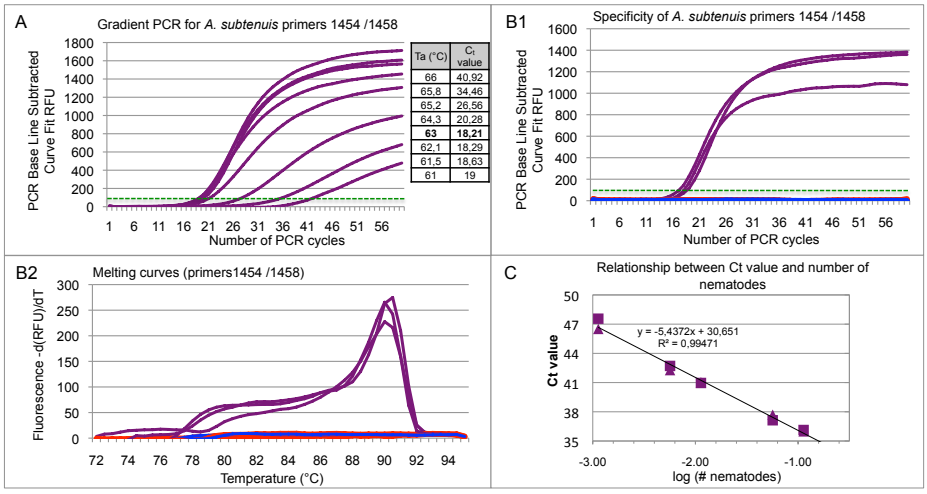

Figure 2. Development of a foliar nematode-specific primer combination. *Aphelenchoides subtenuis* is used as an example to illustrate the procedure followed for primer development. **(A)** All primers were designed to have optimal annealing temperature (T_a) of 63°C, with C_t values rapidly increasing above target temperature plus 1°C. **(B1)** Specificity test of an *A. subtenuis* primer combination with plasmid DNAs from three target species, SSU rDNA fragments from 11 potential false positives (Table 1) and a negative water control. **(B2)** Graph showing the negative first derivatives of the melting curve of the amplicons shown in B1. **(C)** The relationship between C_t-values and numbers of nematodes for quantification of densities. Series of hand-picked individuals of *A. subtenuis* were lysed and amplified with primer combination 1454 /1458. Two times five C_t values were used to define the slope and the Y-intercept of the regression line describing the linear relationship between log (# nematodes) and the corresponding C_t values.

In several cases locked nucleic acids (LNAs) were included to optimize primer specificity. For those who would prefer not to use LNAs, we tested whether conventional primers would give similar specificity. In case of the *A. fragariae* primers 1469 / 1472, removal of a single nucleotide at the 5'-end resulted in primers (1844 / 1847) with the same optimal annealing temperature (63°C) and almost the same specificity (data not shown).

To further verify the specificity of the *Aphelenchoides* primers, target nematodes were added to *Aphelenchoides*-free nematode suspensions from soil. Twenty independent nematode suspensions that had been checked microscopically for the absence of *Aphelenchoides* spp. were used as backgrounds. For each experiment, five hand-picked target nematodes were added to the nematode suspension (~ 1,000 individuals extracted narcissus bulbs with adhering soil), and after DNA extraction the newly developed SSU rDNA-based primers were used to check whether *A. fragariae* and *A. ritzemabosi* could be detected. In the *A. fragariae*-spiked samples, the target could be detected whereas *A. ritzemabosi* primers gave no signal at all (Table 2). In the *A. ritzemabosi*- spiked samples, the targets were always detected, but for three samples an *A. fragariae* signal was found as well (backgrounds 11, 13 and 14). The most plausible explanation for this result would be the presence of a small number (probably one) *A. fragariae* individual in the supposedly *Aphelenchoides*-free nematode suspension (with the detection assay presented here, a single *A. fragariae* gives a C_t value around 37 cycles; data not shown).

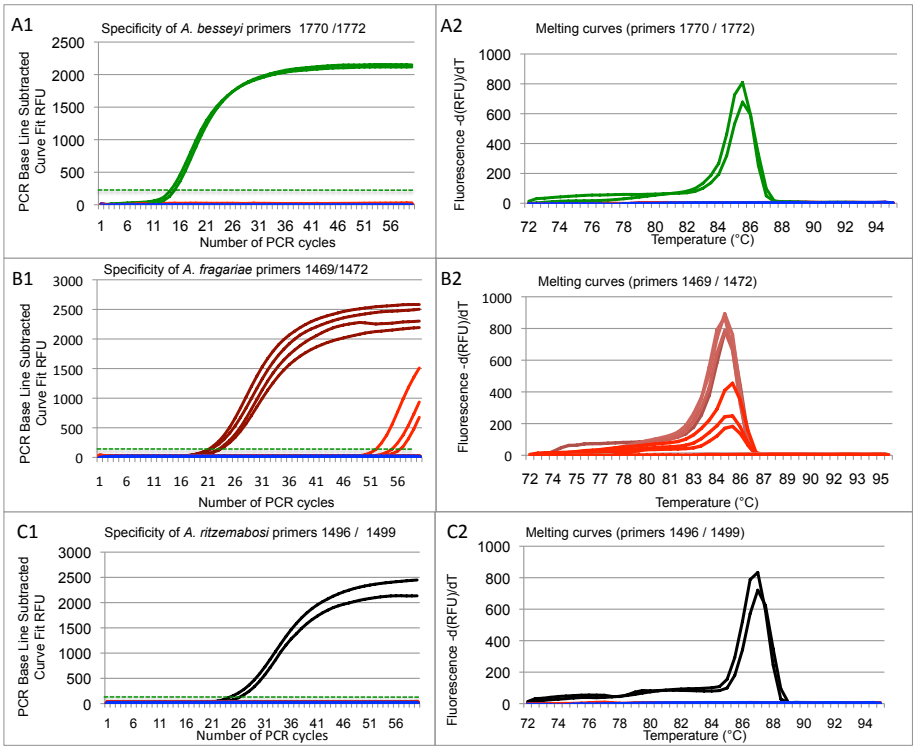

Figure 3. Testing of PCR assays for the detection of three foliar nematode species. **(A1)** Specificity test of an *A. besseyi*-primer combination with plasmid DNAs from target and close non-target species as given in Table 1, and **(A2)** graph showing the negative first derivatives of the melting curve of the amplicons **(B1, 2)** *Idem* for *A. fragariae*. **(C1, 2)** *Idem* for *A. ritzemabosi*.

Discussion

Foliar nematodes – plant-parasitic representatives of the genus *Aphelenchoides* – are problematic in more than one aspect. Although the genus receives most attention because of a few notorious plant parasites, by far most species are – from a plant production point of view – harmless fungivores. The distinction between foliar nematodes and fungal feeding *Aphelenchoides* species on the basis of morphological characteristics is often problematic. This is not only due to the absence of a reliable identification key for this genus, but also to the (very) poor description of numerous nominal species [4]. To assess whether the SSU rDNA gene could be used for the molecular recognition of foliar nematode species, a phylogenetic analysis was made. The resulting tree showed a major, well-supported split among the foliar nematodes; whereas *A. fragariae* and *A. subtenuis* reside in subclade A, two other foliar nematode species – *A. besseyi* and *A. ritzemabosi* – are positioned in subclade B (Fig. 1). In two previous analyses with fewer representatives from this particular group a similar split has been observed. Based on respectively thirteen and eighteen *Aphelenchoides* sequences Chizhov *et al.* [11] and Van Megen *et al.* [8] mentioned two separate clades uniting the plant-parasitic aphelenchids. *A. subtenuis* residing in a sister position vis-à-vis all other subclade A members, is known to infect roots, an atypical characteristic among the plant-parasitic *Aphelenchoides* species. The tail tip morphology seems to support the positioning of *A. subtenuis.* Except for *A. bicaudatus,* the *Aphelenchoides* species represented in subclade A all bear a single, poorly to well-developed mucro ("a sharp point at the end of - in this case - nematode's tail") devoid of any processes.

A remarkable feature of this phylogenetic tree is the positioning of two members of the predatory genus *Seinura* among fungivorous and plant-parasitic relatives. Upon puncturing the cuticle, the prey is immobilized by the injection of pharyngeal gland secretions, and the pre-digested body content is taken up. Possibly, *Seinura* is not the only predaceous nematode among the Aphelenchoidoidea as there is one report about *L. penardi* feeding on other nematodes [17].

Application of real time PCR for the quantitative detection of plant-parasitic nematode species has rapidly gained popularity. Quantitative molecular assays have been developed for several high impact species such as *Pratylenchus penetrans* [18], *P. thornei* [19], the soybean cyst nematode *Heterodera glycines* [20], and the potato cyst nematodes *Globodera rostochiensis* [21] and *G. pallida* [22]. All afore-mentioned assays are based on species-specific motifs in one of the two (non-coding) internal transcribed spacers (ITS1 or ITS2) located within the ribosomal DNA cistron. In a few occasions, authors selected coding regions; for the development of a quantitative assay for the pinewood nematode *Bursaphelenchus xylophilus*, Huang *et al.* [23] preferred the DNA topoisomerase I gene, whereas the small subunit ribosomal DNA was shown to be suitable for the detection of the potato cyst nematode *G. rostochiensis* [6].

Ideally, molecular detection of plant-parasitic nematodes species would be based on a DNA region showing minimal intra-specific and maximal inter-specific variation. Hence, DNA regions that are used to study relationships

among populations are less suitable for species detection. ITS regions were shown to be informative to study the relatedness between *G. pallida* populations [24] [25], isolates of the burrowing nematode *Radopholus similis* [26], and populations of the Columbia lance nematode *Hoplolaimus (Basirolaimus) columbus* [27]. Hence, the use of the ITS regions for species detection purposes requires a thorough inventory of the intraspecific variation. This information would allow for the distinction between population and species-specific polymorphisms, and robust detection assays could be based on the latter. For foliar nematode species detection, we preferred to use a SSU rDNA, a relatively conserved coding region within the ribosomal DNA cistron with for these four *Aphelenchoides* species low intraspecific variation. We showed that this gene harbours sufficient informative nucleotide positions for real time PCR-based detection, and the assays presented here are technically straightforward and easily implementable (*i.e.* requires standard laboratory equipment) in agricultural research and service and inspection laboratories.

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References

1. Hockland, S. (2004) ***Aphelenchoides besseyi*.** EPPO Bulletin 34 (2):303-308.

2. Mor, M., and Spiegel, Y. 1993. **Infection of Narcissus Roots by *Aphelenchoides subtenuis*.** J. Nematol. 25 (3):476-479.

3. Mohammad Deimi, A., Tanha Maafi, Z., Palomares Rius, J. E., and Castillo, P. (2006) ***Aphelenchoides subtenuis* (Cobb, 1926) Steiner & Buhrer, 1932 (Nematoda: Aphelenchoididae) from Iran with morphological and morphometric characterisation.** Nematology 8 (6):903-908.

4. Hunt, D. J. (1993) **Aphelenchida, Longidoridae and Trichodoridae: their systematics and bionomics.** CAB International, Wallingford, UK.

5. De Ley, P., Decraemer, W., and Abebe, E. (2006) **Introduction: Summary of present knowledge and research addressing the ecology and taxonomy of freshwater nematodes.** Pages 3-30 in: Freshwater Nematodes, Ecology and Taxonomy, E. Abebe, I. Andrassy and W. Traunspurger, eds. CABI Publishing, Wallingford.

6. Holterman, M., van der Wurff, A., van den Elsen, S., van Megen, H., Bongers, T., Holovachov, O., Bakker, J., and Helder, J. (2006) **Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown clades.** Mol. Biol. Evol. 23 (9):1792-1800.

7. Zhao, Z., Ye, W., Giblin-Davis, R. M., Li, D., Thomas, K. W., Davies, K. A., and Riley, I. T. (2008) **Morphological and molecular analysis of six aphelenchoidoids from Australian conifers and their relationship to *Bursaphelenchus* (Fuchs, 1937).** Nematology 10 (5):663-678.

8. Van Megen, H., Van Den Elsen, S., Holterman, M., Karssen, G., Mooyman, P., Bongers, T., Holovachov, O., Bakker, J., and Helder, J. (2009) **A phylogenetic tree of nematodes based on about 1200 full-length small subunit ribosomal DNA sequences.** Nematology 11 (6):927-950.

9. Ibrahim, S. K., Perry, R. N., Burrows, P. R., Hooper, D. J. (1994) **Differentiation of Species and Populations of *Aphelenchoides* and of *Ditylenchus angustus* Using a Fragment of Ribosomal DNA** Journal of Nematology 26(4): 412–421.

10. McCuiston, J. L., Hudson, L. C., Subbotin, S. A., Davis, E. L., and Warfield, C. Y. (2007) **Conventional and pcr detection of *Aphelenchoides fragariae* in diverse ornamental host plant species.** J. Nematol. 39 (4):343-355.

11. Chizhov, V. N., Chumakova, O. A., Subbotin, S. A., and Baldwin, J. G. (2006) **Morphological and molecular characterization of foliar nematodes of the genus *Aphelenchoides*: *A. fragariae* and *A. ritzemabosi* (Nematoda: Aphelenchoididae) from the Main Botanical Garden of the Russian Academy of Sciences, Moscow.** Russ. J. Nematol. 14 (2):179-184.

12. Rambaut, A., and Drummond, A. (2007) **Tracer** v1.4. http://beast.bio.ed.ac.uk/Tracer

13. Stamatakis, A. (2006) **RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models.** Bioinformatics 22 (21):2688-2690.

14. Neilson, R., Donn, S., Griffiths, B., Daniell, T., Rybarczyk, K. D., Van den Elsen, S., Mooyman, P., and Helder, J. (2009) **Molecular Tools for Analysing Nematode Assemblages.** Pages 188-207 in: Nematodes as environmental indicators, M. J. Wilson and T. Kakouli-Duarte, eds. CABI, Wallingford.

15. Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A. W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., and Schleifer, K. H. 2004. **ARB: a software environment for sequence data.** Nucl. Acids Res. 32 (4):1363-1371.

16. Cui, R., Zhuo, K., Wang, H., and Liao, J. (2011) ***Aphelenchoides paradalianensis* n. sp. (nematoda: Aphelenchoididae) isolated at Guangzhou, China, in packaging wood from South Korea.** Zootaxa (2864):57-64.

17. McBeth, C. W. (1937) **Observations on a predacious nematode.** Proc. Helminthol. Soc. Washington 4:18.

18. Sato, E., Min, Y. Y., Shirakashi, T., Wada, S., and Toyota, K. (2007) **Detection of the root-lesion nematode, *Pratylenchus penetrans* (Cobb), in a nematode community using real-time PCR.** Jap. J. Nematol. 37 (2):87-92.

19. Yan, G., Smiley, R. W., and Okubara, P. A. (2012) **Detection and quantification of Pratylenchus thornei in DNA extracted from soil using real-time PCR.** Phytopathology 102 (1):14-22.

20. Goto, K., Sato, E., and Toyota, K. (2009) **A novel detection method for the soybean cyst nematode *Heterodera glycines* Ichinohe using soil compaction and real-time PCR.** Nematol. Res. 39 (1):1-7.

21. Nowaczyk, K., Dobosz, R., Kornobis, S., and Obrepalska-Steplowska, A. (2008) **TaqMan real-time PCR-based approach for differentiation between *Globodera rostochiensis* (golden nematode) and *Globodera artemisiae* species.** Parasitol. Res. 103 (3):577-581.

22. Nakhla, M. K., Owens, K. J., Li, W., Wei, G., Skantar, A. M., and Levy, L. (2010) **Multiplex real-time PCR assays for the identification of the potato cyst and tobacco cyst nematodes.** Plant Disease 94 (8):959-965.

23. Huang, L., Ye, J. R., Wu, X. Q., Xu, X. L., Sheng, J. M., and Zhou, Q. X. (2010) **Detection of the pine wood nematode using a real-time PCR assay to target the DNA topoisomerase I gene.** Eur. J. Plant Pathol. 127 (1):89-98.

24. Blok, V. C., Malloch, G., Harrower, B., Phillips, M. S., and Vrain, T. C. (1998) **Intraspecific variation in Ribosomal DNA in populations of the potato cyst nematode Globodera pallida.** J. Nematol.30 (2):262-274.

25. Grenier, E., Bossis, M., Fouville, D., Renault, L., and Mugniéry, D. (2001) **Molecular approaches to the taxonomic position of Peruvian potato cyst nematodes and gene pool similarities in indigenous and imported populations of *Globodera*.** Heredity 86 (3):277-290.

26. Elbadri, G. A. A., De Ley, P., Waeyenberge, L., Vierstraete, A., Moens, M., and Vanfleteren, J. (2002) **Intraspecific variation in *Radopholus similis* isolates assessed with restriction fragment length polymorphism and DNA sequencing of the internal transcribed spacer region of the ribosomal RNA cistron.** Int. J. Parasitol. 32 (2):199-205.

27. Bae, C. H., Szalanski, A. L., and Robbins, R. T. (2009) **Genetic variation of *Hoplolaimus columbus* populations in the United States using PCR-RFLP analysis of nuclear rDNA ITS regions.** J. Nematol. 41 (3):187-193.

28. Nakitandwe, J., Trognitz, F., and Trognitz, B. (2007) **Reliable allele detection using SNP-based PCR primers containing Locked Nucleic Acid: Application in genetic mapping.** Plant Methods 3 (1).

CHAPTER 4

RATHER THAN BY DIRECT ACQUISITION *VIA* LATERAL GENE TRANSFER, GHF5 CELLULASES WERE PASSED ON FROM EARLY PRATYLENCHIDAE TO ROOT-KNOT AND CYST NEMATODES

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Abstract

Plant parasitic nematodes are unusual Metazoans as they are equipped with genes that allow for symbiont-independent degradation of plant cell walls. Among the cell wall-degrading enzymes, glycoside hydrolase family 5 (GHF5) cellulases are relatively well characterized, especially for high impact parasites such as root-knot and cyst nematodes. Interestingly, ancestors of extant nematodes most likely acquired these GHF5 cellulases from a prokaryote donor by one or multiple lateral gene transfer events. To obtain insight into the origin of GHF5 cellulases among evolutionary advanced members of the order Tylenchida, cellulase biodiversity data from less distal family members were collected and analyzed. Single nematodes were used to obtain (partial) genomic sequences of cellulases from representatives of the genera *Meloidogyne, Pratylenchus, Hirschmanniella* and *Globodera.* Combined Bayesian analysis of ≈ 100 cellulase sequences revealed three types of catalytic domains (A, B, and C). Represented by 84 sequences, type B is numerically dominant, and the overall topology of the catalytic domain type shows remarkable resemblance with trees based on neutral (= pathogenicity-unrelated) small subunit ribosomal DNA sequences. Bayesian analysis further suggested a sister relationship between the lesion nematode *Pratylenchus thornei* and all type B cellulases from root-knot nematodes. Yet, the relationship between the three catalytic domain types remained unclear. Superposition of intron data onto the cellulase tree suggests that types B and C are related, and together distinct from type A that is characterized by two unique introns. All Tylenchida members investigated here harbored one or multiple GHF5 cellulases. Three types of catalytic domains are distinguished, and the presence of at least two types is relatively common among plant parasitic Tylenchida. Analysis of coding sequences of cellulases suggests that root-knot and cyst nematodes did not acquire this gene directly by lateral genes transfer. More likely, these genes were passed on by ancestors of a family nowadays known as the Pratylenchidae.

Introduction

Any movement of genetic information, other than by vertical transmission from parents to their offspring via conventional reproduction, is defined as horizontal or lateral gene transfer (HGT or LGT). Although LGT occurs frequently among members of the Archaea and Bacteria, there are only a few probable cases of LGT between prokaryotes and multicellular eukaryotes that have resulted in new functional genes for the recipient. Likely cases of LGT in which the eukaryote is acting as a donor have been described for two mosquito species, *Aedes aegypti* and *Anopheles gambiae* [1]. The transfer of a gene related to malaria sporozoite invasion from mosquito to its endosymbiotic bacterium *Wolbachia pipientis* was demonstrated by Woolfit *et al.* [1]. This gene showed substantial divergence, and the level of expression suggested it to be functional in the new prokaryote host. Inter-domain gene transfers can also happen in the reverse way. The pea aphid *Acyrthisiphon pisum* probably acquired two genes from bacteria by LGT [2]. These laterally transferred genes are expressed in the bacteriocytes, and they contribute to the maintenance of *Buchnera aphidicola,* the aphid's primary symbiont. Donors from multiple domains (bacteria, fungi and plants) are thought to be implicated in the acquisition of at least ten protein-coding sequences by the bdelloid rotifer *Adineta vaga* [3]. A subset of these genes were transcribed and correctly spliced. Interestingly, the authors hypothesized that LGT could be facilitated by mechanisms underlying the desiccation tolerance of this rotifer.

The lateral gene transfer of prokaryotic genes has presumably also played a key role in the evolution of plant parasitism in nematodes. Plant cells are protected by a cell wall, and penetration of this wall is a prerequisite to reach the cytosol. Potato and a soybean cyst nematode (*Globodera rostochiensis* and *Heterodera glycines*) were the first animals shown to harbor symbiont-independent cellulases [4]. These cellulases are classified as members of the glycoside hydrolase family 5 (GHF5). The nematode cellulases appeared to be most similar to bacterial cellulases. In an editorial comment Keen and Roberts [5] suggested that lateral gene transfer may drive the mobility of "pathogenicity islands" (including cellulases) from one organism to the other. Over the last decade, plant parasitic nematodes were shown to harbor a wide spectrum of cell wall-degrading proteins such as pectate lyases [6], polygalacturonase [7], xylanases [8] and expansins [9]. These genes are expressed during infective life stages, and contribute to nematode's ability to exploit plants as a food source.

Bacterivory is generally accepted as the ancestral feeding type of nematodes. A longstanding hypthesis suggests that bacterivores gave rise to fungivorous nematodes, and facultative and obligatory plant parasites arose from fungal feeding ancestors [10]. It is conceivable that the evolution of plant parasitism in nematodes was driven by the lateral transfer of genes via ingestion of the donor (soil bacteria) by the recipient (bacterivorous nematodes) [11]. Mechanisms underlying desiccation tolerance could have facilitated the uptake of prokaryotic DNA [3]. A number of nematode species including

Aphelenchus avenae [12], *Ditylenchus dipsaci* [13], and *Panagrolaimus superbus* [14] can develop into highly drought resistant Dauer larva.

Among nematode genes that could have been acquired via one or multiple HGT events, GHF5 cellulases are best characterized. Recent genome sequencing projects resulted in the identification of large cellulase families in the root-knot nematodes *Meloidogyne incognita* [15, 16] and *Meloidogyne hapla* [17]. These are highly derived (distal) species within the family Meloidogynidae, and to identify possible origin(s) of these genes, cellulase sequence information is required from less derived representatives of this family. Recent morphological and molecular studies based on female gonoduct architecture [18] and small subunit ribosomal DNA sequences [18, 19] suggest that root-knot nematodes originate from - and constitute a subclade within - the genus *Pratylenchus*. By sequencing cellulase genes from *Pratylenchus spp.* (lesion nematodes) and basal root-knot nematode species - the ones that do not belong to one of the subclades I, II and III as defined in 2002 by Tandingan De Ley *et al.* [20] -, we intended to generate clues to establish the evolutionary relationship between members of the Pratylenchidae genera *Pratylenchus* and *Hirschmanniella,* and basal root-knot nematode species such as *Meloidogyne ichinohei, M. mali* and *M ulmi* [19].

Several models have been proposed about HGT event(s) underlying the acquisition of cellulases by plant parasitic and fungivorous nematodes. So far it is unclear whether the distribution of cellulase-encoding genes among Tylenchida is the result of a single HGT event, followed by early single duplication event as suggested by Kyndt *et al.* [21], or the outcome of multiple HGT events. Comparison of the topologies of phylogenetic trees based on SSU rDNA data (*e.g.* [19, 22]) *versus* GHF5 cellulase-based tree might tell us whether the evolution within the Pratylenchidae / Meloidogynidae branch includes one or multiple distinct cellulase lineages. Analysis of 103 paralogs and orthologs of cellulase-encoding gene(s) (fragments) from plant parasitic Tylenchida revealed a major clade with a topology similar to the one revealed by SSU rDNA, a neutral gene. Moreover, a relatively small, divergent subset of cellulases was found that is probably the result of early substrate specificity-driven diversification. Within the catalytic domain types A and B (too few type C sequences are available to make a statement), the overall topology resembles the topologies revealed by neutral ribosomal DNA sequences, and it is hypothesized that root-knot, cyst and lesion nematodes received their cellulases from more ancient Pratylenchidae or even more basal members of Clade 12 [23], rather than by direct lateral gene transfer.

Materials and Methods

Taxon sampling and microscopic identification

Pratylenchus and *Hirschmanniella* species were collected from various habitats throughout The Netherlands and extracted from the soil using standard techniques. Individual nematodes were identified using a light microscope

(Zeiss Axioscope) equipped with differential interference contrast optics. *Globodera pallida* specimens originated from a Dutch population named Pa3 – Rookmaker. *Meloidogyne* species were kindly provided by Dr. Gerrit Karssen from the Plant Protection Service of The Netherlands: *M. ichinochei* (propagated on *Iris levigata;* culture C2312; Japan), *M. artiellia* (sampled from a field with *Triticum aestivum*; culture E8067; Syria), *M. ardenensis* (propagated on *Liguster sp.*; Wageningen) and *M. ulmi* (isolated from *Ulmus* sp.; Wageningen).

Table 1. Overview of PCR primers used for cellulase amplication from individual nematodes

A

CD1 **CD2**

TATP**PPYGQLS**VSGTKLVDSSGQPVQLIGNSLFWHQFQAQYWNAETVKA**LKCNWN**ANVVRAAVGVDLERGYMSDP

ENG1

TTAYNQAVAVIEAAISQGL**YVIVDW**HSHESHVDKAIEFFTKIAKAYGSYPHVLYETFNEPLQGVSWTDILVPYHKKVIAAI

CD4 **ENG2**

RALDSKNVIILGTPT**WCQDV**DIASQNPIKEYKNLMYTFHFYAATHFVNGLGAKLQTAINNGLPI**FVTEYG**TCSADGNGNI

CD6

DTNSISSWWSLMDNLK**ISYLNWAISD**KSETCSALKPGTPAANVGVSSSWTTSGNMVADHDKKKSTGVSCS

B	**Primer sequence 5′→3′ ***		**Primer sequence 5′→3′ ***
Region PPYGQLS (CD1):		**Region WCQDV (CD4):**	
CD1aF	cc**I**cc**I**ta**c**gg**I**caatt**g**tc	CD4aR	tccac**R**tc**c**tggg**a**cca
CD1bF	cc**I**cc**I**ta**t**gg**I**caatt**g**tc	CD4cR	tccac**A**tc**t**tgg**c**acca
CD1cF	cc**I**cc**I**ta**t**gg**I**caatt**a**tc		
CD1PraFa	cc**g**cc**g**ta**t**ggg**c**aa	**Region FVTEYG (ENG2):**	
CD1PraFb	cc**t**cc**c**ta**t**gg**c**caa	ENG2 **(**Rosso *et al*., 1999)	gtIccRtaYTcIgtIacRaa
CD1PraFc	c**g**cc**g**ta**t**ggg**c**aa		
CD1MelF	c**t**cc**a**ta**t**ggg**c**aatt**a**tctgt	**Region ISYLNWAISD (CD6):**	
Region LKCNWN (CD2):		CD6PraFb	tctcctacatcaactgggc
CD2aF	ct**c**aaatgc**aa**ttggaa**c**Kc	CD6aR	gcccag**t**tg**gcg**ta**I**ga**g**a
CD2bF	ct**c**aaatgc**aa**ttggaa**t**Kc	CD6bR	gcccaIttg**gcR**ta**I**ga**a**a
CD2cF	ct**t**aaatgc**aI**ttggaa**t**Kc	CD6cR	gcccaIttg**aIg**ta**M**ga**a**a
CD2dF	ct**t**aaatgc**tI**ttggaa**t**Kc	CD6dR	gcccag**t**tg**aYg**ta**I**ga**g**a
Region YVIVDW (ENG1):		CDGp8R	gcccag**t**tg**agg**ta**c**ga**a**
ENG1 **(**Rosso *et al*., 1999)	taYgtIatcgtIgaYtggca	CD6PraRa	cccag**t**tg**gcg**ta**g**ga
ENG1R	tgccaRtcIacgatIacRta	CD6MelR	tgtttgagatagcccag**t**tg

* I=inosine; K= g or t, M= a or c, R= a or g, Y= c or t. In bold: discriminative nucleotide position.

Legend Table 1. Conserved amino acid motives in GHF5 cellulases from plant parasitic nematodes residing in nematode Clade 12 [23]. Primer design was based on these motives, and all primers used in this study are listed bellow. **A.** The backbone sequence given below is derived from the predicted amino acid sequence of the potato cyst nematode (*Globodera rostochiensis*) cellulase *Gr-eng*-1 (GenBank AF004523, amino acid positions 18 – 324 (mainly catalytic domain). Underlined: part of signal peptide for secretion. **B.** Primer names and primer sequences.

Nematode lysis

Single nematodes were transferred to a 0.2 mL polymerase chain reaction (PCR) tube containing 25 µL sterile water. An equal volume of lysis buffer containing 0.2 M NaCl, 0.2 M Tris-HCl (pH 8.0), 1% (v/v) β-mercaptoethanol and 800 µg/mL proteinase K was added. Lysis took place in a Thermal cycler (MyiQ, Bio-Rad) at 65°C for 2 h followed by 5 min incubation at 100°C. The lysate (crude DNA extract) was used immediately or stored at -20°C.

Amplification of cellulase-coding genes from genomic DNA

Based on publicly available cellulase sequences (cDNA and genomic sequences, see Supplementary Table 1) from lesion, root-knot, and cyst nematodes, six conserved peptide motives were identified within the catalytic domain, namely PPYGQLS (CD1), LKCNWN (CD2), YVIVDW (ENG1), WCQDV (CD4), FVTEYG (ENG2) and ISYLNWAISD (CD6) (for positioning see Table 1). These regions were used as a starting point for the design of *eng*-specific primers (Table 1). The primary aim was to amplify the longest possible fragment, preferably from CD1 to CD6 (230 amino acids, ≈700bp of the coding sequence); however, on some occasions, only shorter cellulase fragments could be amplified (Table 2).

Due to differences in codon usage within the six conserved amino acid motives mentioned above, numerous primer combinations had to be designed and subsequently examined in various combinations. For a quick, first selection of the most effective primer combinations, quantitative PCR was used. For this, 3 µL of template (single nematode lysate) was mixed with relevant primers (end concentrations for both primers 200 nM), and 12.5 µL iQ Absolute Sybr Green Fluorescein Cat. CM-225 (Westburg). The total reaction volume was 25 µL. Thermal cycling took place in the MyiQ thermal cycler (Bio-Rad) under the following conditions: 95 °C for 15 min; followed by 60 cycles at 95 °C for 30 s, 50°C for 1 min and 72 °C for 2 min. In case a possibly applicable amplification signal was produced (criteria: C_t value < 50 cycles, and a melting temperature > 80°C), the amplicon was analyzed on a 1% agarose-gel stained with GelStar (Westburg; 2µl/100ml). For those primer combinations that gave rise to amplification of the expected size products, the annealing temperature was optimized using conventional PCR. These reactions were performed in a final volume of 25 µl and contained 3 µl of a diluted crude DNA extract, 0.1 µM of each PCR primer, and a Ready-To-Go PCR bead (Amersham, Little Chalfont, Buckinghamshire, UK). The following PCR profile was used: 95°C for 5 min followed by 60 x (94°C, 30 sec; specific annealing temperature, 1 min; 72°C, 2 min) and 72°C, 10 min.

Cloning, sequencing and sequence alignment

Gel-purified amplification products (Marligen Bioscience, Ijamsville, MD) were cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA) and sequenced using standard procedures. Newly generated sequences were deposited at GenBank under accession numbers listed in Table 2.

Table 2. Overview of GHF5 cellulase sequences generated in this study from plant parasitic nematodes belonging to the superfamily Hoplolaimoidea

Species name	Individual*	Forward primer (1)	Reverse primer (1)	Fragment length (bp)	Fragment length after removal putative introns (bp)	Gene name (2)	GenBank Acc. No.
Meloidogyne ardenensis	1	CD1cF	CD6cR	1228	**727**	***Mard-eng-1***	JN052024
Meloidogyne artiellia	1	CD1MelF	CD6MelR	936	**731**	***Mart-eng-1***	JN052025
Meloidogyne ichinochei	1	CD2aF	CD4aR	673	343	*Mic-eng-1*	JN052026
M. ichinochei	2	CD2aF	CD4aR	673	343	*Mic-eng-2*	JN052027
Hirschmanniella gracilis	1	CD1aF	CD6bR	950	**728**	***Hgr-eng-1***	JN052061
H. gracilis	1	CD1aF	CD6bR	866	**728**	***Hgr-eng-2***	JN052062
H. gracilis	1	CD1aF	CD6bR	950	**728**	***Hgr-eng-3***	JN052063
Hirschmanniella loofi	1	CD1bF	ENG1R	282	255	*Hl-eng-1*	JN052057
H. loofi	1	CD1bF	CD4aR	560	487	*Hl-eng-2*	JN052058
H. loofi	1	ENG1	CD6aR	983	461	*Hl-eng-3*	JN052059
H. loofi	1	ENG1	CD6aR	554	431	*Hl-eng-4*	JN052060
Pratylenchus crenatus	1	CD1PraFa	CD6aR	820	**721**	***Pcr-eng-1***	JN052031
P. crenatus	2	CD1PraFb	CD6PraRa	819	**723**	***Pcr-eng-2***	JN052030
P. crenatus	3	ENG1F	CD6aR	543	449	*Pcr-eng-3*	JN052029
Pratylenchus neglectus	1	ENG1	CD6aR	508	452	*Pn-eng-1*	JN052032
P. neglectus	2	CD1PraFc	CD6PraRa	791	**735**	***Pn-eng-2***	JN052033
P. neglectus	2	CD1PraFc	CD6PraRa	789	**733**	***Pn-eng-3***	JN052034
Pratylenchus penetrans	1	CD2cF	CD6aR	1514	588	*Pp-eng-3*	JN052035
P. penetrans	1	CD2cF	CD6aR	695	587	*Pp-eng-4*	JN052036
P. penetrans	1	CD1bF	CD4aR	739	484	*Pp-eng-5*	JN052037
P. penetrans	2	CD1PraFb	CD6PraRb	841	**733**	***Pp-eng-6***	JN052038
Pratylenchus convalariae (3)	1	CD1PraFb	CD6aR	874	**739**	***Pcon-eng-1***	JN052028
Pratylenchus pratensis	1	CD1bF	ENG1R	256	256	*Ppr-eng-1*	JN052040
P. pratensis	1	CD2bF	CD6dR	1124	592	*Ppr-eng-2*	JN052039
P. pratensis	1	CD2dF	CD4aR	407	349	*Ppr-eng-3*	JN052041
P. pratensis	1	CD1aF	CD4aR	489	489	*Ppr-eng-4*	JN052043

P. pratensis	1	ENG1	CD6aR	507	452	*Ppr-eng-5*	JN052042
P. pratensis	2	CD1PraFb	CD6PraRa	801	**750**	***Ppr-eng-6***	JN052044
Pratylenchus thornei	1	CD2bF	CD6dR	678	587	*Pt-eng-1*	JN052045
P. thornei	2	CD1PraFc	CD6PraRb	824	**733**	***Pt-eng-2***	JN052046
Pratylenchus vulnus	1	CD2cF	CD6aR	639	587	*Pv-eng-1*	JN052047
P. vulnus	1	CD2bF	CD6dR	1001	614	*Pv-eng-2*	JN052048
P. vulnus	1	CD2bF	CD6dR	639	587	*Pv-eng-3*	JN052049
P. vulnus	1	CD2aF	CD4aR	349	349	*Pv-eng-4*	JN052050
P. vulnus	1	ENG1	CD6aR	958	449	*Pv-eng-5*	JN052051
P. vulnus	1	ENG1	CD6aR	604	312	*Pv-eng-6*	JN052052
P. vulnus	1	ENG1	CD6aR	505	452	*Pv-eng-7*	JN052053
P. vulnus	2	CD1aF	CD6bR	1031	**728**	***Pv-eng-8***	JN052054
P. vulnus	3	CD1PraFb	CD6aR	790	**733**	***Pv-eng-9***	JN052055
P. vulnus	3	CD1PraFb	CD6PraRa	792	**735**	***Pv-eng-10***	JN052056
Globodera pallida	1	CD1aF	CD4cR	1144	481	*Gp-eng-1*	JN052064
G. pallida	1	CD2aF	CD6cR	852	589	*Gp-eng-2*	JN052065
G. pallida	1	CD2aF	CD6cR	853	590	*Gp-eng-3*	JN052066
G. pallida	2	CDGp2F	CD6cR	1561	**709**	***Gp-eng-4***	JN052067
G. pallida	2	CDGp2F	CDGp8R	706	**706**	***Gp-eng-5***	JN052068

Legend Table 2. Overview of GHF5 cellulase sequences generated in this study from plant parasitic nematodes belonging to the superfamily Hoplolaimoidea (as defined by Siddiqi [35]). Single nematodes were used for the amplification of putative cellulase fragments. Occasionally, multiple fragments were amplified from an individual, such as in case of *Pratylenchus neglectus* 2 (*Pn-eng-2 and -3*). (1) Primers sequences are given in Table 1. (2) gene names in bold indicate fragments spanning almost the full catalytic domain (CD1 - CD6). (3) Small subunit ribosomal DNA data suggest that *Pratylenchus convallariae* is identical to *P. penetrans* [19].

Intron positions in the genomic sequences were identified on the basis of information about exon-intron structure of publicly available sequences (all the ones in the Supplementary Table 1 harboring at least one intron). The newly obtained nucleotide sequences as well as those derived from GenBank were translated into amino acids and aligned using the ClustalW algorithm as implemented in BioEdit 5.0.9 [24]. The protein alignment was improved manually and translated back into nucleotides. The final nucleotide alignment consisted of 103 sequences, of which 45 were generated in this study. More than half of these sequences (66 out of 103) span almost the full catalytic domain (from CD1 to CD6).

RNA extraction and cDNA cellulase amplification

In order to support the chosen intron extraction approach, cDNA cellulase sequence for *G. pallida* was synthesized. For this purpose 100 individuals of *G. pallida* were collected into a 0.2 mL PCR tube containing 25 µL of sterile water and lysed as specified above. The lysate was used immediately for the RNA extraction according to RNeasy Micro kit protocol (Qiagen). Total RNA end concentration was approximately 7ng of RNA/µL of water. 3 µL of the five times diluted RNA was mixed with CDGp2F and CDGp8R primers (Table 2; end concentrations for both primers 200 nM), 20 units of RNAse inhibitor (Invitrogen) and the components of the SuperScript III One-Step RT-PCR with Platinium *Taq* kit (12 µL of 2X reaction Mix and 2 µL of the SuperScriptTM III RT/ Platinium *Taq* Mix). This reaction of 25 µL in total, was used for the specific cDNA fragment amplification under the following conditions: 60 °C for 30 min, 94 °C for 2 min; followed by 60 cycles at 94 °C for 15 s, 60°C for 30 s and 68 °C for 1 min and finished with one cycle of 68 °C for 5 min. As a result of this experiment the Gp-eng-5 sequence was acquired.

Phylogenetic analysis

The Bayesian phylogeny was constructed with the program MrBayes 3.1.2 using a site-specific model. Data were partitioned by codon position and gamma distribution of rate variation with a proportion of invariable sites was used. Four independent runs were set with 4 Markov chains per run. The program was run for 5 million generations. Stabilization of the likelihood and parameters were checked with the program Tracer v1.4 [25] and the burnin was defined as 120,000 generations. For the construction of the maximum likelihood of the cellulase tree the RAxML-HPC BlackBox program [26] available at the CIPRES Science Gateway V. 3.1 was used. The program FindModel (http://hcv.lanl.gov/content/sequence /findmodel/findmodel.html from the HCV sequence database) was used to determine the best phylogenetic model, and the following parameters were applied: estimated proportion of invariable sites (GTRGAMMA + I). To find best tree using maximum likelihood search, bootstrapping halted automatically and printed branch lengths. Cellulases from *Aphelenchus avenae,* a predominantly fungivorous species, were used as outgroup as this species does not belong to the Tylenchida (all other species used in this study belong to this order), and - on the basis of SSU rDNA data - resides at the very base of Cade 12.

Results and Discussion

Identification of new cellulase genes in nematodes

Genomic DNA from individuals from seven *Pratylenchus* (Pratylenchidae), two *Hirschmanniella* (Pratylenchidae), three basal *Meloidogyne* (Meloidogynidae) and one *Globodera* (Heteroderidae) species were tested for the presence of GHF5 cellulases. In all of the nematodes we identified at least one GHF5 cellulase gene (Table 2). Recently, it was shown that GHF5 cellulases are not the only type of cellulases present among members of the Tylenchida (Bauters *et al.*, pers. comm.). Except for the very basal *Meloidogyne* species *M. ichinochei* and *Hirschmanniella loofi*, we obtained at least one complete CD1-CD6 (Table 2) sequence for every species under investigation.

Genome sequencing of the distal tropical *Meloidogyne* species *M. incognita* not only revealed the presence of multiple (21) genes encoding putative cellulases, but also showed distinct clusters of GHF5 sequences within a single species [15]. Here we demonstrate that this is not unique for distal *Meloidogyne* species. *Pratylenchus pratensis* harbors multiple cellulase genes, whereas both type A or type B catalytic domains (as coined by [21]; Table 2) are represented. Similar results were obtained for *Pratylenchus vulnus* (Fig. 1). The inventory of all currently available catalytic domain sequences points at a numerical dominance of the type B over type A and C catalytic domains. For the soybean cyst nematode *Heterodera glycines*, the substrate specificities of cellulases with type A, B and C catalytic domains were tested [27]. Hg-ENG5 (Type C) and Hg-ENG6 (Type A) differed greatly from the most abundant type B cellulases: their depolymerizing activity on carboxymethylcellulose was strongly reduced (respectively ≈40% and ≈20% of the activities of HG-ENG-1 and -4 (both belonging to type B cellulases)), whereas both degrade xylan and crystalline cellulose (Hg-ENG5 showing two fold higher activity than Hg-ENG6). By contrast, the latter two substrates were not significantly degraded by Hg-ENG-1 and -4 [27]. As such differences have been reported for other GHF5 cellulases as well (*e.g.* [28]), we hypothesize that the different types of catalytic domains could point at differences in substrate specificities.

Phylogenetic analyses

Bayesian inference-based phylogenetic analysis of 103 coding sequences (of which 45 were generated in this study) for - at least - a major part of the catalytic domain of GHF5 cellulases resulted in the distinction of three major types of catalytic domains (A, B, and C, see Fig. 1). This systematics elaborates on the evolutionary model proposed by Kyndt *et al.* [21]; catalytic domain C was originally presented as a well-supported group of cellulases nested within the type B clade [21].

Among cellulases with a type B catalytic domain (comprising 84 out of the 103 sequences), we observed a topology that shows a remarkable overall similarity with the one presented by Holterman *et al.* [19] on the basis of a neutral gene, *viz.* SSU rDNA. Also on the basis of type B catalytic domain sequences *M. artiellia* and *M. ichinochei* appear at the base of the family Meloidogynidae, and just as observed on the basis of SSU rDNA sequences the Meloidogynidae appear as an

elaborate subclade nested within the genus *Pratylenchus.* Unlike SSU rDNA sequences that gave no clear answer about the nature of the link between the Pratylenchidae and the Meloidogynidae, a robust sister relationship was observed between a specific *Pratylenchus* species, *P. thornei*, and all representatives of the genus *Meloidogyne.*

Ribosomal DNA-based phylogenetic analysis revealed a sister relationship between lesion and root-knot nematodes on the one hand, and cyst nematodes, Hoplolaimidae and Rotylenchulidae on the other (*e.g.* [19]). Remarkably, all catalytic domains from the lesion nematode *P. crenatus,* and subsets from the *P. vulnus* and *P. penetrans* cellulases reside in a sister position with regard to all type B cyst nematode cellulases. Although the number of sequences included is considerably smaller, we observed a similar pattern for the type A catalytic domain. Firstly, a sister relationship was established here between all type A catalytic domain sequences from root-knot nematodes and a cellulase from *P. vulnus*, whereas a similar relationship was observed for a soybean cyst and a reniform nematode cellulase on the one hand, and *P. pratensis* on the other (Fig 1). Hence, at least some lesion nematode species are equipped with both root-knot and cyst nematode-like cellulases.

The positioning of *Ditylenchus spp.* is based only on one genomic and two CDSs sequences, and should be considered as a consequence of the virtual absence of cellulases data from other, more basal Tylenchida. The current positioning is not well supported, and will not be discussed.

Exon-intron structure

The number of introns in the catalytic domains of the nematode cellulases varied between zero and seven. For the intron identifiers, we followed the nomenclature proposed by Kyndt *et al.* [21]. As a consequence of the increase in the number and the diversity of catalytic domains additional predicted intron positions were found. To label new intron positions without uprooting the existing systematics, we used identifiers such as 1½ and 5½ for introns positioned between introns 1 and 2, introns 5 and 6, *etc.* (see Supplementary Table 2).

Most of the *in silico* predicted introns in the newly generated cellulase sequences were located at positions equivalent to the positions reported before for plant parasitic cyst and root-knot nematodes ([29], [16], [27] and [21]) and the outgroup *Aphelenchus avenae* [30]. Identifiers 1½, 12½, 15½ were added as - up to now - unique introns in cellulase gDNAs from *A. avenae*, whereas 4½ and 5½ were used to indicate new intron positions in *Hl-eng-1* and *Pv-eng-2*. Among the newly generated cellulase sequences the largest intron was found in *Gp-eng-1*; intron 2 with a length of 563 bp. Numerous occasions of intron gain and loss were observed in all three main types of cellulase catalytic domains tree (A, B and C). Particular introns appear to be characteristic for catalytic domain types: Type A typically contains introns 4 and 14, whereas all type B and C catalytic domains investigated so far have lost intron 18. Type C representatives share the presence of intron 2. However, this feature is not unique as it is typical for the type B cellulases from cyst nematodes as well. Among root-knot nematode type B cellulase the presence of intron 1 appears as a common characteristic. The two

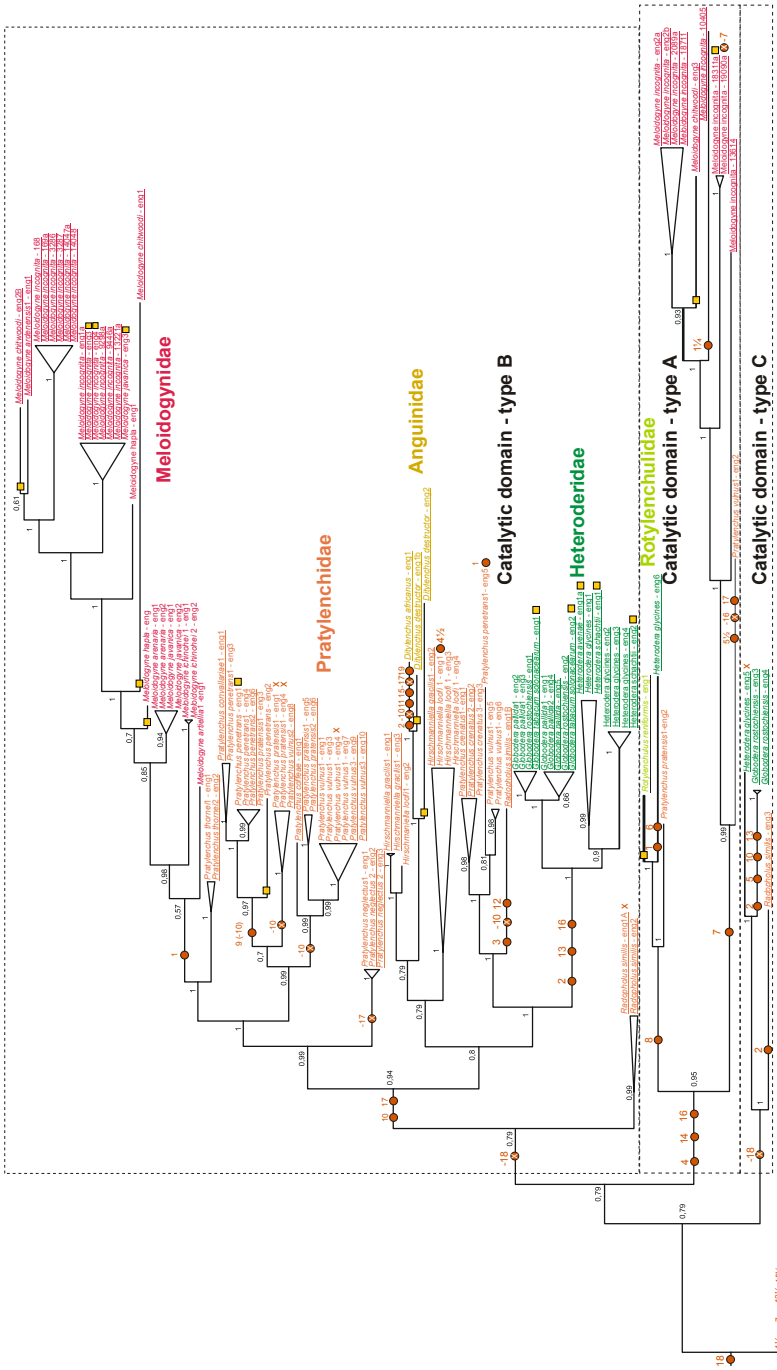

Figure 1. Bayesian tree of GHF5 catalytic domains from members of the nematode order Tylenchida. Genomic and coding sequences (indicated by a yellow box at the base of the relevant branch) from (partial) cellulase catalytic domains were analyzed. Sequences covering the catalytic domain from CD1 to CD6 (as defined in Table 1) are underlined (non underlined sequences are slightly shorter). Identical colors are used for members of the same nematode family. The tree is rooted with genomic cellulase sequences from the fungivorous nematode *Aphelenchus avenae* (infraorder Tylenchomorpha). Posterior probabilities are given next to each node. Orange circles with or without a bright cross are used to indicate the presence or absence of an intron. An orange cross behind a sequence is used to indicate that the generated piece of a sequence was intronless. Intron numbering is essentially according to Kyndt *et al.* [21]. Branch length is calculated in MrBayes, and the scale bar below represents branch length (as number of DNA substitutions/site).

other cellulase catalytic domains with an intron at position 1 were found in a type B catalytic domain *Pp-eng-5* from *Pratylenchus penetrans* and interestingly, in a type A catalytic domain *Hg-eng-6* from soybean cyst nematodes.

Intron phase distribution

The intron phase distribution in the catalytic domain of nematode GHF5 cellulases was biased towards phase 0; 16 out of the 24 intron positions (66%) were inserted in between two codons. Respectively, two and four phase 1 (after the first base of a codon) and phase 2 (after the second base of a codon) introns were identified, whereas intron positions 7 and 17 occurred in two phases (0 and 1). To some extend a bias towards the phase 0 introns was to be expected as the overall frequencies of intron phases 0, 1 and 2 in *Caenorhabditis elegans* are roughly 50%, 25% and 25% [31]. This phase bias seems to be stronger in the case of cellulase catalytic domain from Tylenchida.

In case of mixed phase intron 7, phase 1 was observed only for *Aphelenchus avenae*, a fungivorous nematode that can feed on root hair or epidermal cells of plants as well. Contrary to all other taxa investigated here, *A. avenae* does not belong to the order Tylenchida, though it is included in the infraorder Tylenchomorpha ([32]). A phase 0 variant of intron 7 is found among a subset of the type A catalytic domains: the ones present in *Meloidogyne incognita* (with one exception: *Minc 19090a*), and in *Pratylenchus vulnus*. Among the 47 taxa harboring mixed phase intron 17, 13 were in phase 1, and 34 in phase 0. Phase 1 appeared to be a typical characteristic for type B and type C catalytic domains of cyst nematodes. Hence, it was also present in *eng3* and *eng4* from *G. rostochiensis*, two otherwise highly distinct cellulases. The only other case of an intron 17 in phase 1 was observed for *eng-1B* from the banana root nematode *Radopholus similis* (family Pratylenchidae). This could be seen as a confirmation of a recent SSU rDNA-based analysis suggesting a (unexpected) close relatedness between *Radopholus* and cyst nematodes [19].

Conclusions

Addition of 45 new genomic sequences from the catalytic domain of cellulases from plant parasitic members of the order Tylenchida, followed by phylogenetic analyses further develops our understanding of the evolution of cellulases within a nematode order that harbors most economically high impact plant parasites. Three distinct types of catalytic domains were distinguished, and we hypothesize that types of catalytic domains reflect distinct substrate preferences. Numerous plant parasitic nematode species were shown to harbor two types of GHF5 cellulases. *Heterodera glycines,* soybean cyst nematode, is the only example of a plant parasite equipped with all three types of catalytic domains distinguished so far.

All Clade 12 members of the phylum Nematoda analyzed to date harbor one or multiple GHF5 cellulases. This also holds for basal representatives that are not fully dependent on plants as sole food source. *Aphelenchus spp.*, on the basis of

SSU rDNA data suggested to be sister to all Tylenchida [22], is primarily mycetophagous (fungal cell walls do not contain cellulose), but can also grow and multiply on various plant species [33]. It is noted that the necromenic nematode species *Pristionchus pacificus* (Clade 9, Diplogasteridae) is harbouring seven celulase genes all belonging to GHF5 ([34]). Hence, although GHF5 cellulases are widespread within Clade 12, they are not exclusively present in this clade.

Within the order Tylenchida, the family Anguinidae includes the most ancestral representatives of the order Tylenchida included in this paper, and *Ditylenchus destructor,* a member of this family and the causal agent of dry rot in potato tuber, is also known to feed on fungal hyphae. Recently, another early branching representative of the Tylenchida, *Deladenus siridicola* - a nematode with a mycetophagous and an insect parasitic life cycle, was shown to harbour GHF5 cellulases (dr. Bernard Slippers and co-workers, pers. comm.). Hence, all members of Clade 12 seem to harbor GHF5 cellulases, even ones that according to literature do not feed on plants. Presuming GHF5 cellulases were indeed acquired by lateral gene transfer, the most parsimonious explanation of the current cellulase tree would be the acquisition of such a gene by an ancient representative of the Pratylenchidae. Though we realize that current datasets are too fragmented to make a strong statement, our results are compatible with a scenario in which a GHF5 cellulase was acquired by the common ancestor of *Aphelenchus* and all Tylenchida, followed by one or multiple gene duplications and subsequent diversification.

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References

1. Woolfit M, Iturbe-Ormaetxe I, McGraw EA, O'Neill SL: **An ancient horizontal gene transfer between mosquito and the endosymbiotic bacterium Wolbachia pipientis**. *Molecular Biology and Evolution* 2009, **26**(2):367-374.

2. Nikoh N, Nakabachi A: **Aphids acquired symbiotic genes via lateral gene transfer**. *BMC Biology* 2009, **7**.

3. Gladyshev EA, Meselson M, Arkhipova IR: **Massive horizontal gene transfer in bdelloid rotifers**. *Science* 2008, **320**(5880):1210-1213.

4. Smant G, Stokkermans J, Yan YT, de Boer JM, Baum TJ, Wang XH, Hussey RS, Gommers FJ, Henrissat B, Davis EL *et al*: **Endogenous cellulases in animals: Isolation of beta-1,4-endoglucanase genes from two species of plant-parasitic cyst nematodes**. *Proceedings of the National Academy of Sciences of the United States of America* 1998, **95**(9):4906-4911.

5. Keen NT, Roberts PA: **Plant parasitic nematodes: Digesting a page from the microbe book**. *Proceedings of the National Academy of Sciences of the United States of America* 1998, **95**(9):4789-4790.

6. Popeijus H, Overmars H, Jones J, Blok V, Goverse A, Helder J, Schots A, Bakker J, Smant G: **Enzymology: Degradation of plant cell walls by a nematode**. *Nature* 2000, **406**(6791):36-37.

7. Jaubert S, Laffaire JB, Abad P, Rosso MN: **A polygalacturonase of animal origin isolated from the root- knot nematode Meloidogyne incognita**. *Febs Letters* 2002, **522**(1-3):109-112.

8. Mitreva-Dautova M, Roze E, Overmars H, De Graaff L, Schots A, Helder J, Goverse A, Bakker J, Smant G: **A symbiont-independent endo-1,4-β-xylanase from the plant-parasitic nematode Meloidogyne incognita**. *Molecular Plant-Microbe Interactions* 2006, **19**(5):521-529.

9. Qin L, Kudla U, Roze EHA, Goverse A, Popeijust H, Nieuwland J, Overmars H, Jones JT, Schots A, Smant G *et al*: **A nematode expansin acting on plants**. *Nature* 2004, **427**(6969):30.

10. Maggenti AR: **Nemic relationships and the origin of plant parasitic nematodes**. In: *Plant parasitic nematodes.* Edited by Zuckerman BM, Mai WF, Rohde RA, vol. 1. New York: Academic Press Inc.; 1971: 65-81.

11. Ford Doolittle W: **You are what you eat: A gene transfer ratchet could account for bacterial genes in eukaryotic nuclear genomes**. *Trends in Genetics* 1998, **14**(8):307-311.

12. Reardon W, Chakrabortee S, Pereira TC, Tyson T, Banton MC, Dolan KM, Culleton BA, Wise MJ, Burnell AM, Tunnacliffe A: **Expression profiling and cross-species RNA interference (RNAi) of desiccation-induced transcripts in the anhydrobiotic nematode Aphelenchus avenae**. *BMC Molecular Biology* 2010, **11**.

13. Wharton DA, Aalders O: **Desiccation stress and recovery in the anhydrobiotic nematode Ditylenchus dipsaci (Nematoda: Anguinidae)**. *European Journal of Entomology* 1999, **96**(2):199-203.

14. Shannon AJ, Tyson T, Dix I, Boyd J, Burnell AM: **Systemic RNAi mediated gene silencing in the anhydrobiotic nematode Panagrolaimus superbus**. *BMC Molecular Biology* 2008, **9**.

15. Abad P, Gouzy J, Aury JM, Castagnone-Sereno P, Danchin EGJ, Deleury E, Perfus-Barbeoch L, Anthouard V, Artiguenave F, Blok VC *et al*: **Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita***. *Nature Biotechnology* 2008, **26**(8):909-915.

16. Danchin EGJ, Rosso MN, Vieira P, De Almeida-Engler J, Coutinho PM, Henrissat B, Abad P: **Multiple lateral gene transfers and duplications have promoted plant parasitism ability in nematodes**. *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**(41):17651-17656.

17. Opperman CH, Bird DM, Williamson VM, Rokhsar DS, Burke M, Cohn J, Cromer J, Diener S, Gajan J, Graham S *et al*: **Sequence and genetic map of *Meloidogyne hapla*: A compact nematode genome for plant parasitism**. *Proceedings of the National Academy of Sciences of the United States of America* 2008, **105**(39):14802-14807.

18. Bert W, Leliaert F, Vierstraete AR, Vanfleteren JR, Borgonie G: **Molecular phylogeny of the Tylenchina and evolution of the female gonoduct (Nematoda : Rhabditida)**. *Molecular Phylogenetics and Evolution* 2008, **48**(2):728-744.

19. Holterman M, Karssen G, Van den Elsen S, Van Megen H, Bakker J, Helder J: **Small Subunit rDNA-Based Phylogeny of the Tylenchida Sheds Light on Relationships Among Some High-Impact Plant-Parasitic Nematodes & the Evolution of Plant Feeding**. *Phytopathology* 2009, **99**(3):227-235.

20. Tandingan De Ley I, De Ley P, Vierstraete A, Karssen G, Moens M, Vanfleteren J: **Phylogenetic analyses of *Meloidogyne* small subunit rDNA**. *Journal of Nematology* 2002, **34**(4):319-327.

21. Kyndt T, Haegeman A, Gheysen G: **Evolution of GHF5 endoglucanase gene structure in plant-parasitic nematodes: No evidence for an early domain shuffling event**. *BMC Evolutionary Biology* 2008, **8**(1).

22. Van Megen H, Van Den Elsen S, Holterman M, Karssen G, Mooyman P, Bongers T, Holovachov O, Bakker J, Helder J: **A phylogenetic tree of nematodes based on about 1200 full-length small subunit ribosomal DNA sequences**. *Nematology* 2009, **11**(6):927-950.

23. Holterman M, van der Wurff A, van den Elsen S, van Megen H, Bongers T, Holovachov O, Bakker J, Helder J: **Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown clades**. *Molecular Biology and Evolution* 2006, **23**(9):1792-1800.

24. Hall TA: **BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT**. *Nucleic Acids Symposium Series* 1999, **41**:95-98.

25. Rambaut A, Drummond A: **Tracer v1.4**. In., 1.4 edn: http://beast.bio.ed.ac.uk/ Tracer 2007.

26. Stamatakis A, Hoover P, Rougemont J: **A rapid bootstrap algorithm for the RAxML web servers**. *Systematic Biology* 2008, **57**(5):758-771.

27. Gao B, Allen R, Davis EL, Baum TJ, Hussey RS: **Developmental expression and biochemical properties of a b-1,4-endoglucanase family in the soybean cyst nematode, Heterodera glycines**. *Molecular Plant Pathology* 2004, **5**(2):93-104.

28. Feng Y, Duan CJ, Pang H, Mo XC, Wu CF, Yu Y, Hu YL, Wei J, Tang JL, Feng JX: **Cloning and identification of novel cellulase genes from uncultured microorganisms in rabbit cecum and characterization of the expressed cellulases**. *Applied Microbiology and Biotechnology* 2007, **75**(2):319-328.

29. Yan YT, Smant G, Stokkermans J, Qin L, Helder J, Baum T, Schots A, Davis E: **Genomic organization of four beta-1,4-endoglucanase genes in plant-parasitic cyst nematodes and its evolutionary implications**. *Gene* 1998, **220** (1-2):61-70.

30. Karim N, Jones JT, Okada H, Kikuchi T: **Analysis of expressed sequence tags and identification of genes encoding cell-wall-degrading enzymes from the fungivorous nematode Aphelenchus avenae**. *BMC Genomics* 2009, **10**.

31. Coghlan A, Wolfe KH: **Origins of recently gained introns in *Caenorhabditis***. *Proceedings of the National Academy of Sciences of the United States of America* 2004, **101**(31):11362-11367.

32. De Ley P, Blaxter ML: **Systematic Position and Phylogeny**. In: *The Biology of Nematodes*. Edited by Lee DL. London, UK: Taylor & Francis; 2002: 1-30.

33. Hunt DJ: **Aphelenchida, Longidoridae and Trichodoridae: their systematics and bionomics**. Wallingford, UK: CAB International; 1993.

34. Schuster LN, Sommer RJ: **Expressional and functional variation of horizontally acquired cellulases in the nematode Pristionchus pacificus**. *Gene* 2012, **506**(2):274-282.

35. Siddiqi MR: **Tylenchida: parasites of plants and insects**. Wallingford, UK: CABI publishing; 2000.

Supplementary information

Supplementary Table S1. List of GHF5 endoglucanase sequences from plant parasitic nematodes from public databases used in this paper.

Taxon/cellulase name	GenBank Acc No	Data available	Fragment used for phylogenetics	Number of introns within CD1-CD6 region	Intron positions and lengths within CD1-CD6 region
Nematoda					
Aphelenchus avenae					
Aa-eng-1	AB495300	DNA, cDNA	CD1-CD6	5	SQ-WM 319; EAG 47; LQ-VD 40; TGE 43; KWF 85
Aa-eng-2	AB495302	DNA, cDNA	CD1-CD6	5	SQ-WM 337; EAG 44; LQ-VD 41; TGE 43; KWF 73
Ditylenchus africanus					
Da-eng-1	EU180235	DNA, cDNA	CD1-CD6	4	IQ-AL 67; FTQ 110; SQ-DV 103; NQ-IS 183
Ditylenchus destructor					
Dd-eng1-b	FJ430142	cDNA	CD1-CD6	-	-
Dd-eng-2	FJ374266	cDNA	CD1-CD6	-	-
Meloidogyne arenaria					
Ma-eng-1	AF323097	partial DNA	ENG1-ENG2	2	QA-IN 230;NK-GV 47;
Ma-eng-2	AF323098	partial DNA	ENG1-ENG2	2	QA-IN 355;NK-GV 46;
Meloidogyne chitwoodi					
Mc-eng-1	JN052069	cDNA	CD1-CD6	-	-
Mc-eng-2	JN052070	cDNA	CD1-CD6	-	-
Mc-eng-3	JN052071	cDNA	CD1-CD6	-	-
Meloidogyne hapla					
Mh-eng	AY277718	partial cDNA	ENG1-ENG2	-	-
Mh-eng-1	AF323096	partial DNA, cDNA	ENG1-ENG2	2	QA-IE 58; NN-GA 41;
Meloidogyne incognita					
Mi-eng-1a	AF323087	DNA, cDNA	CD1-CD6	3	KGS 369; QA-IE 71; NK-GA 45;
Mi-eng-2(a)	AF323088	DNA, cDNA	CD1-CD6	5	NV-VR 216; IA-NG 115; LP-VI 240; LMY 297; DFW 120;
Mi-eng-2(b)	AF323086	cDNA	CD1-CD6	-	
Mi-eng-3	AY422836	cDNA	CD1-CD6	-	
Mi-eng-4	AY422837	cDNA	CD1-CD6	-	

Mi9446a	Danchin et al.	cDNA	CD1-CD6	3		KGS; QA-IE; NK-GA;
Mi168	Danchin et al.	cDNA	CD1-CD6	3		KGN; KA-KE; DN-NV;
Mi3286	Danchin et al.	cDNA	CD1-CD6	3		KGN; KA-KE; DN-NV;
Mi14048	Danchin et al.	cDNA	CD1-CD6	3		KGN; KA-KE; DN-NV;
Mi169a	Danchin et al.	cDNA	CD1-CD6	3		KGN; KA-KE; NN-NV;
Mi3287	Danchin et al.	cDNA	CD1-CD6	3		KGN; KA-KE; NN-NV;
Mi14047a	Danchin et al.	cDNA	CD1-CD6	3		KGN; KA-KE; NN-NV;
Mi13221a	Danchin et al.	cDNA	CD1-CD6	3		KGS; QA-IE; NK-GA;
Mi9298a	Danchin et al.	cDNA	CD1-CD6	3		KGS; QA-IE; NK-GA;
Mi10405	Danchin et al.	cDNA	CD1-CD6	6		KLLR, NV-IR; ID-NG; LE-AI; TLY; KFL;
Mi18711	Danchin et al.	cDNA	CD1-CD6	5		NV-VR; IA-NG; LP-VI; LMY; DFW;
Mi19090a	Danchin et al.	cDNA	CD1-CD6	4		NI-IR; LW-AI; IMH; DFW;
Mi13614	Danchin et al.	cDNA	CD1-CD6	5		NV-IR; IE-NG; MK-AI; LMY; FLY;
Mi20089a	Danchin et al.	cDNA	CD1-CD6	5		NV-VR; MT-NG; LA-RV; LLY; DFW;
Meloidogyne javanica						
Mj-eng-1	AF323099	partial DNA	ENG1-ENG2	2		QA-IN 350; NK-GV 47;
Mj-eng-2	AF323100	partial DNA	ENG1-ENG2	2		QA-IN 457; NK-GV 46;
Mj-eng-3	AM231138	cDNA	CD1-CD6	-		-
Heterodera schachtii						
Hs-eng-1	AJ299386	cDNA	CD1-CD6	-		-
Hs-eng-2	AJ299387	cDNA	CD1-CD6	-		-
Heterodera avenae						
Ha-eng-1a	FJ839965	cDNA	CD1-CD6	-		-
Heterodera glycines						
Hg-eng-1	AF052733	DNA, cDNA	CD1-CD6	5		VK-AL 85; EA-VK 49; KK-VI 52; LMY 297; NGL 78;
Hg-eng-2	AF052734	DNA, cDNA	CD1-CD6	5		VK-AL 388; KA-IE 247; KK-VI 45; LMY 297; KGL 57;
Hg-eng-3	AF056048	DNA, cDNA	CD1-CD6	5		VK-AL 387; KA-IE 247; KK-VI 45; LMY 297; KGL 57;
Hg-eng-4	AY325809	DNA, cDNA	CD1-CD6	5		VK-AL 198; KA-IE 247; KK-VI 46; LMY 297; KGL 57;
Hg-eng-5	AY336935	DNA, cDNA	CD1-CD6	0		-
Hg-eng-6	AY163572	DNA, cDNA	CD1-CD6	7		KGT 920; DJ-VRA333; EQ-EY 47; CIY 259; VK-VI 160; IMY 120;NWY 95

			CD1											ENG1
Catalytic domain type	GenBank ID	Cellulase\Intron	1	1½	2	3	4	4½	5	5½	6	7	8	9
C	JN052066	Gp-eng-3												
C	JN052067	Gp-eng-4			VK-AL 522									
C	AY336935	Hg-eng-5												
C	EF693943	Rs-eng-3			VK-QI 54									
B	AF323098	Ma-eng-2												
B	AF323097	Ma-eng-1												
B	JN052024	Mard-eng-1	KDN 343											
B	JN052025	Mart-eng-1	KGT 70											
B	JN052026	Mic-eng-1												
B	JN052027	Mic-eng-2												
B	AF323096	Mh-eng-1												
B	AF323087	Mi-eng-1a	KGS 369											
B	AF323099	Mj-eng-1												
B	AF323100	Mj-eng-2												
B	JN052028	Pcon-eng-1												SQ-AI 55
B	JN052035	Pp-eng-3												SQ-AI 526
B	JN052036	Pp-eng-4												SQ-AI 58
B	JN052038	Pp-eng-6												SQ-AI 58
B	JN052041	Ppr-eng-3												SQ-AI 58
B	EU176871	Pc-eng-1												
B	JN052040	Ppr-eng-1												
B	JN052043	Ppr-eng-4												
B	JN052042	Ppr-eng-5												
B	JN052044	Ppr-eng-6												
B	JN052047	Pv-eng-1												
B	JN052049	Pv-eng3												
B	JN052050	Pv-eng-4												
B	JN052053	Pv-eng-7												
B	JN052054	Pv-eng-8												
B	JN052055	Pv-eng-9												
B	JN052056	Pv-eng-10												
B	JN052032	Pn-eng-1												
B	JN052033	Pn-eng-2												
B	JN052034	Pn-eng-3												
B	JN052031	Pcr-eng-1												
B	JN052030	Pcr-eng-2												
B	JN052029	Pcr-eng-3												
B	JN052037	Pp-eng-5	SGT 146											
B	JN052045	Pt-eng-1												
B	JN052046	Pt-eng-2												
B	JN052051	Pv-eng-5												
B	JN052052	Pv-eng-6												
B	JN052061	Hgr-eng-1												
B	JN052062	Hgr-eng-2												
B	JN052063	Hgr-eng-3												
B	JN052057	Hl-eng-1						LA-GY 27						
B	JN052058	Hl-eng-2												
B	JN052059	Hl-eng-3												
B	JN052060	Hl-eng-4												
B	JN052064	Gp-eng-1			VK-AL 563									
B	JN052065	Gp-eng-2												
B	AF056110	Gr-eng-1			VK-AL 308									
B	AF056111	Gr-eng-2			VK-AL 742									
B	AF408154	Gr-eng-3			VN-RI 354				TQ-RS 64					
B	AF408157	Gr-eng-4			VN-RI 271				TQ-KS 65					
B	AF052733	Hg-eng-1			VK-AL 85									
B	AF052734	Hg-eng-2			VK-AL 388									
B	AF056048	Hg-eng-3			VK-AL 387									
B	AY325809	Hg-eng-4			VK-AL 198									
B	EF693940	Rs-eng-1A												
B	EF693941	Rs-eng-1B				QQ-LK 106								
B	EF693942	Rs-eng-2												
A	AF323088	Mi-eng-2a					NV-VRA 216					IA-NG 115		
A	JN052048	Pv-eng-2					NV-IR 55			ES-AP 25		IA-NG 90*		
A	JN052039	Ppr-eng-2					DI-VR 274						CMY 51	
A	AY163572	Hg-eng-6	KGT 920				DI-VR 333				EQ-EY 47		CIY 259	
Not yet classified	AB495300	Aa-eng-1		SQ-WM 319								EAG 47		
Not yet classified	AB495302	Aa-eng-2		SQ-WM 337								EAG 44		
Not yet classified	EU180235	Da-eng-1			IQ-AL 67									

= no introns could be found in this protein fragment
= phase 0
= phase 1
= phase 2
* = GC-AG type
Underlined = sequences generated in this study
? = unknown data

Supplementary Table S2. Schematic overview of the (predicted) introns in genomic sequences from the GHF5 endoglucanase genes. For the intron identifiers, we adhered to the nomenclature proposed by Kyndt *et al.* (2008) [20]. Identifiers such as 1½ and 5½ were used for novel introns positioned between introns 1 and 2, 5 and 6, etc. The color scheme for the phase of the introns is explained below this Table. Length of the introns (in bp) is given inside each box.

							CD4				ENG2		CD6		Linker	CBM2
10	11	12	12½	13	14	15		15½	16	17	18	19	20	21	22	23
KA-IE 50				KK-VI 64*					LMY 101	NGL 48						
KA-LE 41				KK-VI 52*					LMY 145	NGL 48						
										LGL 113				NGV 50		
QA-IN 355										NK-GV 46*						
QA-IN 230										NK-GV 47*						
KS-IE 112										NK-GI 46*						
KA-IE 66										NK-GV 69						
QA-II 330																
QA-II 330																
QA-IE 58										NN-GA 41						
QA-IEF 71										NK-GA 45				KNTGN 47	TGN 49	G-QQ 59
QA-IN 350										NK-GV 47*						
QA-IN 457										NK-GV 46*						
										NK-GV 80						
										NK-GV 397						
										NK-GV 50						
										NK-GV 50						
										NK-KV 48*				NGV 46		56
										NK-KV 55						
										NK-KV 51						
										NT-KA 52						
										NT-KA 52						
										NK-KV 53						
										NK-NV 303*						
										NK-KV 57						
										NK-KV 57						
QA-IA 56																
QA-IA 56																
QA-IA 56																
QA-EA 53										NN-GL 46						
QA-EA 52										NN-GL 44						
QA-VT 50										NN-GI 44						
QA-VAF 109																
QA-IN 43										NN-GA 48						
QA-IN 43										NN-GA 48						
QA-VA 409										DN-GL 100						
?-VA										DN-?						
SA-AS 137										NN-GI 85						
QA-VS 60										NA-GL 81						
SA-VS 137										NN-GI 85						
SA-VS 73																
QA-VE 459										NN-GL 63						
SA-VT 53										TK-GL 70						
KA-AE 47				KK-VI 53*												
KA-IE 50				KK-VI 64*					LMY 101	NGL 48						
KA-IE 53				KK-VI 63*					LMY 101	NGL 93			AN-VG 51	TGV 53		AG-TT 214
KA-VE 49				KK-VI 56*					LMY 102	NGL 67			AN-VG 51	TGV 49		
SA-KE 62				KT-MV 111						LGL 50				NGV 113		
SA-KE 61				KT-MV 111						LGL 50				NGV 114		
EA-VK 49				KK-VI 52*					LMY 297	NGL 78*			AN-VG 50	TGV 46		SG-TT 76
KA-IE 247				KK-VI 45					LMY 297	KGL 57			AN-VG 133	TGV 150		
KA-IE 247				KK-VI 45					LMY 297	KGL 57			AN-VG 133	TGV 150		
KA-IE 247				KK-VI 46					LMY 297	KGL 57			AN-VG 133			
																SG-TT 48
		TK-VS 46								NGLP 72			ST-VG 44	NGV 106		SG-TT 247
														NGV 56		
					LP-VI 240				LMY 297		DFW 120			NGI 39		
					IK-AI 56					IN-GL 29*	LWW 132					
					IK-AI 84				IMY 46		EWY 76					
					VK-VI 160				IMY 120		NWY 95					
			LQ-VD 40					TGE 43			KWFA 85					
			LQ-VD 41					TGE 43			KWFA 73					
	FTQ 110					SQ-	DV 103					NQ-IS 183				SG-QT 149

CHAPTER 5

BOTH SSU RDNA AND RNA POLYMERASE II DATA RECOGNIZE THAT ROOT-KNOT NEMATODES AROSE FROM MIGRATORY PRATYLENCHIDAE, BUT PROBABLY NOT FROM ONE OF THE ECONOMICALLY HIGH-IMPACT LESION NEMATODES

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Abstract

Already in 2000, Siddiqi [1] formulated a hypothesis stating that root-knot nematodes (*Meloidogyne* spp.) constitute a branch arising from yet another important group of plant parasites, the migratory Pratylenchidae. This hypothesis was solely based on morphological characteristics. Ribosomal DNA (rDNA) sequence analysis supports this hypothesis in its broad sense, but the more precise question about the identity of a migratory Pratylenchidae representative being closest to the most basal *Meloidogyne* species could not be addressed due to a lack of backbone resolution [2]. Here we present an extended small subunit rDNA sequence analysis, and a data set of partial RNA polymerase II sequences from Pratylenchidae and basal Meloidogynidae. Our data point at members of the genus *Pratylenchus* as being closest to the common ancestor of the root-knot nematodes, but it was not possible to unequivocally identify a candidate lesion nematode species. *Pratylenchus* is a species-rich genus (> 100 species), and we suggest that the species closest to the most basal root-knot nematode should be sought outside the group of the relatively well-characterized, agronomically relevant *Pratylenchus* species.

Introduction

Within the phylum Nematoda, the order Tylenchida harbors the largest and agronomically most important groups of plant parasites. However, plant parasitism is not the only trophic group in this speciose clade as the insect and mite-parasitic suborder Hexatylina is nested in it. In terms of economic impact three Tylenchid groups are most relevant: root-knot (*Meloidogyne* spp.), lesion (*Pratylenchus* spp.) and cyst nematodes (*Heterodera* and *Globodera* spp.). Root-knot and cyst nematodes are sedentary endoparasites that induce the formation of a feeding site in the roots of their host plant, whereas lesion nematodes are migratory endoparasites that continuously enter and leave the plant root. Hence also from an application-oriented point of view it could be relevant to pinpoint in more detail the evolutionary relationship between the three families, Meloidogynidae, Pratylenchidae and Heteroderidae, in which these genera curently reside.

On the basis of (nearly) full length small subunit ribosomal DNA (SSU rDNA) sequences, a sister relationship was inferred between members of the families Meloidogynidae and Pratylenchidae on the one hand, and Heteroderidae and Hoplolaimidae on the other [2]. Despite the fact that more and more data point at a close evolutionary relationship between lesion and root-knot nematodes, we don't have a detailed picture yet as most of the research efforts are dedicated to a few highly distal members of the genus *Meloidogyne* such as *M. incognita, M. hapla* and *M. chitwoodi.*

In his overview on Tylenchida, Siddiqi [1] distinguished two Meloidogynidae subfamilies: Meloidogyninae and Nacobboderinae. Since Plantard *et al.* [3] rejected the genus *Spartonema* on the basis of ribosomal DNA data, the first subfamily is monotypic (just *Meloidogyne*). The Nacobboderinae are constituted by two genera: *Meloinema* and *Bursadera.* Detailed study of the architecture of the female gonoduct prompted [4] to suggest that *Meloinema* is more close to the Hoplolaimidae (and - thus - relatively unrelated to the Meloidogynidae). As virtually nothing is known about the phylogenetic positioning of the other genus, *Bursadera*, no further attention will be paid to the Nacobboderinae. Within Pratylenchidae, Siddiqi [1] recognized four subfamilies, namely Hirschmanniellinae, Nacobbinae, Radopholinae, and Pratylenchinae. Ribosomal DNA sequences suggest that the Pratylenchidae do not constitute a monophyletic family: Hirschmanniellinae (*Hirschmanniella*) and Pratylenchinae (*Pratylenchus* and *Zygotylenchus*) are closely related, but members of the subfamilies Nacobbinae and Radopholinae were shown to be more related to the Telotylenchidae (*Nacobbus*), to the cyst nematodes (*Radopholus similis*) and to the Merliniidae (*Pratylenchoides*) [2, 5-7]. Regarding the Heteroderidae only representatives of the circumfenestrate cyst nematodes (Heteroderinae) will be considered herein as data on the two other subfamilies, Meloidoderinae and Ataloderinae, are scarce.

A hypothesis stating that root-knot nematodes evolved from ancestors of the subfamilies Hirschmanniellinae or Pratylenchinae is based on a comparative study on the female gonoduct morphology [6] and SSU rDNA data [2]. The

resolution of the relevant nodes did not allow a more precise statement. A detailed study on the basis of a non-neutral gene, β-1,4-endoglucanases belonging to glycoside hydrolase family 5, among representatives of the Pratylenchidae, Heteroderidae and Meloidogynidae resulted in a well-supported sister relationship between a member of the Pratylenchinae, namely *Pratylenchus thornei,* and the genus *Meloidogyne* [8]. A hypothesis about *Meloidogyne* being a branch originating 'from ancestors similar to the contemporary migratory Pratylenchidae' (quote from Siddiqi [1] p. 372) is evidently not new, but not widely accepted. An additional pathogenicity-unrelated molecular analysis is essential to test and specify this hypothesis as it could confirm or question the identity of a living representative of the migratory Pratylenchidae (e.g. *Pratylenchus thornei*) as being closest to the common ancestor of the root knot nematodes.

To this end additional full length SSU rDNA sequences were collected and analyzed from a greater diversity of *Pratylenchus* species. In parallel, sequences were generated from another gene, in this case of a fragment of the largest subunit of RNA polymerase II (*rpb1*). Contrary to ribosomal genes, *rpb1* is coding for a protein (other set of constraints on evolution). A part of this gene has been used before by Baldwin [9] and Kiontke [10] to elucidate relationships among Rhabditidae. With regard to plant parasitic nematodes, Lunt [11] used a 661 bp exon of *rpb1* to investigate origins of asexuality in parthenogenetic root-knot nematodes. This study concentrated on more distal members of the genus *Meloidogyne* (species belonging to Clades I, II and III as defined by Tandingan De Ley [12]). Here, we concentrated on basal root-knot nematodes and coding sequences from the largest subunit of the RNA polymerase II gene (*rpb1*) that were generated from four *Meloidogyne,* six *Pratylenchus* and one *Hirschmanniella* species. We compared the resulting phylogenies with one generated from an extended (as compared to Holterman *et al.* [2]) SSU rDNA data set. Similarly to Lunt [11] we concentrated on the central part of the *rpb1* gene, but a larger exon fragment (appr. 1200 bp, harboring up to 5 introns) was amplified. The relationships between the representatives of *Pratylenchidae* and *Meloidogynidae* as revealed by *rpb1* sequence data are compared with the topology of an extended SSU rDNA tree, and discrepancies and similarities are discussed.

Materials and Methods

Taxon sampling, identification and DNA extraction

Meloidogyne species were kindly provided by Prof. Gerrit Karssen from the Plant Protection Service of The Netherlands: *M. ichinochei* (propagated on *Iris levigata;* culture C2312; Japan), *M. artiellia* (propagated on *Triticum aestivum*; culture E8067; Syria), *M. ardenensis* (propagated on *Liguster sp.*; Wageningen), and *M. ulmi* (propagated on *Ulmus sp.*; Wageningen). *Globodera rostochiensis* specimens originated from a Dutch population Ro_1 – "Mierenbos" (Wageningen). *Pratylenchus*, *Hirschmaniella* and the rest of species used for building the SSU rDNA phylogeny

were collected from various habitats throughout The Netherlands and extracted from the soil using standard techniques. Individual nematodes were subsequently identified using a light microscope (Zeiss Axioscope) equipped with DIC optics. Selected nematodes were transferred to a 0.2 mL Eppendorf tube containing 25 µL sterile water. An equal volume of lysis buffer containing 0.2 M NaCl, 0.2 M Tris-HCl (pH 8.0), 1% (v/v) β-mercaptoethanol and 800 µg/mL proteinase K was added. Lysis took place in a Thermal cycler (Bio-Rad) at 65 °C for 2h followed by 5 min incubation at 95 °C. The lysate (crude DNA extract) was used immediately for a (q)PCR or stored at –20 °C.

rpb1-specific primer design

The primers were designed based on publicly available sequences ([13]; [9]; [14]; [10, 11, 15]; *C. elegans* NM068122 sequence from Nematode Sequencing Project, Wellcome Trust, Sanger Institute). Seven conserved peptide regions, namely RP2-1 to RP2-7, from RNA polymerase II large subunit (*rpb1*) were identified and used to design a range of RNA polymerase II-specific primers. Majority of the sequences generated in this study span approximately 1200 bp (excluding introns). For sequences from *M. ulmi*, *M. ichinohei* 2, *H. gracilis* and *G. rostochiensis* (used as an outgroup) slightly shorter sequences were amplified (600-800 bp). Primer sequences used for generation of the required amplicons are given in Table 1, while relevant GenBank accessions in Table 2.

Table 1. Overview of PCR primers and the annealing temperatures (Ta) used for the amplification of *rpb1* sequences from individual nematodes. The number behind a species name was assign to differentiate particular individuals belonging to the same species.

Specimen identifier	Forward Primer 5′-3′	Reverse Primer 5′-3′	Ta
Meloidogyne *M. ardenensis* 1,2	RP2Mel1F GACCAAAAYTTGCCiAC	RP2Mel4R TTCiGGATGCATRTGGAA	53
M. artiellia 1,2	RP2Mel2F GAGAGiGAATTCAARTTGGA	RP2Mel3R TGGTTTGTGCATGTACAC	57
M. ichinohei 1		RP2Mel4R TTCiGGATGCATRTGGAA	53
M. ichinohei 2	RP2-3Fb TiGAYGGTATGTGGGTTGA	RP2-5Rb GGRTCiAAGTCiGGCATTTC	54
M. ulmi 1	RP2-4Fb CAiCCiGGWGAGATGGT	RP2-6Ra GCiGTTCCRTCiGTTTC	54
Pratylenchus *P. crenatus* 1a, 2	RP2Pcr1F CGAACAACCAGCTCTT	RP2Prat2R GTiGGCTTGTGCATGTA	56
P. neglectus 1, 2	RP2Pn1F AAGCCAAACAACCAGC		
P. thornei 1, 2			
P. penetrans 1, 2	RP2Pp1F GATCAGAATGTTCCAATCC		
P. pratensis 1, 2	RP2Ppr1F ACCAAAATGTGCCCATAT		
P. vulnus 1, 2	RP2Pv1F GAATGTACCCATATTAAAGCC		
Hirschmanniella *H. gracilis* 1, 2	RP2Hg1F CACAAAGTTTGGGCGA		
Globodera *G. rostochiensis* 1a	RP2-2Fb TTCCAYGCiTGGGtGG	RP2Hg2R ACTTTGGCAATGGACTC	57
G. rostochiensis 1b	RP2-3Fc TiGAYGGTATGTGGGTGGA	RP2-5Rd GGRTCiAAGTCiGGCACTTC	50

i=inosine

Amplification target fragments

SSU rDNA sequences were acquired according to protocols described in Holterman e*t al.* [16] and Van Megen [5].

Due to third codon variation (not affecting the amino acid composition) a number of primer combinations had to be used for the amplification of the relevant *rpb1* fragments. An overview of the primer combinations is given in Table 2. For first selection of the most effective primer combinations, quantitative PCR was used. Three µL of template from single nematode lysate was mixed with relevant primers (end concentrations for both primers 200 nM), and 12.5 µL iQ Absolute Sybr Green Fluorescein Cat. CM-225 (Westburg). The total reaction volume was 25 µL. Thermal cycling was performed in a MyiQ (Bio-Rad) under the following conditions: 95 °C for 15 min, followed by 60 cycles at 95 °C for 30 s, 50°C for 1 min and 72 °C for 2 min. In case a possibly correct amplicon was produced (criteria: Ct value < 50 cycles, and a melting temperature > 80°C), it was analyzed on a 1% agarose-gel stained with ethidium bromide. For selected primer combinations, the annealing temperature was optimized using conventional PCR. A single PCR reaction was performed in a final volume of 25 µl and contained 3 µl of a diluted crude DNA extract, 0.2 µM of each PCR primer, and a Ready-To-Go PCR bead (Amersham, Buckinghamshire, UK). The following PCR profile was used: 95 °C for 5 min followed by 60 x (94 °C, 30 sec; specific annealing temperature, 30 sec; 72 °C, 1 min) and 72 °C, 10 min.

Cloning, sequencing and sequence alignment

Gel-purified amplification products (Marligen Bioscience, Ijamsville, MD) were cloned into a TOPO TA vector (pCR®2.1, Invitrogen, Carlsbad, CA) and sequenced using standard procedures. Newly generated sequences were deposited at GenBank under accession numbers listed in Table 1 in case of *rpb1* gene and Table S1 in case of the SSU sequences. Intron positions in the obtained *rpb1* genomic fragments were identified based on information about exon-intron structure of publicly available sequences.

The newly obtained *rpb1* nucleotide sequences combined with relevant public data (GenBank accessions) were translated into amino acids and aligned using the ClustalW algorithm as implemented in BioEdit 5.0.9 [17]. The alignment was manually improved and translated back into nucleotides. The final *rpb1* alignment consisted of 38 sequences, of which 23 were generated in this study. The *Globodera pallida rpb1* sequence (used as an outgroup) was extracted from the Sanger Institute website, contig >1017577 (www.sanger.ac.uk /resources/downloads/helminths/globodera-pallida.html).

Phylogenetic analysis

The program MrBayes 3.1.2 was used for the Bayesian inference-based analysis of nearly full-length SSU rDNA sequences from 90 taxa. We applied 4by4 nucleotide model and General Time Reversible (GTR) substitution model with gamma-distributed rate variation across sites and a proportion of invariable sites. Four independent runs were used with four Markov chains per run. The

program was run for 4.5 million generations with a sample frequency of 200 generations. The first 450,000 generations were discarded as burnin. The program Tracer v1.4 [18] was used to check the stabilization of likelihood and parameters.

For the Bayesian phylogeny of the *rpb1* tree partition "by_codon" was specified and GTR model with gamma distribution of rate variation with a proportion of invariable sites was applied. Four independent runs were made with 4 Markov chains per run. The program was run for 1,000,000 generations. Stabilization of the likelihood and parameters was checked with the program Tracer v1.4 and as the result the first 60,000 generations were discarded as burnin. For the construction of the maximum likelihood of the second *rpb1* tree the RAxML-HPC BlackBox program available at the CIPRES Science Gateway V. 3.1 was used (http://www.phylo.org/sub_sections/portal/). The following parameters were chosen: estimated proportion of invariable sites (GTRGAMMA + I), finding best tree using maximum likelihood search, bootstrapping halted automatically and printed branch lengths.

Results and Discussion

Phylogenetic relationships based on SSU ribosomal DNA data

To gain more insight into the relationship between lesion and basal root-knot nematodes, additional (nearly) full-length SSU rDNA sequences were generated. As shown in Figure 1, this more extensive phylogenetic analysis resulted in some interesting findings. *P. penetrans, P. convallariae* and *P. fallax* are robustly placed in a single subclade, and it could be worthwhile to investigate whether the species status of *P. convallariae* and *P. fallax* should be continued. Moreover, the current analysis points at a sister relationship between a member of major clade c [19]; here represented by *P. thornei* and *P. neglectus*) and all *Meloidogyne* representatives, but the posterior probability (0.84) is below the robust support threshold (0.95). It is remarkable to see that at least one characteristic is shared between these two lesion nematode species and the two most basal *Meloidogyne* representatives: the virtual absence of males.

However, the backbone resolution among the lesion nematode species was barely improved in comparison to an earlier study by Holterman *et al*. [2]. On the basis of the current data it was concluded that SSU rDNA probably does not offer the resolution required to address detailed questions about the evolutionary relationship between lesion and root-knot nematodes. Hence, it was decided to investigate the resolution offered by another neutral gene, *rpb1* (encoding the largest subunit of the RNA polymerase II). A part of this gene (≈ 660 bp) has been used by Lunt [11] to study the evolution of (a)sexuality among root-knot nematodes.

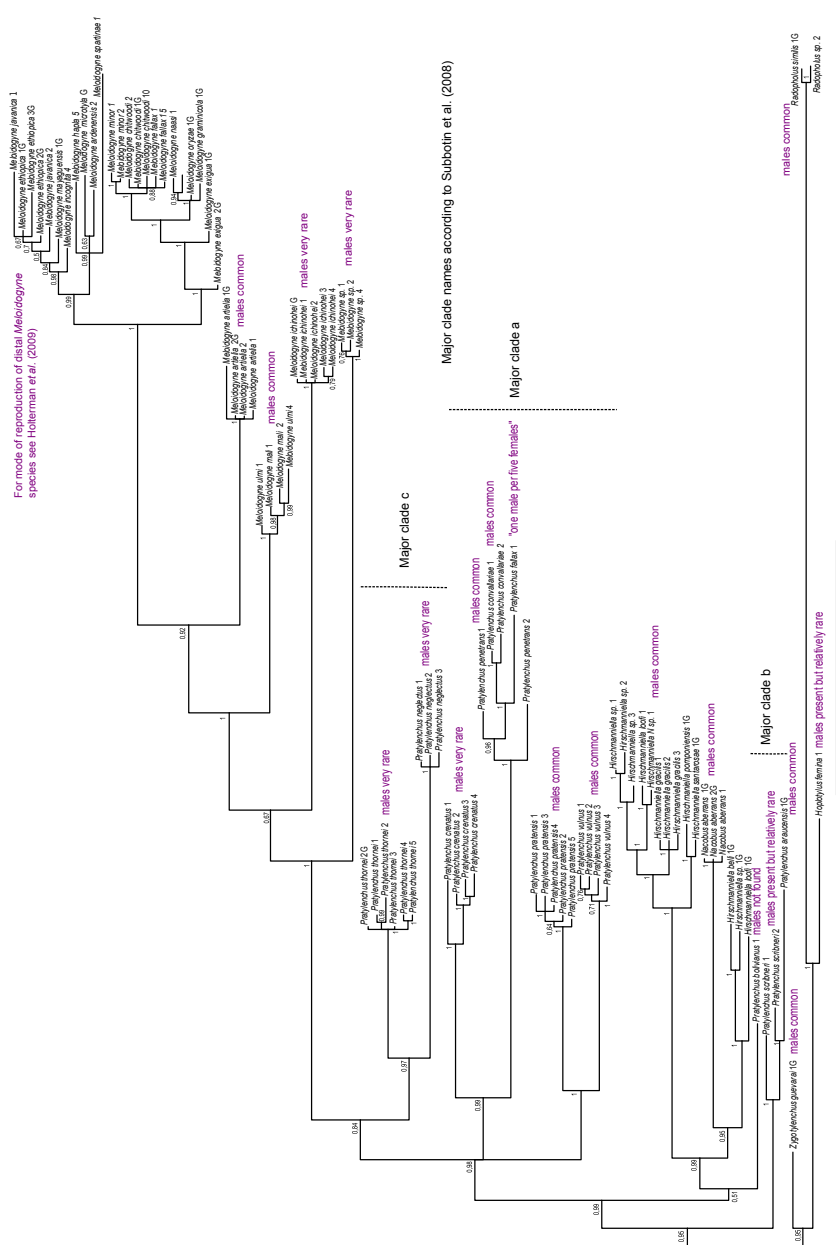


Figure 1. A Bayesian inference of a Pratylenchidae and Meloidogynidae tree based on SSU rDNA sequences. The numbers next to nodes are posterior probabilities. Posterior probabilities > 0.95 are considered to be robust. Occurrence of males in populations is marked next to each species. For grouping of *Pratylenchus* species, major clade identifiers as proposed by Subbotin *et al.* [19] is used.

rpb1 sequence amplification

Contrary to SSU rDNA, *rpb1* is a protein-coding gene, and the variability of the third codon position (often without consequences for the amino acid composition) interfered with the design of general Pratylenchidae primers for the gene. Hence, contrary to rRNA coding genes, numerous PCR primers (Table 1) had to be tested in order to amplify ≈ 1,200 bp *rpb1* fragments from Pratylenchidae and Meloidogynidae species. A few primers appeared to be relatively versatile; *e.g.* RP2Prat2R could be used as a reverse primer for all *Pratylenchus* species tested in this study (Table 1). In total 23 new *rpb1* sequences were generated. The potato cyst nematode *Globodera rostochiensis* was selected as an outgroup, and remarkably two different sequences were found within one individual pre-parasitic juvenile (Fig. 2). For some species such as *M. ulmi*, *H. gracilis* and *G. rostochiensis* only shorter sequences (600-800 bp) could be amplified.

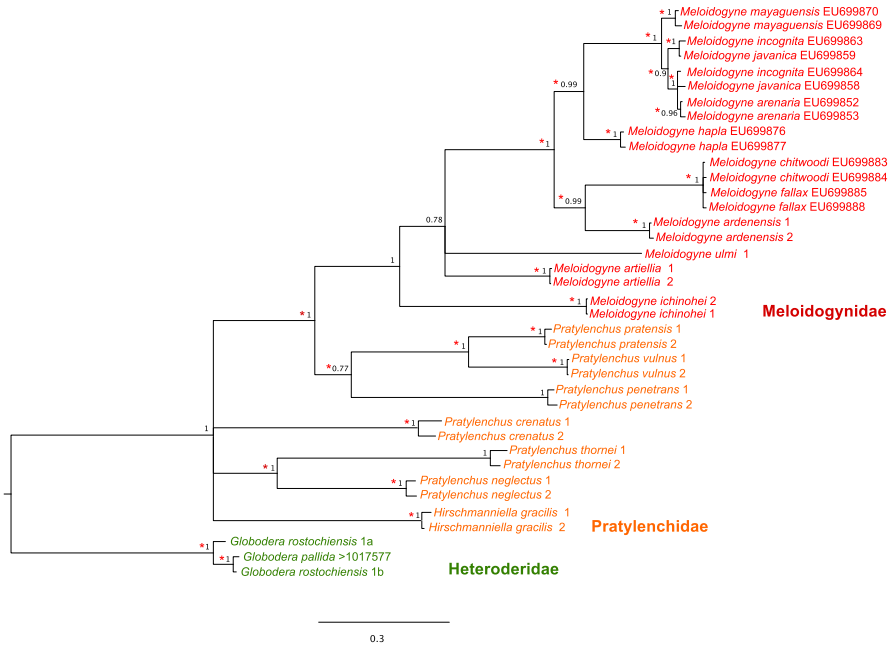


Figure 2. A Bayesian inference of a Pratylenchidae and Meloidogynidae tree based on partial sequences of the largest subunit of the RNA polymerase II gene (*rpb1*) (see Table 2). The numbers next to nodes are posterior probabilities. Posterior probabilities > 0.95 are considered to be robust. Asterisks refer to nodes that are supported by maximum likelihood analysis as well.

Phylogenetic analysis of rpb1 sequences

The Bayesian and Maximum likelihood phylogenetic analysis of 38 *rpb1* genomic sequences confirmed the monophyletic positioning of the Meloidogynidae as a distinct branch within representatives of the Pratylenchidae (Fig. 2). Within the genus *Pratylenchus* the close sister relationships between *P. pratensis* and *P. vulnus*,

and between *P. thornei* and *P. neglectus* were confirmed (Fig. 1, Fig. 2). However, no additional support was found for evolutionary relatedness of *P. penetrans* and *P. crenatus* as suggested by SSU rDNA sequence data (Fig. 1). With regard to the origin of the genus *Meloidogyne,* our *rpb1*-based phylogenetic analysis suggested for an alternative hypothesis saying that *P. pratensis* and *P. vulnus* (with or without *P. penetrans*) could be closest to the base of the root-knot nematodes. It is noted that the analysis of *rpb1* sequences reinforced the most basal positioning of *M. ichinochei* among the root-knot nematodes. The positioning of two other basal root-knot nematodes, namely *M. ulmi* and *M. artiellia,* could not be established.

A.

	Pcr1a	Pcr1b	Pcr1c	Pcr1d	Pcr2	Pcr3
Pcr1a	0.0000					
Pcr1b	0.0252	0.0000				
Pcr1c	0.0265	0.0299	0.0000			
Pcr1d	0.0031	0.0154	0.0215	0.0000		
Pcr2	0.0342	0.0253	0.0401	0.0266	0.0000	
Pcr3	0.0202	0.0061	0.0184	0.0170	0.0250	0.0000

B.

	I	II	III	IV	V	VI	VII
Pcr1a	53	50	45	45	45	ND	ND
Pcr1b	53	49	46	46	ND	ND	ND
Pcr1c	53	50	43	49	ND	ND	ND
Pcr1d	ND	ND	ND	45	45	53	49
Pcr2	53	50	40	43	45	ND	ND
Pcr3	ND	ND	ND	47	45	48	44

Figure 3. A. Distances between variants of *rpb1* gene fragments within *P. crenatus* (as inferred by DNA Dist, BioEdit). B. Intron lengths in the different variants of the *rpb1* gene fragment within *P. crenatus*. The digit after the "Pcr" abbreviation (1, 2 or 3) stands for distinct individuals. The lowercase letter following the digit identifies the intra-individual variants. Lengths of coding sequence: Pcr1a and Pcr2 ~ 1170 bp (GenBank KC822441, KC822442), Pcr1d and Pcr3 954 bp (KC822446, KC822443), Pcr1b and Pcr1c 886 bp (KC822444, KC822445). "ND" stands for no data available.

rpb1 sequences characteristics - predicted intron composition and intraspecific variation

Five intron positions were predicted to be present in the genomic *rpb1* sequences (Table 2). *P. penetrans* lacks an intron in position II, while two other related *Pratylenchus* species, *P. thornei* and *P. neglectus*, have the absence of intron V in common. Interestingly, all the *rpb1* sequences from the root-knot nematode species seem to lack intron III.

In case of the lesion nematode *P. crenatus, rpb1* fragments were amplified from three individuals (*P. crenatus* 1, 2 and 3). Six variants of the *rpb1* gene fragments were observed. Four of those sequences (*Pcr*1a, *Pcr*1b, *Pcr*1c and *Pcr*1d) were amplified from a single individual (of whitch only *Pcr*1a (*P.crenatus* 1) was included in Fig. 2). Within a region of about 660 bp, six single nucleotide polymorphisms (SNP's) were found. Sequence distances between these gene fragments as well as their intron compositions can be found in Fig. 3. It is noted

that four SNPs were located within intron IV (data not shown). *P. crenatus* reproduces asexually (males are extremely rare [20]), and just like other parthenogenetic lesion root species the total number of chromosomes could be in a range between 20 and 32, whereas n= 5, 6 or 7 is typical for sexually reproducing lesion nematode species [21]. As a single copy of the *rpb1* gene in a genome was expected, the presumed polyploidy nature of *P. crenatus* could be an explanation for the remarkable number of *rpb1* variants that were found in *P. crenatus* individuals.

Table 2. Schematic overview of intron structure of *rpb1* genomic sequences from representatives of the Pratylenchidae and the Meloidgynidae. The roman digits in a column's header symbolize a predicted intron. Absence of the intron is marked with a dash. Capital letters within the known sequence area (gray, not in scale) stand for intron flanking amino acid residues, whereas the digits next to them represent an intron length (in base pairs). The maximal amplified length of *rpb1* sequence in this study was about 1,200 bp. (i.e. *M. ardenensis* 1). Only shorter sequences could be amplified from *M. ulmi, M. ichinohei* 2, *H. gracilis* and *G. rostochiensis* (in italics).

Specimen identifier	GenBank ID	I	II	III	IV	V	Length coding regions (in bp)	
Meloidogyne *M. ardenensis* 1	KC822435	IT-NS42	DL-SP49	-	LK-GL43	GE-DQ49	1191	This paper
M. ardenensis 2	KC822434	IT-NS42	DL-SP49	-	LK-GL43	GE-DQ49	1191	
M. arenaria	EU699852				LK-GL40		675	Lunt (2008)
M. arenaria	EU699853				LK-GL40		675	
M. artielia 1	KC822436	IS-NS67	DM-SP66	-	LK-GL216	SE-DQ187	1140	This paper
M. artielia 2	KC822437	IS-NS67	DM-SP66	-	LK-GL216	SE-DQ187	1140	
M. chitwoodi	EU699883				LK-GL45		675	Lunt (2008)
M. chitwoodi	EU699884				LK-GL45		675	
M. hapla	EU699876				LK-GL42		675	
M. hapla	EU699877				LK-GL42		675	
M. ichinohei 1	KC822438	IS-NS45	DL-SP58	-	LK-GL65	GE-DQ46	1146	This paper
M. ichinohei 2	KC822439	*IS-NS45*	*DL-SP58*	-	*LK-GL65*		*867*	
M. incognita	EU699863				LK-GL40		675	Lunt (2008)
M. incognita	EU699864				LK-GL40		675	
M. javanica	EU699858				LK-GL40		675	
M. javanica	EU699859				LK-GL40		675	
M. fallax	EU699885				LK-GL45		675	
M. fallax	EU699888				LK-GL45		675	
M. enterolobii	EU699869				LK-GL40		675	
M. enterolobii	EU699870				LK-GL40		675	
M. ulmi 1	KC822440				*LK-GL703*	*GE-EQ614*	*653*	This paper
Pratylenchus *P. crenatus* 1	KC822441	IA-NS53	DL-NP50	CT-AI45	LK-GL45	GE-EQ45	1170	
P. crenatus 2	KC822442	IA-NS53	DL-NP50	CT-AI40	LK-GL43	GE-EQ45	1170	
P. neglectus 1	KC822447	IT-NS67	DL-SP69	CT-AI66	LK-GL80	-	1173	
P. neglectus 2	KC822448	IT-NS67	DL-SP54	CT-AI66	LK-GL79	-	1173	
P. penetrans 1	KC822449	IT-NS50	-	CN-AI52	LK-GS47	GE-EQ57	1191	
P. penetrans 2	KC822450	IT-NS55	-	CN-AI52	LK-GS54	GE-EQ57	1191	
P. pratensis 1	KC822451	IT-NS46	DL-SP52	CT-AI50	LK-GS46	GE-EQ48	1185	
P. pratensis 2	KC822452	IT-NS46	DL-SP52	CT-AI50	LK-GS46	GE-EQ48	1185	
P. thornei 1	KC822453	IT-NS49	DM-SP47	CT-AI49	LK-GS49	-	1179	
P. thornei 2	KC822454	IT-NS53	DM-SP65	CT-AI60	LK-GS46	-	1179	
P. vulnus 1	KC822455	IT-NS44	DL-SP123	CT-AI46	LK-GL45	GE-EQ46	1185	
P. vulnus 2	KC822456	IT-NS44	DL-SP123	CT-AI46	LK-GL45	GE-EQ46	1185	
Hirschmanniella *H. gracilis* 1	KC822457				LK-GV52	GE-EL57	612	
H. gracilis 2	KC822458				LK-GV52	GE-EQ57	612	
Globodera *G. pallida* contig >*1017577* *		IT-NS54	DL-SP46	DQ-AI51	LK-GL45	GE-EQ132	1191	*G. pallida* sequencing consortium
G. rostochiensis 1a	KC822459	*IT-NS55*	*DL-SP46*	*DQ-AI47*	*LK-GI44*		*867*	This paper
G. rostochiensis 1b	KC822460	*IT-NS55*	*DL-SP46*	*DQ-AI47*	*LK-GI44*		*867*	

*Contig >101577 spans 1,521 bp. A 'T' at position 452, and an 'A' at position 1,177 (part of an A 7-mer) were removed from alignment.

On the relationship between lesion and basal root-knot nematodes

An expanding diversity and number of full length SSU and partial LSU rDNA sequences confirms the close relationship between lesion and root-knot nematodes, the latter being a group branching for the lesion nematodes ([2, 5, 22, 23]). However, more detailed information about this connection, in this case the representatives of each of these families being closest to this genus transition, could not be identified with the current ribosomal DNA sequence information. Recently, the analysis of catalytic domains of GHF5 cellulases pointed at *P. thornei* being a lesion nematode species close to genus transition [8], but analysis of connection with yet another gene, a fragment of *rpb1*, could not further underpin this hypothesis. This could be due to the fact that we have sampled only a too small part of the *Pratylenchus* biodiversity. According to Siddiqi [1] there are 68 *Pratylenchus* species, 12 *species inquirendae*, and 4 species referred to as *nomina nuda*. Apparently, the living representative of the lesion nematodes closest to the most basal representatives of the root-knot nematodes does not reside among the agronomically most important *Pratylenchus* species.

References

1. Siddiqi MR: **Tylenchida: parasites of plants and insects**. Wallingford, UK: CABI publishing; 2000.

2. Holterman M, Karssen G, Van den Elsen S, Van Megen H, Bakker J, Helder J: **Small Subunit rDNA-Based Phylogeny of the Tylenchida Sheds Light on Relationships Among Some High-Impact Plant-Parasitic Nematodes & the Evolution of Plant Feeding**. *Phytopathology* 2009, **99**(3):227-235.

3. Plantard O, Valette S, Gross MF: **The root-knot nematode producing galls on *Spartina alterniflora* belongs to the genus Meloidogyne: Rejection of *Hypsoperine* and *Spartonema* spp**. *Journal of Nematology* 2007, **39**(2):127-132.

4. Bert W, Claeys M, Borgonie G: **The comparative cellular architecture of the female gonoduct among tylenchoidea (Nematoda: Tylenchina)**. *Journal of Nematology* 2006, **38**(3):362-375.

5. Van Megen H, Van Den Elsen S, Holterman M, Karssen G, Mooyman P, Bongers T, Holovachov O, Bakker J, Helder J: **A phylogenetic tree of nematodes based on about 1200 full-length small subunit ribosomal DNA sequences**. *Nematology* 2009, **11**(6):927-950.

6. Bert W, Leliaert F, Vierstraete AR, Vanfleteren JR, Borgonie G: **Molecular phylogeny of the Tylenchina and evolution of the female gonoduct (Nematoda : Rhabditida)**. *Molecular Phylogenetics and Evolution* 2008, **48**(2):728-744.

7. Sturhan D: **Contribution to a revision of the family Merliniidae Ryss, 1998,with proposal of Pratylenchoidinae subfam. n., *Paramerlinius* gen. n., *Macrotylenchus* gen. n. and description of *M. hylophilus* sp. n. (Tylenchida)**. *J Nematode Morphol Syst* 2012, **15**(2):127-147.

8. Rybarczyk-Mydłowska K, Ruvimbo Maboreke H, Van Megen H, Van den Elsen S, Mooyman P, Smant G, Bakker J, Helder J: **Rather than by direct acquisition via lateral gene transfer, GHF5 cellulases were passed on from early Pratylenchidae to root-knot and cyst nematodes**. *BMC Evolutionary Biology* 2012, **12**.

9. Baldwin JG, Frisse LM, Vida JT, Eddleman CD, Thomas WK: **An evolutionary framework for the study of developmental evolution in a set of nematodes related to *Caenorhabditis elegans***. *Molecular Phylogenetics and Evolution* 1997, **8**(2):249-259.

10. Kiontke K, Barrière A, Kolotuev I, Podbilewicz B, Sommer R, Fitch DHA, Félix MA: **Trends, Stasis, and Drift in the Evolution of Nematode Vulva Development**. *Current Biology* 2007, **17**(22):1925-1937.

11. Lunt DH: **Genetic tests of ancient asexuality in root knot nematodes reveal recent hybrid origins**. *BMC Evolutionary Biology* 2008, **8**(1).

12. Tandingan De Ley I, De Ley P, Vierstraete A, Karssen G, Moens M, Vanfleteren J: **Phylogenetic analyses of *Meloidogyne* small subunit rDNA**. *Journal of Nematology* 2002, **34**(4):319-327.

13. Aboobaker AA, Blaxter ML: **Use of RNA interference to investigate gene function in the human filarial nematode parasite *Brugia malayi***. *Molecular and Biochemical Parasitology* 2003, **129**(1):41-51.

14. Baldwin JG, Giblin-Davis RM, Eddleman CD, Williams DS, Vida JT, Thomas WK: **The buccal capsule of *Aduncospiculum halicti* (Nemata: Diplogasterina): An ultrastructural and molecular phylogenetic study**. *Canadian Journal of Zoology* 1997, **75**(3):407-423.

15. Kiontke K, Gavin NP, Raynes Y, Roehrig C, Piano F, Fitch DHA: ***Caenorhabditis* phylogeny predicts convergence of hermaphroditism and extensive intron loss**. *Proceedings of the National Academy of Sciences of the United States of America* 2004, **101**(24):9003-9008.

16. Holterman M, van der Wurff A, van den Elsen S, van Megen H, Bongers T, Holovachov O, Bakker J, Helder J: **Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown clades**. *Molecular Biology and Evolution* 2006, **23**(9):1792-1800.

17. Hall TA: **BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT**. *Nucleic Acids Symposium Series* 1999, **41**:95-98.

18. Rambaut A, Drummond A: **Tracer v1.4**. In., 1.4 edn: http://beast.bio.ed.ac.uk/ Tracer 2007.

19. Subbotin SA, Ragsdale EJ, Mullens T, Roberts PA, Mundo-Ocampo M, Baldwin JG: **A phylogenetic framework for root lesion nematodes of the genus *Pratylenchus* (Nematoda): Evidence from 18S and D2-D3 expansion segments of 28S ribosomal RNA genes and morphological characters**. *Molecular Phylogenetics and Evolution* 2008, **48**(2):491-505.

20. Karssen G, Brinkman H: **On the male of *Pratylenchus crenatus* Loof, 1960 (Nematoda: Pratylenchidae)**. *Nematology* 2003, **5**(3):479.

21. Roman J, Triantaphyllou AC: **Gametogenesis and reproduction of seven species of *Pratylenchus***. *Journal of Nematology* 1969, **1**(4):357-362.

22. Palomares-Rius JE, Castillo P, Liébanas G, Vovlas N, Landa BB, Navas-Cortés JA, Subbotin SA: **Description of *Pratylenchus hispaniensis* n. sp. from Spain and considerations on the phylogenetic relationship among selected genera in the family Pratylenchidae**. *Nematology* 2010, **12**(3):429-451.

23. Palomares-Rius JE, Castillo P, Liébanas G, Vovlas N, Landa BB, Navas-Cortés JA, Subbotin SA: **Description of *Pratylenchus hispaniensis* n. sp. from Spain and considerations on the phylogenetic relationship among selected genera in the family Pratylenchidae**. *Nematology* 2010, **12**(3):429-451.

Supplementary information

Supplementary Table S1. Newly generated SSU sequences used in this study.

Genus	species	Abbreviation	GenBank ID
Hirschmanniella	*cf. belli*	HirsBel1GZ	EF029856
Hirschmanniella	*gracilis*	HirsGra1	EU669959
Hirschmanniella	*gracilis*	HirsGra2	KC875381
Hirschmanniella	*gracilis*	HirsGra3	KC875384
Hirschmanniella	*loofi*	HirsLoo1	KC875382
Hirschmanniella	*loofi*	HirsLoo1G	EU306353
Hirschmanniella	*n. sp.1*	HirsNSp1	KC875394
Hirschmanniella	*pomponiensis*	HirsPom1G	EF029854
Hirschmanniella	*santarosae*	HirsSan1G	EF029855
Hirschmanniella	*sp*	HirsSp1G	EF029857
Hirschmanniella	*sp. 1*	HirsSp1	AY284614
Hirschmanniella	*sp. 2*	HirsSp2	AY284615
Hirschmanniella	*sp. 3*	HirsSp3	AY284616
Hirschmanniella	*sp. 1*	HirsSp1G	EF029857
Hoplotylus	*femina*	HoplFem1	KC875396
Meloidogyne	*ardenensis*	MeloArd2	EU669946
Meloidogyne	*artiellia*	MeloArt1	KC875392
Meloidogyne	*artiellia*	MeloArt1G	AF248477
Meloidogyne	*artiellia*	MeloArt2	KC875391
Meloidogyne	*artiellia*	MeloArt2G	AF442192
Meloidogyne	*chitwoodi*	MeloChi10	EU669932
Meloidogyne	*chitwoodi*	MeloChi1G	AF442195
Meloidogyne	*chitwoodi*	MeloChi2	AY593884
Meloidogyne	*ethiopica*	MeloEth1G	AY942630
Meloidogyne	*ethiopica*	MeloEth2G	FJ559408
Meloidogyne	*ethiopica*	MeloEth3G	GQ324703
Meloidogyne	*exigua*	MeloExi1G	AY942627
Meloidogyne	*exigua*	MeloExi2G	AY942627
Meloidogyne	*fallax*	MeloFal1	AY593895
Meloidogyne	*fallax*	MeloFal15	EU669936
Meloidogyne	*graminicola*	MeloGraG	AF442196
Meloidogyne	*hapla*	MeloHap5	AY593893
Meloidogyne	*ichinohei*	MeloIch1	EU669953
Meloidogyne	*ichinohei*	MeloIch2	EU669954
Meloidogyne	*ichinohei*	MeloIch3	KC875385
Meloidogyne	*ichinohei*	MeloIch4	KC875386
Meloidogyne	*ichinohei*	MeloIchG	AF442191
Meloidogyne	*incognita*	MeloInc4	KC875376
Meloidogyne	*javanica*	MeloJav1	EU669938
Meloidogyne	*javanica*	MeloJav2	AY268121
Meloidogyne	*mali*	MeloMal1	EU669948
Meloidogyne	*mali*	MeloMal2	EU669949
Meloidogyne	*enterolobii*	MeloMay1G	AY942629
Meloidogyne	*microtyla*	MeloMicG	AF442198
Meloidogyne	*minor*	MeloMin1	AY593899
Meloidogyne	*minor*	MeloMin2	EU669937
Meloidogyne	*naasi*	MeloNaa1	AY593900
Meloidogyne	*oryzae*	MeloOry1G	AY942631
Meloidogyne	*sp. 2* (on *Sansevieria*)	MeloSp2	EU669951

Meloidogyne	*sp. 4* (on *Sansevieria*)	MeloSp4	EU669952
Meloidogyne	*sp.1* (on *Sansevieria*)	MeloSp1	EU669950
Meloidogyne	*spartinae*	MeloSpa1G	EF189177
Meloidogyne	*ulmi*	MeloUlm1	EU669947
Meloidogyne	*ulmi*	MeloUlm4	KC875395
Nacobbus	*aberrans*	NacoAbe1	KC875388
Nacobbus	*aberrans*	NacoAbe1G	AF442190
Nacobbus	*aberrans*	NacoAbe2G	AJ966494
Pratylenchus	*araucensis*	PratAra1G	FJ154950
Pratylenchus	*bolivianus*	PratBol1	KC875390
Pratylenchus	*convallariae*	PratCon1	EU669957
Pratylenchus	*convallariae*	PratCon2	FJ969136
Pratylenchus	*crenatus*	PratCre1	AY284610
Pratylenchus	*crenatus*	PratCre2	EU669920
Pratylenchus	*crenatus*	PratCre3	EU669921
Pratylenchus	*crenatus*	PratCre4	EU669922
Pratylenchus	*fallax*	PratFal	KC875393
Pratylenchus	*neglectus*	PratNeg1	EU669923
Pratylenchus	*neglectus*	PratNeg2	EU669924
Pratylenchus	*neglectus*	PratNeg3	KC875378
Pratylenchus	*penetrans*	PratPen1	EU669925
Pratylenchus	*penetrans*	PratPen2	EU669926
Pratylenchus	*pratensis*	PratPra1	AY284611
Pratylenchus	*pratensis*	PratPra2	KC875387
Pratylenchus	*pratensis*	PratPra3	KC875379
Pratylenchus	*pratensis*	PratPra4	KC875380
Pratylenchus	*pratensis*	PratPra5	KC875377
Pratylenchus	*scribneri*	PratScr1	EU669927
Pratylenchus	*scribneri*	PratScr2	EU669958
Pratylenchus	*thornei*	PratTho1	AY284612
Pratylenchus	*thornei*	PratTho2G	AJ966499
Pratylenchus	*thornei*	PratTho3	EU669928
Pratylenchus	*thornei*	PratTho4	EU669929
Pratylenchus	*thornei*	PratTho5	EU669930
Pratylenchus	*vulnus*	PratVul1	EU669955
Pratylenchus	*vulnus*	PratVul2	EU669956
Pratylenchus	*vulnus*	PratVul3	KC875389
Pratylenchus	*vulnus*	PratVul4	KC875383
Radopholus	*similis*	RadoSim1G	AJ966502
Radopholus	*sp 2*	RadoSp2	FJ040398
Zygotylenchus	*guevarai*	ZygoGue1G	AF442189

CHAPTER 6
GENERAL DISCUSSION

I. Aim

The main objective of this thesis was to investigate phylogenetic relationship within ecologically and economically important groups of nematodes. The groups under investigation were Dorylaimida and Mononchida (two orders which members are highly sensitive to environmental disturbances), foliar nematodes (fungivorous and plant-parasitic *Aphelenchoides* species) and representatives of two of the economically most relevant genera within the order Tylenchida. Additionally, emphasis was put on the use of a variety of molecular markers to study nematode evolution.

II. Phylogenetic relationships in the selected groups of nematodes

In this thesis we adhered to the division of the phylum Nematoda into 12 major clades as proposed by Holterman *et al.* [1] and van Megen *et al.*, [2] on the basis of (nearly) full length SSU rDNA sequences. The ability of nematode to exploit higher plants as a main food source has arisen at least four times, and facultative and/or obligate plant parasites can be found in the orders Triplonchida (Clade 1), Dorylaimida (Clade 2), Aphelenchida (families Aphelenchoididae and Aphelenchidae) (Clade 10 and Clade 12, respectively) and Tylenchida (Clade 12). Only the plant parasitic family Trichodoridae from Clade 1 was not included in this research. Nematodes from the subclass Dorylaimia (Chapter 2), more specifically members of the orders Mononchida and Dorylaimida, were included because of their sensitivity to disturbances. As such these taxa can be used as ecological indicators.

Within the group of nematodes studied in Chapter 2, the subclass Dorylaiminia, the SSU rDNA gene revealed good resolution within the order Mononchida, an order dominated by predatory nematodes. However, the resolution within the speciose order Dorylaimida was poor due to high sequence conservation. In an attempt to find more phylogenetic signal, a switch was made to the large subunit (LSU) rDNA, another gene residing on the rDNA cistron. Analysis of a more variable fragment (appr. 1,000 bp) at the 5′ end of the LSU rDNA genes distinguished 12 subclades of Dorylaimida. As identifiers the subclades were labeled D1-D9 and PP1-PP3. Intentionally, we just gave short, taxonomically uninformative labels to these subclades as they are based on the sequence information of a single gene only, and since this analysis was made on the basis of only a limited number of representatives of this speciose order. This division shows (very) little similarity to classical (= morphology-based) Dorylaimida systematics. The deeper phylogenetic relationships within this particular order

are far from established. Members of this order show a mosaic of morphological characteristics, and our inability to confirm the nature of Dorylaimid families such as the Qudsianematidae, the Nordiidae and the Dorylaimidae was not unexpected. The poor resolution offered by the SSU rDNA gene for this particular order is exceptional, and is not observed anywhere else phylum-wide rDNA based phylogenetic trees of nematodes. Common denominators of the members of this order are their habitat; they exclusively live in soil (no marine representatives), and their sensitivity to environmental disturbances. We hypothesize that diversification within this order is the result of rapid speciation. Similar phenomena have been observed in other animal group such as echinoderms (a marine group including starfish, sea urchins and sea cucumbers) [3] and feather mites [4]. Remarkably, SSU rDNA sequences offered good resolution within another major order in the subclass Dorylaimia, the Mononchida. Members of this order are mainly predatory nematodes, nematodes feeding on other nematodes, and the molecular phylogenetics of this order appeared to be quite similar to the current morphological classification. Only the family Mylonchulidae appeared to be polyphyletic.

Another taxonomically problematic group studied in this thesis (Chapter 3) is the family Aphelenchoididae. In an authoritative monograph on Aphelenchida, Longidoridae and Trichodoridae, dr. D.J. Hunt stated that "many nominal (*Aphelenchoides*) species are inadequately characterized for reliable recognition and the genus is in urgent need for revision" [5]. Members of the genus *Aphelenchoides* are predominantly fungivorous, but some species can feed on higher plants as well. A few plant parasitic *Aphelenchoides* species have a considerable economical impact and in this respect the causal agent of white tip in rice, *Aphelenchoides besseyi* (on EPPO A2 quarantine list), should be mentioned. However, no proper identification key is available for the genus *Aphelenchoides*, and it is easily conceivable that the export of plants or plant materials is stopped by custom agencies because of the presence of fully innocent fungivorous *Aphelenchoides* species. In Chapter 3, we addressed this issue and tried to resolve the relationships between truly fungivorous *Aphelenchoides* species and their facultatively plant-parasitic relatives. Due to a biased availability of properly identified material, the facultative plant parasites are over-represented as compared to the true fungivores. Numerous fungivorous *Aphelenchoides* species described in literature could not be found, and are, hence, not included in Chapter 3. Although we had to deal with an essentially incomplete molecular framework, our data clearly showed the plant parasitic foliar nematodes do not create a monophyletic group. They were spread over two subclades (A and B). *A. fragariae* and *A. subtenuis*, parasites of respectively strawberry and flower bulb species, were residing in one subclade. On the other hand *A. besseyi*, *A. ritzemabosi* and *A. paradalianensis* were robustly positioned in another branch. Similarly, representatives of the genus *Laimaphelenchus* and many unknown *Aphelenchoides* species were also found in the both subclades. Although it is outside the scope of this thesis to propose alternative systematic schemes, it is clear that the genus

Aphelenchoides as it is defined nowadays is not monophyletic, and our data could contribute to the justification of a drastic revision of this species-rich genus.

In the last two chapters (4 and 5) we focus on the relationship within the most economically significant plant parasitic representatives of the order Tylenchida, namely the members of the families *Pratylenchidae*, *Meloidogynidae* and *Heteroderidae*. Within these chapters ample attention was paid to yet another systematic controversy: the supposed evolutionary relationship between lesion and root-knot nematodes. So far the relationship between these families was predominantly based on ribosomal DNA sequence data (*e.g.* [6] and the female gonoduct morphology [7]). In these two chapters, two neutral and one parasitism-related gene was taken into consideration (SSU rDNA, *rpb1* and β-1,4-endoglucanase, respectively). All three phylogenetic markers pointed at a similar deep topology: root-knot nematodes have evolved from ancient representatives of the family *Pratylenchidae.* Sister to this monophyletic subclade, cyst nematode arose (together with members of the Hoplolaimidae). We tried to identify the lesion nematode species closest to the most basal representative of the root-knot nematodes, and β-1,4-endoglucanase data suggest *Pratylenchus thornei* to be closest to the origin of the root-knot nematode subclade. The basal positioning of *M. artiellia* and *M. ichinohei* within the *Meloidogyne* clade, is confirmed by both cellulase and *rpb1* data. However, comparison of sequence data from the largest subunit of RNA polymerase II did not result in an unequivocal confirmation of *Pratylenchus thornei* as being the closest living representatives of the common ancestor of all root-knot nematodes (Chapter 5).

III. Molecular detection of ecologically and economically important groups of nematodes

So far the Small subunit ribosomal DNA (SSU rDNA) gene is the only gene that allowed for making a phylum-wide molecular framework for nematodes. We tried in vain to do the same with the slightly more variable 5'-end fragment of LSU rDNA, but were unable to properly align sequences from Clade 12 (Tylenchida) with in essence corresponding fragments from Clade 2 (Dorylaimida). Target nematodes, irrespective whether these are animal, human or plant-plant parasites, or environmental indicators, are part of biodiverse nematode assemblages, and for the generation of assays to detect these targets in complex DNA backgrounds, a phylum-wide framework is extremely helpful. So far, various detection methods have been proposed: direct sequencing [8], PCR DGGE [9], T-RFLP [10] and quantitative PCR [1] [11] [12]. Using the quantitative PCR-based methods, it was possible to identify presence and quantify individuals from both single and from many nematode species in parallel. The first two experimental chapters (Chapter 2 and 3) of this thesis show how such real-time quantitative PCR-based identification and monitoring of environmentally (Mononchida) and economically (Aphelenchoidae) important groups of nematodes can be applied in practice. The main principles of such approach

have been already described in detail by Neilson *et al.* [13]. In case of Mononchida five subclades were identified (M1-M5), for which specific primer combinations were selected. Subsequently, they allowed for the identification of ecologically significant (as these predators belong the highest tophic level in the soil food web) nematodes belonging to the genera: *Mylonchulus* (M1); *Mononchus* (M2); *Clarcus, Prionchulus and Coomansus* (M3); *Anatonchus and Miconchus* (M4); *Bathyodontus* (M5). Study on *Aphelenchoides* (Chapter 2) constituted a continuation of our work on qPCR-based nematode detection in complex DNA backgrounds. Newly designed framework for four foliar nematode species: *Aphelenchoides besseyi, A. fragariae, A. ritzemabosi and A. subtenuis,* (Chapter 2) which are very difficult to distinguish from economically unimportant, fungivorous representatives of the genus *Aphelenchoides*, can be a powerful tool for screening of plant material and soil. Taking the *A. subtenuis* as an example we showed how the nematode's presence could not only be identified by engaging especially designed species specific primers, but also be quantified by making use of the linear relationship between the output of the qPCR reaction, the C_t value, and the ^{10}log of the number of target nematodes.

IV. Evolution of β-1,4-endoglucanases in Tylenchida

So far we concentrated on neutral genes; genes unrelated to a plant-parasitic life style. In earlier studies on distal Tylenchida such as cyst and root-knot nematodes, these pathogens appeared to be unusually well-equipped with non-neutral, cell wall-degrading enzymes [14][15]. The word 'unusually' is appropriate in this context as only a very few herbivores are able to degrade plant cell walls themselves (most of them make use of bacterial endosymbionts). The term 'non-neutral' is used here as the availability of cell wall-degrading enzymes is directly linked to the ability of nematodes to parasitize plants, a derived lifestyle. Various indications have been presented suggesting that nematodes acquired cell wall-degrading enzymes from soil bacteria by horizontal genes transfer [16]. If this is correct, one might wonder whether this is the result of one or multiple horizontal gene transfer events within the order Tylenchida.

The study on one specific category of cell wall degrading enzymes, β-1,4-endoglucanases ('cellulases'), from more basal *Meloidogyne* species and a range of representatives of the family *Pratylenchidae* (Chapter 4) widened our insights in the origin and the diversification of these enzymes in Clade 12. Results presented in Chapter 4 point at a scenario in which cellulases evolved from early lesion nematodes to those that are found now in more distal *Heteroderidae* and *Meloidogynidae*. Three types of catalytic domains can be distinguished - A, B and C (see Fig. 1 in Chapter 4) - but the phylogenetic relationships between them is still unresolved. It is noticed that some of the noncoding elements are unique for particular cellulase types. For instance presence of introns 4, 14 and 16 was only observed in a type A catalytic domains. Based on the phylogenetic analysis in Chapter 4, and additionally

phylogenetic analyses on intron distribution (Fig 1; gives good support only for the division into the type A and B catalytic domains.), I propose a model of GHF5 intron-exon evolution within Tylenchida (Fig 2).

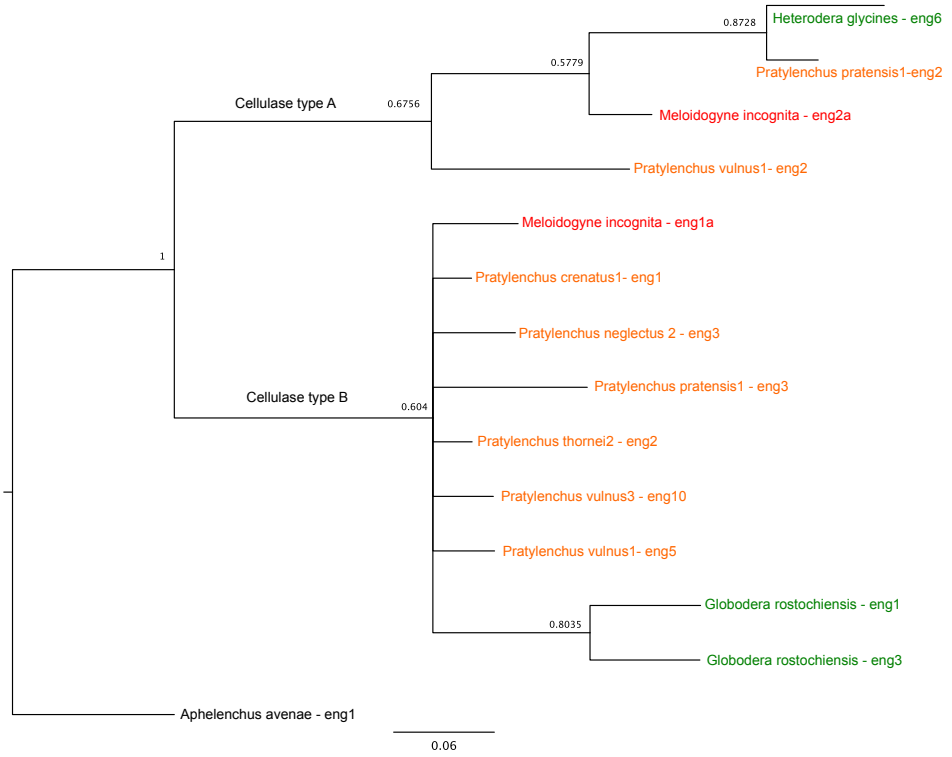


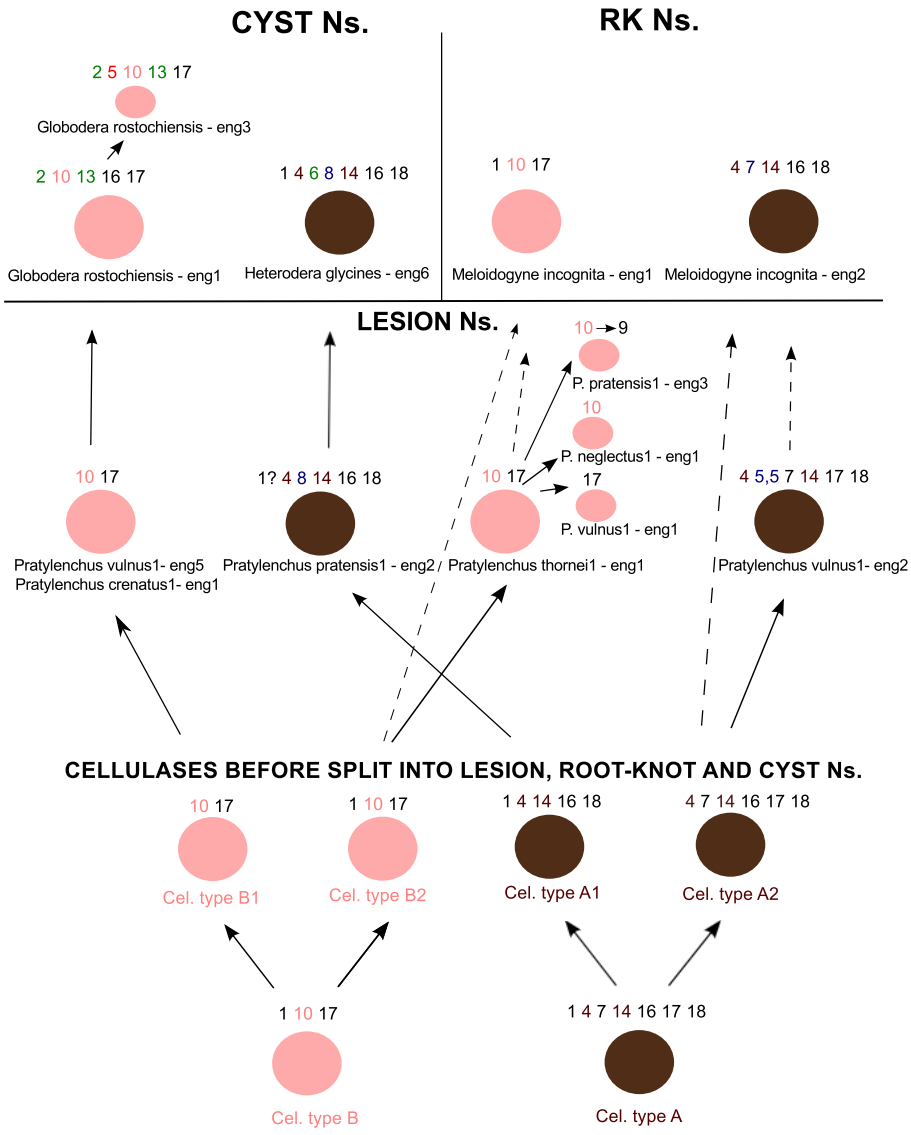
Figure 1. Bayesian tree of GHF5 catalytic domains from members of the nematode order Tylenchida based on sequence intron composition (Intron presence or absence was coded by binary scoring: 1/0).

This evolution model is based on the analysis of GHF5 cellulase catalytic domains (at most CD1-CD6 region) from lesion, root-knot and cyst nematodes. This model assumes occurrence of at least four ancient cellulase subtypes, namely A1, A2, B1 and B2, derived from the primary A and B types. Two main arguments supporting this theory are: first, the bifurcation within both A and B cellulases into root-knot-like ant cyst nematode-like cellulases (Fig 1. Chapter 4) and second, the specific intron composition for each of those four subtypes. The first argument is visualized by the twofold positioning of lesion nematode cellulases (either next to the RKN or in close proximity to the cyst nematodes; Fig 1 from Chapter 4) within both, type A and B cellulases which could imply the *Pratylenchidae* might harbor descendants of not two (like it was suggested in case of cyst and RKNs) but four ancient cellulase copies (Fig. 2). Consequently, *Pratylenchus* cellulases, from each of the two types (A and B) diverged into two

subtypes: A1, A2, B1 and B2. Both of the subtypes of the A type contain introns 4, 14 and 18. However, the A1 one represented by the *Pratylenchus pratensis*1-eng2 clusters with a cyst nematode cellulose from *Heterodera glycines*, and additionally harbors introns 8 and 16 while the subtype A2 (*P. vulnus*1-eng2) clusters with RKN cellulases and contains introns 5.5, 7 and 17.

Subtype B1 of lesion nematodes is represented by cellulases that are positioned next to cyst nematodes, and contain the characteristic introns 10 and 17. The second subtype B2 is associated with RKNs, and consists of cellulase forms that may contain both introns 10 and 17. Cellulases devoid of one or both of these introns can also be found here. Noticeably, the presence of intron 10 is characteristic only for members of the cellulose catalytic type B. Probably this intron has been acquired by an ancient form of this cellulases type. And analogically the same might have happened for introns 4 and 14 that are typical in all representatives of the type A cellulases. On the other hand intron 17 can be found within representatives of both types A and B; and interestingly, it is in phase 0 in all RKNs and lesion nematodes but in phase 1 in all cyst nematodes (suppl. Table 2, chapter 4). According to Ruvinsky and Ward [17] "Changes in intron phase patterns indicate changes occurring in genes, which may or may not affect corresponding proteins." This may indicate that this intron is of ancient origin and that some changes have occurred in the ancestors of lesion and RKNs on the one hand and cyst nematodes on the other. Also intron 1 seems to be of ancient origin: it is characteristic for the RKN group, type B but it interestingly can be also found in the sequence *Heterodera glycines*-eng6 (cellulase type A). *P. pratensis*-eng2 cellulase clustering with the *H. glycines*-eng6 may be also harboring this intron. Unfortunately, this sequence lacks its front part. Assuming that introns might only be very rarely gained at homologous sites in different lineages [17], cellulases from RKNs and *Pratylenuchs* might have shared common ancestors, which contained those introns, but *Pratylenchus* species seem to have lost them. Within type B, intron 16 occurs only in the cyst nematodes, and it is most likely to be of ancient origin because it is present in almost all representatives (excluding *P.vulnus*-eng2) of the type A. Interestingly, introns 7 and 18 are characteristic for type A cellulases but are also present in the outgroup –*Aphelenchus sp.* Intron 7 is in phase 0 in RKNs and the *P. vulnus*-eng2 but in phase 1 in *Aphelenchus sp.*, which indicates that the *Aphelenchoides* sequences might be older and those of type A could be a result of such gene change in their descendants. Consequently, the fact that only type A cellulases share introns with those from *Aphelenchus avenae* could also suggest that the A type is older that the type B cellulases.

As we focused only on an interior part of the catalytic domain, we cannot say much about the evolution of CBM2 including possible domain shuffling. However, we believe that the obtained cellulase fragments can be easily used as a start for the whole genomic cellulase sequences acquisition.



Arrows show a direction of a cellulase gene change. Dashed arrows indicate alternative scenarios.
Digits in different colors represent gradualy acquired introns:
I) Introns 1, 7, 16,17 and 18 are of most ancient origin.
IIa) Introns 4 and 14 - first newly acquired introns of cel. type A.
IIb) Intron 10-first newly acquired intron of cel. type B.
III) Insertion of introns 8 (and 5,5) within one of cel. type A indicates split between RKN like and cyst nematode like celulases.
IV) Introns 2, 13 (type B) and 6 (type A) introns characteristic for evolution of only cyst nematode cellulases.
V) Intron 5 - newest intron in cyst nematodes.

Figure 2. Hypothesis of GHF5 (Cel. = cellulase) evolution (CD1-CD6 fragment) within cyst, root-knot (RK) and lesion nematodes (Ns)

V. Evolution of plant parasitism in most economically important representatives of Tylenchida

With regard to plant parasitic nematodes, Tylenchida is by far the most species-rich order. Apart from this biodiversity, it is also the only lineage where their endoparasitic representatives can be found. An abiding hypothesis says that these sophisticated forms, represented by the root-knot and cyst nematodes, are the final resultants of the evolution from fungal feeders through simple ectoparasites. The phylogenetic analysis based on the SSU rDNA, β-1,4-endoglucanase and *rpb1* gene (Chapter 4 and 5) confirmed earlier studies [6][7][18] implying the origins of more complex *Meloidogyne spp.* from simpler parasitic lesion nematodes. However, this research was based on more distal, economically important tylenchids; therefore, it was not possible to analyze whether this order evolved directly from fungal feeding ancestors. On the other hand, these researches try to find the most probable candidate for the ancestor of the root-knot nematodes by employing in the analysis more basal *Meloidogyne* species. Next to the analyses of phylogenetic trees based on neutral genes (pathogenicity unrelated) a novelty here is the study of the phylogeny of β-1,4-endoglucanase (Chapter 4), a gene which is particularly involved in the parasitic behavior of those nematodes. Although, the β-1,4-endoglucanase analysis and the SSU rDNA tree (Chapter 5) might indicate *Pratylenchus thornei* or its ancestral form as the origin of the root-knot nematodes, this was not confirmed by the *rpb1* tree (Chapter 5). At the same time, it is hard to establish whether root-knot nematodes derive from sexual or asexual ancestor. Males are rarely found among *P. thornei* and *P. neglectus* while *P. penetrans*, *P. pratensis* and *P. vulnus* which have been position at the base of the root-knot nematodes clade in the *rpb1* tree, represent sexual type of reproduction. Additionally, high support for the sexual origin of the *Meloidogyne* is shown by the positioning of the *P. vulnus*1-eng2 sequence in the catalytic domain – type A part of the cellulase tree. This ambiguity may consequently imply that the research of the ancient (a)sexuality of the *Meloidogyne* species should be actually searched in the origins of Pratylenchidae themselves.

VI. Use of β-1,4-endoglucanases and *rpb1* markers in molecular evolution of nematodes

As it has been showed in Chapter 2 for the Dorylaimida group, SSU rDNA does not always give a satisfactory resolution on all phylogenetic levels. It resolves well deep relationships on distantly related genera and species [1][19], but showing to high level of sequence conservation it is not always generating sufficient phylogenetic signal to establish relationship between closely related taxa. In this case other markers are employed. In most cases a switch to a gene encoding the neighboring, slightly more variable Large Subunit ribosomal DNA (LSU rDNA) results in obtaining the desired resolution. The two ribosomal genes show; nevertheless, close evolutionary relatedness. Together with ITS1,

5.8S and ITS2 regions inbetween they reside in a single cistron. However, since gene-based phylogenies not always relate to organismal trees, one or two related genes are often not enough to study evolution of many species. Species information from other, preferably less related genes would be more desirable. In this thesis we employ two less popular phylogenetic markers to investigate closer phylogenetic relationship between economically important root-knot and lesion nematodes. We choose them because some information and sequences were already available for significant number of species and we could use them as a starting point for our research. RNA polymerase II is an enzyme that, similarly to the two ribosomal genes encoding for ribosomal subunits, is involved in DNA - protein information flow processes. Consequently, those genes can also be found in every nematode species but they function on different biological stages. Ribosomes translate mRNA into polypeptides, while RNA polymerase II is an enzyme that catalyzes transcription of DNA to synthetize precursors of mRNA and most snRNA and miRNA. One of the things that differ those two genes is that rDNA genes are translated into intronless rRNA, while RNA polymerase is a protein that on genomic level contains noncoding elements. Together with the fact that for organism it is more important to sustain the protein sequence rather than the genomic one unchanged, may hamper the use of protein-encoding genes as phylogenetic marker. On the other hand RNA polymerase is a single-copy gene [20]. It is advisable to use such sequences as phylogenetic markers because it eliminates the risk of comparing paralogs instead of orthologs. 55 rDNA gene copies have been identified in *C. elegans* genome [21]; however, the process of intrachromosomal homogenization ensures that all the copies are identical. This contrasts with the highly diversified glycoside hydrolase family 5 cellulases, which occurs almost exclusively in plant parasitic nematodes (not identified in *C. elegans*). These cell wall-degrading enzymes demonstrate high level of sequence versatility and on this stage it is hard to distinguish paralogous sequence from the ortologous ones. However, as can be noticed in Fig. 1 of Chapter 4, type A, B and C cellulases are most likely paralogous and additionally one can notice that most cellulases group with an agreement to genus they derive from, which can be informative in terms of the evolution of particular genera.

Concluding remarks

Over the last decade we have seen enormous progress in the establishment of evolutionary relationships between and within major nematode lineages based on molecular data. So far, most attention was paid to two ribosomal genes; the small (SSU) and the large (LSU) subunit of the ribosomal DNA. For the transition of the resulting gene trees into entities that could be referred to as 'close to true organismal trees', multiple genes should be employed. Preferably, rRNA enconding sequences should be combined with protein-coding nuclear

and mitochondrial genes. However, as shown in this PhD thesis, this is easier said than done. Very few genes show a mosaic pattern that allows straightforward amplification from a wide range of nematodes - from Enoplida to Tylenchida - and at the same time harbour sufficient phylogenetic signal to resolve deep as well as more shallow relationships within this speciose phylum. As they are coding for ribozymes, both the small and the large subunit of the rDNA can be amplified from a wide range of nematode species, but for phylum-wide studies only SSU rDNA sequences can be aligned properly (LSU rDNA is too variable). In this thesis a possibility to use other neutral (*rbp1*), and plant pathogenicity-related (GHF5 cellulase) markers is explored, and dozens of primers had to be designed and tested to amplify genes and gene fragments from taxonomically related groups of nematodes. Other have explored possibilities offered by mitochondrial genes such as COI and COII, but so far none of them has the characteristics of SSU rDNA: (1) easily amplifiable from a wide range of taxa, while (2) offering phylogenetic resolution at a wide range of taxonomic levels.

With the advent of affordable next generation sequencing technologies, it will be possible to check the robustness of current phylogenetic schemes. A technogical bottleneck still awaiting a proper solution is the quantity of starting material. It is currently good common practise to use single nematodes (≈ 0.2 ng DNA) as a point of departure for phylogenetic research. After all, this is for most species the only guarantee that a single genotype is used. Once 'whole genome amplification kits' really do what their name suggests, whole genome and or whole transcriptome sequencing approaches will offer enormously powerfull handles to really understand and reconstruct the evolution of this fascinatingly diverse group of animals.

References

1. Holterman M, van der Wurff A, van den Elsen S, van Megen H, Bongers T, Holovachov O, Bakker J, Helder J: **Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown clades**. *Molecular Biology and Evolution* 2006, **23**(9):1792-1800.

2. Van Megen H, Van Den Elsen S, Holterman M, Karssen G, Mooyman P, Bongers T, Holovachov O, Bakker J, Helder J: **A phylogenetic tree of nematodes based on about 1200 full-length small subunit ribosomal DNA sequences**. *Nematology* 2009, **11**(6):927-950.

3. Pisani D, Feuda R, Peterson KJ, Smith AB: **Resolving phylogenetic signal from noise when divergence is rapid: A new look at the old problem of echinoderm class relationships**. *Molecular Phylogenetics and Evolution* 2012, **62**(1):27-34.

4. Knowles LL, Klimov PB: **Estimating phylogenetic relationships despite discordant gene trees across loci: The species tree of a diverse species group of feather mites (Acari: Proctophyllodidae)**. *Parasitology* 2011, **138**(13):1750-1759.

5. Hunt DJ: **Aphelenchida, Longidoridae and Trichodoridae: their systematics and bionomics**. Wallingford, UK: CAB International; 1993.

6. Holterman M, Karssen G, Van den Elsen S, Van Megen H, Bakker J, Helder J: **Small Subunit rDNA-Based Phylogeny of the Tylenchida Sheds Light on Relationships Among Some High-Impact Plant-Parasitic Nematodes & the Evolution of Plant Feeding**. *Phytopathology* 2009, **99**(3):227-235.

7. Bert W, Leliaert F, Vierstraete AR, Vanfleteren JR, Borgonie G: **Molecular phylogeny of the Tylenchina and evolution of the female gonoduct (Nematoda : Rhabditida)**. *Molecular Phylogenetics and Evolution* 2008, **48**(2):728-744.

8. Floyd R, Abebe E, Papert A, Blaxter M: **Molecular barcodes for soil nematode identification**. *Molecular Ecology* 2002, **11**(4):839-850.

9. Okada H, Oba H: **Comparison of nematode community similarities assessed by polymerase chain reaction-denaturing gradient gel electrophoresis (DGGE) and by morphological identification**. *Nematology* 2008, **10**(5):689-700.

10. Donn S, Neilson R, Griffiths BS, Daniell TJ: **A novel molecular approach for rapid assessment of soil nematode assemblages – variation, validation and potential applications.** *Methods in Ecology and Evolution* 2012, **3**:12-23.

11. Yan G, Smiley RW, Okubara PA: **Detection and quantification of *Pratylenchus thornei* in DNA extracted from soil using real-time PCR**. *Phytopathology* 2012, **102**(1):14-22.

12. Vervoort MTW, Vonk JA, Mooijman PJW, van den Elsen SJJ, van Megen HHB, Veenhuizen P, Landeweert R, Bakker J, Mulder C, Helder J: **SSU Ribosomal DNA-Based Monitoring of Nematode Assemblages Reveals Distinct Seasonal Fluctuations within Evolutionary Heterogeneous Feeding Guilds**. *PLoS ONE* 2012, **7**(10):e47555.

13. Neilson R, Donn S, Griffiths B, Daniell T, Rybarczyk KD, Van den Elsen S, Mooyman P, Helder J: **Molecular Tools for Analysing Nematode Assemblages**. In: *Nematodes as environmental indicators*. Edited by Wilson MJ, Kakouli-Duarte T. Wallingford: CABI; 2009: 188-207.

14. Smant G, Stokkermans J, Yan YT, de Boer JM, Baum TJ, Wang XH, Hussey RS, Gommers FJ, Henrissat B, Davis EL *et al*: **Endogenous cellulases in animals: Isolation of beta-1,4-endoglucanase genes from two species of plant-parasitic cyst nematodes**. *Proceedings of the National Academy of Sciences of the United States of America* 1998, **95**(9):4906-4911.

15. Rosso MN, Favery B, Piotte C, Arthaud L, De Boer JM, Hussey RS, Bakker J, Baum TJ, Abad P: **Isolation of a cDNA encoding a beta-1,4-endoglucanase in the root-knot nematode Meloidogyne incognita and expression analysis during plant parasitism**. *Molecular Plant-Microbe Interactions* 1999, **12**(7):585-591.

16. Haegeman A, Jones JT, Danchin EGJ: **Horizontal gene transfer in nematodes: A catalyst for plant parasitism?** *Molecular Plant-Microbe Interactions* 2011, **24**(8):879-887.

17. Ruvinsky A, Ward W: **A gradient in the distribution of introns in eukaryotic genes**. *Journal of Molecular Evolution* 2006, **63**(1):136-141.

18. Subbotin SA, Sturhan D, Chizhov VN, Vovlas N, Baldwin JG: **Phylogenetic analysis of Tylenchida Thorne, 1949 as inferred from D2 and D3 expansion fragments of the 28S rRNA gene sequences**. *Nematology* 2006, **8**:455-474.

19. Blaxter ML, De Ley P, Garey JR, Liu LX, Scheldeman P, Vierstraete A, Vanfleteren JR, Mackey LY, Dorris M, Frisse LM *et al*: **A molecular evolutionary framework for the phylum Nematoda**. *Nature* 1998, **392**(6671):71-75.

20. Longet D, Archibald JM, Keeling PJ, Pawlowski J: **Foraminifera and Cercozoa share a common origin according to RNA polymerase II phylogenies**. *International Journal of Systematic and Evolutionary Microbiology* 2003, **53**(6):1735-1739.

21. Ellis RE, Sulston JE, Coulson AR: **The rDNA of *C. elegans*: Sequence and structure**. *Nucleic Acids Research* 1986, **14**(5):2345-2364.

SUMMARY

Nematodes are probably the most abundant Metazoans on our planet; in soil and sediments these small animals are present in densities of millions of individuals per square meter. If this is true, one might ask why roundworms, the common name of nematodes, are hardly known to the general public. The answer to this question relates to their size, most individuals are smaller than 1 mm, and their colorless nature. As most species live in an environment without light, colors (and eyes) are of no use. In fact nematodes are inconspicuous animals. If people are aware of nematodes, it is because of the damage they inflict on humans and animals such as elephantiasis (*Wucheria bancrofti*) and ascariasis (in humans *Ascaris lubricoides*; in pigs *Ascaris suum*), and on plants such as potato (potato cyst nematode) and tomato (root-knot nematodes). To a far lesser extent it is known that nematodes – actually the majority – are key players in the soil and sediment food web, and as such they can be used as indicators for the biological condition of soils and sediments.

In this PhD thesis I concentrated on terrestrial nematodes belonging to four orders: Dorylaimida (Clade 2 according to Holterman et al. 2006 [1], Van Megen *et al.* 2009 [2]), Mononchida (Clade 2), Aphelenchida (Clades 10 and 12) and Tylenchida (Clade 12), which represent animals of ecological and economical relevance. The orders Dorylaimida and Mononchida are dominated by families with *c-p* values of 4 or 5 (extreme *c*olonizer = 1, extreme *p*ersister = 5; see Bongers 1990 [3]). Members of families belonging to these two order are highly sensitive the environmental disturbances, and as such they are informative as biological indicators. The order Aphelenchida harbors numerous facultative plant-parasitic species. In the absence of a host plant, most of these species are able to feed on fungi as an alternative food source. This is in contrast to the distal representatives of the order Tylenchida that are invariably obligate parasites of higher plants.

Establishment of evolutionary relationships between and within these orders, as well as management of those organisms in soil is hampered by a common reason: the lack of informative morphological characters. In other words, terrestrial nematodes very much look alike. Nematode morphology can be observed microscopically using a high magnification (preferably up to 1,000 times) and requires specialist knowledge. And even when this would be available, it should be noted that several of the important groups lack reliable identification keys. To overcome these problems, there is a need for establishing dependable molecular tools for nematode detection and identification on the one hand, and employment of good phylogenetic markers to resolve their phylogenetic relationships, on the other. Recently, due to the significant accumulation of molecular data, small subunit ribosomal DNA (SSU rDNA)-based assays have been proposed for the identification and quantification of nematode species in complex DNA backgrounds (such as nematode suspensions). Here, it is demonstrated, how such real-time quantitative PCR-based identification and monitoring of nematodes can be applied in practice. On

the other hand, ribosomal genes are also widely used phylogenetic markers for resolving, especially deep, phylogenetic relationship between nematodes. However, it is realized that multiple independent phylogenetic markers are required for the transition of current rDNA-based gene trees into general organismal trees. Moreover, the application of additional phylogenetic markers allows for a better resolution of the relationships between recently evolved clades. In order to study phylogeny of agriculturally relevant representatives of the order Tylenchida, β-1,4-endonucleases (cellulases belonging to glycoside hydrolase family 5) and the largest subunit of RNA polymerase II (RPB1) have been employed next to SSU rDNA data.

Stress-sensitive nematode orders Dorylaimida and Mononchida have a high potential for soil health assessment. The SSU rDNA-based analysis of these two orders resulted in two highly distinct phylogenies. Relationships among the Mononchida, an order dominated by carnivorous nematodes, were to some extent in accordance with the classical nematode systematics. It is noted that the families Mylonchulidae, Mononchidae and Anatonchidae are not monophyletic. Nevertheless, it was possible to design (combinations of) family-specific primers for rDNA-based molecular detection. On the contrary, resolution of the SSU rDNA tree of the Dorylaimida was extremely poor, except for the plant-parasitic family Longidoridae and the mainly predaceous family Nygolaimidae. Analysis of a 1,000 bp fragment of the 5′ region of large subunit (LSU) rDNA resulted in an improved resolution. Twelve subclades (D1-D9 and PP1- PP3) were distinguished and this topology was only in slight agreement with the classical systematics of the suborder Dorylaimina. The poor resolution generated by SSU rDNA sequence analysis within this species-rich suborder is remarkable; it has not been observed in any other suborder in the phylum Nematoda. Possibly, Dorylaimina diversification is the result of rapid speciation events.

A plant-parasitic lifestyle apparently accelerates the rate of change of rDNA genes. This was not only true for the obligate plant-parasitic Longidoridae, but also for the facultative plant-parasitic Aphelenchoididae. Most members of the genus *Aphelenchoides* are fungivores, but a few of them feed on higher plants as well. As they feed on above-ground parts of higher plants they are usually called 'foliar nematodes'. Species such as *Aphelenchoides besseyi*, *A. fragariae* and *A. ritzemabosi* parasitize on ornamental plants in greenhouses and nurseries, and some field crops such as rice or strawberry. Moreover, *A. subtenuis* causes serious damage infecting flower bulbs. Identification of foliar nematode species, and the distinction between plant-parasitic species and other, mostly harmless, fungal feeding representatives of the genus *Aphelenchoides* is hampered by the scarcity of informative morphological characters and lack of well-established systematics. Based on nearly full-length SSU rDNA sequences, a phylogenetic tree was generated, where the four target species appeared as distinct, well-supported, monophyletic groups. The presence of species-specific DNA motifs made it possible to design PCR primers for the detection and quantification of the foliar nematode species in complex DNA backgrounds such as plant material and soil samples.

The order Tylenchida is dominated by obligatory plant-parasitic nematode taxa. Economically high-impact species such as the lesion (*Pratylenchus spp.*), root-knot (*Meloidgyne spp.*) and cyst (*Heterodera, Globodera*) nematodes are positioned in the most distal parts of Clade 12, a speciose clade dominated by the representatives of the order Tylenchida. Within this group I investigated the sequence diversification of cellulases, a non-neutral, plant pathogenicity-related genes. Unlike by far most other animals, nematodes do not depend on endosymbionts for the production of cell wall-degrading enzymes. Among a repertoire of these proteins, glycoside hydrolase family 5 (GHF5) cellulases are best studied [4]. It is hypothesized that they were acquired by one or multiple horizontal gene transfer (HGT) events. Moreover, the nature of the donor - hypothesized to be a plant-parasitic soil bacterium - and the recipient, possibly a bacterivorous nematode is fully unclear. Using a range of primers, partial GHF5 cellulase sequences spanning the core catalytic domain were amplified and sequenced from basal *Meloidogyne,* and a range of *Pratylenchus* and *Hirschmanniella* species. Phylogenetic analysis of more than 100 partial GHF5 cellulase sequences resulted in a division of the enzymes' catalytic domains into three types (A, B, C). Type B was numerically dominant, and notably the *P. thornei* cellulase was positioned sister to all type B root-knot nematode cellulases. Moreover, the overall topology of the catalytic domain B-type showed remarkable resemblance with trees based on rDNA sequences. This analysis suggests that most likely the cellulases were passed on by ancestors of a family nowadays known as the Pratylenchidae, and root-knot and cyst nematodes did not acquire these genes directly by lateral genes transfer.

To further elucidate the relationship between a part of the family Pratylenchidae (namely the subfamilies Pratylenchinae and Hirschmanniellinae) and the Meloidogynidae, two neutral (= pathogenicity-unrelated) genes were taken into consideration: SSU rDNA and a part of the largest subunit of the RNA polymerase II gene (*rpb1*). Both morphological [5] and molecular data [6] seem to point at root-knot nematodes being a subclade branching from the migratory endoparasites *Pratylenchidae*. Extension of the SSU rDNA data set - more sequences from a broader range of species – did not result in a well-resolved relationship between the Pratylenchidae and the Meloidogynidae. A switch to another gene that was previously exploited to investigate relationships within the genus *Meloidogyne* [7], a fragment of the largest subunit of RNA polymerase II sequences, did not provide us with more robust information about the evolutionary transition between lesion and root-knot nematodes. The genus *Pratylenchus* comprises more than 100 species. It is referred to as a stenomorphic genus since only a few subtle characteristics are used for species identification. In the current study, only a subset of these species was taken to consideration; predominantly species that are relatively well-characterized as pathogens in agro-ecosystems. It was postulated that the *Pratylenchus* species closest to the basal root-knot nematodes should be sought among the less well-known and agronomically less relevant lesion nematode species.

References

1. Holterman M, van der Wurff A, van den Elsen S, van Megen H, Bongers T, Holovachov O, Bakker J, Helder J: **Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown clades**. *Molecular Biology and Evolution* 2006, **23**(9):1792-1800.

2. Van Megen H, Van Den Elsen S, Holterman M, Karssen G, Mooyman P, Bongers T, Holovachov O, Bakker J, Helder J: **A phylogenetic tree of nematodes based on about 1200 full-length small subunit ribosomal DNA sequences**. *Nematology* 2009, **11**(6):927-950.

3. Bongers T: **The maturity index - an ecological measure of environmental disturbance based on nematode species composition**. *Oecologia* 1990, **83**(1):14-19.

4. Haegeman A, Jones JT, Danchin EGJ: **Horizontal gene transfer in nematodes: A catalyst for plant parasitism?** *Molecular Plant-Microbe Interactions* 2011, **24**(8):879-887.

5. Bert W, Leliaert F, Vierstraete AR, Vanfleteren JR, Borgonie G: **Molecular phylogeny of the Tylenchina and evolution of the female gonoduct (Nematoda : Rhabditida)**. *Molecular Phylogenetics and Evolution* 2008, **48**(2):728-744.

6. Holterman M, Karssen G, Van den Elsen S, Van Megen H, Bakker J, Helder J: **Small Subunit rDNA-Based Phylogeny of the Tylenchida Sheds Light on Relationships Among Some High-Impact Plant-Parasitic Nematodes & the Evolution of Plant Feeding**. *Phytopathology* 2009, **99**(3):227-235.

7. Lunt DH: **Genetic tests of ancient asexuality in root knot nematodes reveal recent hybrid origins**. *BMC Evolutionary Biology* 2008, **8**(1).

SAMENVATTING

Nematoden vormen naar alle waarschijnlijkheid de meest talrijke diergroep op aarde; in bodems en in sedimenten zijn deze kleine dieren aanwezig in dichtheden van miljoenen individuen per vierkante meter. Als dit inderdaad zo is, dan kan men zich afvragen waarom deze diergroep niet bij een breder publiek bekend is. Het antwoord is gelegen in de beperkte grootte, meestal minder dan 1 mm, en in de afwezigheid van kleuren. Kleur is in dit geval niet zinvol, aangezien de meeste nematoden leven in de afwezigheid van licht. Nematoden zijn eigenlijk zeer onopvallende dieren. Als mensen er al ooit van gehoord hebben, dan is dit meestal in relatie tot parasieten van dieren en mensen zoals *Wucheria bancrofti*, de veroorzaker van elefantiasis, en *Ascaris lubricoides* en *A. suum*, de veroorzakers van ascariasis bij respectievelijk mensen en varkens. Daarnaast zijn er nog enkele breed bekende parasieten op veel verbouwde gewassen zoals aardappel (aardappelcystenaaltjes) en tomaat (wortelknobbelaaltjes). Echter, het is niet algemeen bekend dat de niet-parasitaire nematoden - veelal de meerderheid - een essentiële rol spelen in het bodemvoedselweb. Deze groep wordt gebruik als indicator voor de biologisch gezondheid van bodems en sedimenten.

In dit proefschrift heb ik mij geconcentreerd op terrestrische nematoden die behoren tot vier ordes: Dorylaimida (Clade 2 volgens Holterman *et al*. 2006 [1], Van Megen et al. 2009 [2]), Mononchida (Clade 2), Aphelenchida (Clades 10 en 12) en Tylenchida (Clade 12), die rondwormen representeren van economische en/of ecologische importantie. The ordes Dorylaimida en Mononchida worden gedomineerd door families met *c-p* waarden van 4 of 5 ("extreme colonizer" = 1, "extreme persister" = 5; see Bongers 1990 [3]). Leden van de families die tot deze ordes behoren zijn sterk gevoelig voor milieuverstoringen, en zijn daarom informatief als bio-indicator. De orde Aphelenchida behelst een groot aantal facultatieve plantenparasieten. In afwezigheid van een waardplant kunnen deze soorten zich veelal voeden op een alternatieve voedselbron, in dit geval schimmels. Dit in tegenstelling tot de distale vertegenwoordigers van de vierde orde, de Tylenchida, waarvan de leden zonder uitzondering obligate plantenparasieten zijn.

De vaststelling van evolutionaire relaties binnen en tussen deze ordes evenals de manipulatie van deze dieren in de bodem wordt geremd om een gemeenschappelijke reden: het gebrek aan informatieve morfologische kenmerken. Met andere woorden, terrestrische nematoden lijken erg veel op elkaar. De morfologie van nematoden wordt veelal microscopisch bekeken (bij een 1000 maal vergroting), en identificatie van deze dieren vereist specialistische kennis. Zelf wanneer dit goed zou werken is soort-identificatie voor sommige groepen lastig door het ontbreken van robuuste identificatie sleutels. Om dit te ondervangen zijn adequate moleculaire gereedschappen nodig. Deze kunnen zowel dienen voor soorts-detectie en identificatie, als voor de ontrafeling van fylogenetische relaties.

Dankzij de beschikbaarheid van een brede moleculaire data set is het gebruik van het 'small subunit' ribosomaal DNA in zwang geraakt voor de

identificatie en de kwantificering van nematoden soorten tegen complexe DNA achtergronden (zoals bijvoorbeeld een nematodensuspensie). In dit proefschrift laten we zien hoe 'real time' (kwantitatieve) PCR in de praktijk gebruikt kan worden voor identificatie en monitoring doeleinden. Aan de andere kant kan ditzelfde gen worden toegewend als fylogenetisch merker, in het bijzonder voor de vaststelling van diepe fylogenetische relaties. Echter, het is duidelijk dat voor het maken van organismale bomen, meerdere fylogenetische merkers gebruikt moeten worden. Daarnaast zou het gebruik van extra merkers kunnen resulteren in een lokaal verbeterde resolutie. Teneinde tot een beter beeld te komen aangaande de relaties tussen agronomisch relevante leden van de orde Tylenchida hebben we ervoor gekozen om, naast SSU rDNA sequenties, gebruik te maken van β-1,4-endonucleases (cellulases die behoren tot glycoside hydrolase familie 5) en het grootste subunit van RNA polymerase II (RPB1).

De stress-gevoelige ordes Dorylaimida and Mononchida zijn potentieel erg bruikbaar voor het inschatten van biologische bodemkwaliteit. Fylogenetisch analyse van deze twee groepen met behulp van SSU rDNA leidde tot twee zeer verschillende fylogenieën. De relaties tussen de Mononchida, een orde die gedomineerd wordt door carnivore aaltjes, komt voor een aanzienlijk deel overeen met de klassieke nematode systematiek. Hierbij moet worden aangetekend dat de families Mylonchulidae, Mononchidae en Anatonchidae niet monofyletische lijken te zijn. Desalniettemin was het mogelijk om (combinatie van) PCR primers te ontwikkelen voor de detective van deze families. Met uitzondering de plantenparasitaire familie Longidoridae en de voornamelijk carnivore familie Nygolaimidae, geldt dit niet voor de op SSU rDNA gebaseerde Dorylaimida boom. Analyse van een 1.000 bp groot fragment van het large subunit (LSU) van het rDNA resulteerde in een veel betere resolutie. Twaalf subclades (D1-D9 en PP1- PP3) konden worden onderscheiden, en deze indeling was maar in beperkte mate in overeenstemming van de klassieke systematiek binnen de suborder Dorylaimina. De magere resolutie die het SSU rDNA gen opleverde in deze soortrijke orde is opmerkelijk; dit was nog niet eerder waargenomen voor een nematoden orde. Mogelijkerwijs is deze magere resolutie het resultaat van een snelle speciatie.

Een plantenparasitaire levensstijl lijkt de veranderingssnelheid van rDNA genen te bevorderen. Dit geldt niet alleen voor de obligaat plantenparasitaire Longidoridae maar ook voor de facultatief plantenparasitaire Aphelenchoididae. De meeste leden van het genus *Aphelenchoididae* voeden zich op schimmels, maar een aantal van hen kan zich ook voeden op hogere planten. Aangezien zij zich voeden op bovengrondse plantendelen worden deze groep wel aangeduid van de bladaaltjes. Soorten zoals *Aphelenchoides besseyi*, *A. fragariae* and *A. ritzemabosi* worden aangetroffen als parasieten van sierplanten in kassen, en in sommige open-grond gewassen zoals rijst en aardbei. Voor *A. subtenuis* geldt dat ze aanziende schade kunnen berokkenen aan bolgewassen. De identificatie van bladaaltjes en, meer in het bijzonder, het onderscheid tussen deze groep en verwante, niet-schadelijke schimmel-etende *Aphelenchoides* soorten wordt bemoeilijkt door een gebrek aan informatieve

kenmerken alsmede de instabiele systematiek van deze groep. Op basis van SSU rDNA sequenties werd een fylogenetische boom van deze groep gemaakt en de vier doelorganismen komen hierin naar voren als aparte monofyletische groepen. De aanwezigheid van soort-kenmerkende DNA motieven maakte het mogelijk om PCR primers te ontwikkelen voor de specifieke detective van bladaaltjessoorten tegen complexe DNA achtergronden zoals plant material of bodemmonsters.

De orde Tylenchida wordt gedomineerd door een groot aantal obligaat plantenparasitaire taxa. Economisch belangrijke soorten zoals wortellesie (*Pratylenchus spp.*), wortelknobbel (*Meloidgyne spp.*) en cystenaaltjes (*Heterodera, Globodera*) bevinden zich in de meest distale gedeeltes van Clade 12, een soortrijke clade die gedomineerd wordt door representanten van de orde Tylenchida. Binnen deze groep heb ik de sequentie diversificatie bekeken van cellulases; niet-neutrale, aan plant pathogeniciteit gerelateerde genen. Anders dan bij deze meeste dieren is de aanmaak van celwand-afbrekende enzymen bij nematoden niet afhankelijk van de aanwezigheid van endosymbionten. Binnen het spectrum van celwandafbrekende eiwitten zijn de cellulases van glycoside hydrolase familie 5 (GHF5) het best bestudeerd [4]. Aangenomen wordt dat nematoden deze genen verworven hebben middels een of meerdere horizontale genoverdracht gebeurtenissen. Echter, de aard van de donor, waarschijnlijk een plantenparasitaire bodembacterie, en de ontvanger, waarschijnlijk een bacterie-etende nematodensoort, zijn vooralsnog onbekend.

Met behulp van een aantal PCR primers zijn GHF5 cellulase fragmenten geïsoleerd en gekarakteriseerd van *Meloidogyne, Pratylenchus* en *Hirschmanniella* soorten die het catalytische domein van dit gen omvatten. Fylogenetische analyse van meer dan 100 GHF5 cellulase fragmenten resulteerde in een onderverdeling van de catalytische domeinen in drie groepen (A, B, en C). Type B kwam het meeste voor, en opmerkelijk genoeg stond een *P. thornei* cellulase aan de basis van alle type B cellulases van de wortelknobbelaaltjes. Daarnaast, bleek dat de topologie van de type B cellulases een opmerkelijk overeenkomst vertoonde met een boom die gebaseerd was op rDNA data. Deze analyse suggereert dat cellulases niet rechtstreeks verkregen zijn door wortelknobbel- of cystenaaltjes maar dat ze zijn doorgegeven door voorouders van een groep die we nu kennen als de Pratylenchidae.

Voor een verdere opheldering van de relatie tussen een deel van de family Pratylenchidae (namelijk de subfamilies Pratylenchinae en Hirschmanniellinae) en de Meloidogynidae, werden twee neutrale (= niet aan parasitisme gerelateerde) genen nader bestudeerd: SSU rDNA en een deel van het grootste subunit van het RNA polymerase II gen (*rbp1*). Zowel morfologische [5] als moleculaire data [6] suggereren dat wortelknobbelaaltjes een aftakking vormen van de mobiele endoparasieten binnen de Pratylenchidae. Uitbreiding van de SSU rDNA data set - meer sequenties van een bredere range aan soorten – bracht niet meer gedetailleerd inzicht in de relatie tussen de Pratylenchidae en de Meloidogynidae. Beschouwing van een ander gen dat eerder met succes was toegepast binnen het genus *Meloidogyne* [7], een fragment

van het grootste subunit van RNA polymerase II, verschafte ook niet meer inzicht in de transitie van vroege wortellesie-aaltjes naar wortelknobbelaaltjes.

Het geslacht *Pratylenchus* omvat meer dan 100 soorten. Het wordt aangeduid al een stenomorf geslacht waarmee wordt gezegd dat deze soorten qua morfologie erg op elkaar lijken, en dat soorts-identificatie gebaseerd is op een klein aantal, zeer subtiele kenmerken. In dit onderzoek is slechts een deel van de beschreven soorten meegenomen, in meerderheid de soorten die van belang zijn in agro-ecosystemen. Op basis van de huidige data veronderstellen we de *Pratylenchus* soort die het nauwst verwant is aan de meest basale representanten van de wortelknobbelaaltjes gezocht moet worden binnen de minder bekende, agronomisch nauwelijks relevante wortellesie-aaltjes soorten.

Referenties

1. Holterman M, van der Wurff A, van den Elsen S, van Megen H, Bongers T, Holovachov O, Bakker J, Helder J: **Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown clades**. *Molecular Biology and Evolution* 2006, **23**(9):1792-1800.

2. Van Megen H, Van Den Elsen S, Holterman M, Karssen G, Mooyman P, Bongers T, Holovachov O, Bakker J, Helder J: **A phylogenetic tree of nematodes based on about 1200 full-length small subunit ribosomal DNA sequences**. *Nematology* 2009, **11**(6):927-950.

3. Bongers T: **The maturity index - an ecological measure of environmental disturbance based on nematode species composition**. *Oecologia* 1990, **83**(1):14-19.

4. Haegeman A, Jones JT, Danchin EGJ: **Horizontal gene transfer in nematodes: A catalyst for plant parasitism?** *Molecular Plant-Microbe Interactions* 2011, **24**(8):879-887.

5. Bert W, Leliaert F, Vierstraete AR, Vanfleteren JR, Borgonie G: **Molecular phylogeny of the Tylenchina and evolution of the female gonoduct (Nematoda : Rhabditida)**. *Molecular Phylogenetics and Evolution* 2008, **48**(2):728-744.

6. Holterman M, Karssen G, Van den Elsen S, Van Megen H, Bakker J, Helder J: **Small Subunit rDNA-Based Phylogeny of the Tylenchida Sheds Light on Relationships Among Some High-Impact Plant-Parasitic Nematodes & the Evolution of Plant Feeding**. *Phytopathology* 2009, **99**(3):227-235.

7. Lunt DH: **Genetic tests of ancient asexuality in root knot nematodes reveal recent hybrid origins**. *BMC Evolutionary Biology* 2008, **8**(1).

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Till next time!

Yours,
Kasia

Curriculum vitae

Katarzyna Diana Rybarczyk-Mydłowska was born 11.08.1982 in Warsaw, Poland. In 2006 she graduated from Warsaw University of Life Sciences, faculty of Horticulture and Landscape Architecture. Her master thesis entitled "Construction of transgenic tomatoes var. Micro-Tom with silenced cytochrome P450 gene" concerned a functional investigation of plant genes involved in responses to nematode infection. The thesis was awarded the second place price at the faculty. During the academic year 2005/2006, she participated the "Erasmus" student exchange programme at Wageningen University in The Netherlands. During this time, in the laboratory of Nematology, she was discovering the other side of the plant-nematode interactions story, completing a minor thesis entitled "Ribosomal DNA-based detection of the root-knot nematode species *Meloidogyne naasi* and *M. minor*". After graduating from the Polish university she came back to Wageningen (2006) and was involved in research focused on molecular detection of nematodes from orders Dorylaimida and Mononchida. Subsequently, she started her PhD research October, 2007, the result of which is this thesis. Currently, she continues her adventure with nematodes at Museum and Institute of Zoology of the Polish Academy of Sciences in Warsaw.

LIST OF PUBLICATIONS

Refered journals and book chapters

Holterman, M.; **Rybarczyk, K.**; Elsen, S. van den; Megen, H. van; Mooijman, P.; Santiago, R.; Bongers, T.; Bakker, J.; Helder, J. (2008) *A ribosomal DNA-based framework for the detection and quantification of stress-sensitive nematode families in terrestrial habitats* Molecular Ecology Resources *8. - p. 23 – 34*

Helder, J.; Elsen, S. van den; Mooijman, P.; **Rybarczyk, K.;** Pomp, H.; Holterman, M.; Megen, H. van; Bongers, T.; Bakker, J. (2008) *Het gebruik van DNA-barcodes voor de routinematige analyse van nematodengemeenschappen als indicator voor biologische bodemkwaliteit* Gewasbescherming 39 (Suppl.) - p. 8 S - 9 S.

Neilson, R.; Donn, S.; Griffiths, B.; Daniell, T.; **Rybarczyk, K.**; Elsen, S. van den; Mooijman, P.; Helder, J. (2009) *Molecular Tools for Analysing Nematode Assemblages*; Chapter 8 in: *Nematodes as Environmental Indicators,* Eds. Wilson, M.J.; Kakouli-Duarte, T.; CAB International, p. 188-207

Rybarczyk-Mydłowska, K.; Mooyman, P.; Megen, H. van; Elsen, S. van den; Vervoort, M.; Veenhuizen, P.; Doorn J. van; Dees, R.; Karssen, G.; Bakker, J.; Helder, J. (2012) *Small Subunit Ribosomal DNA-Based Phylogenetic Analysis of Foliar Nematodes (Aphelenchoides spp.) and Their Quantitative Detection in Complex DNA Backgrounds* Phytopathology Vol. 102 No.12 pp. 1153-1160

Rybarczyk-Mydłowska, K.; Maboreke, H.; Megen, H. van; Elsen, S. van den; Mooijman, P.; Smant, G.; Bakker, J.; Helder, J. (2012) *Rather than by direct acquisition via lateral gene transfer, GHF5 cellulases were passed on from early Pratylenchidae to root-knot and cyst nematodes* BMC Evolutionary Biology 2012, 12:221

Rybarczyk-Mydłowska, K.; Megen, H. van; Elsen, S. van den; Mooijman, P.; Karssen, G.; Bakker, J.; Helder, J. (2013) *Both SSU rDNA and RNA polymerase II data recognize that root-knot nematodes arose from migratory Pratylenchidae, but probably not from one of the economically high-impact lesion nematodes* Nematology 2013 00 pp. 1-12

Conference and symposia proceedings

Holterman, M.; **Rybarczyk, K.**; Elsen, S. van den; Megen, H. van; Mooijman, P.; Santiago, R.; Bongers, T.; Bakker, J.; Helder, J. (2007) *A framework for the detection and quantification of stress-sensitive nematode families in terrestrial habitats using ribosomal DNA* Zeist, The Netherlands: Netherlands Scientific Symposium Soil & Water, 06-07.06.2007

Rybarczyk, K.; Megen, H. van; Elsen, S. van den; Mooijman, P.; Smant, G.; Helder, J. (2009) *Towards the identification of one or multiple common ancester(s) of cellulase genes within the nematode order Tylenchida* Toledo, Spain: COST872, NEMAGENICS Exploiting genomics to understand plant-nematode interactions, 25-28.05.2009

Vervoort, M.; Mooijman, P.; Megen, H. van; Elsen, S. van den; **Rybarczyk-Mydłowska, K.**; Vonk, J.; Mulder, C.; Ruiter, P. de; Bakker, J.; Helder, J. (2010) *Use of DNA barcodes for the monitoring of seasonal fluctuations in nematode communities* Amsterdam, The Netherlands: 3rd National Ecogenomics Day, 2010-04-21

Rybarczyk-Mydłowska, K.; Megen, H. van; Elsen, S. van den; Mooijman, P.; Smant, G.; Bakker, J.; Helder, J. (2010) *In search of the evolutionary origin of cellulase genes within the plant parasite dominated nematode order Tylenchida* Hinxton, Cambridge, UK: Scientific Conference on Evolutionary Biology of Caenorhabditis and other Nematodes, 5-8.06.2010

Helder, J.; Karssen, G.; Bert, W.; Megen, H. van; Elsen, S. van den; Mooijman, P.; **Rybarczyk-Mydłowska, K.**; Bakker, J. (2010) *Root-knot nematodes - a success story among the plant parasitic nematodes - constitute & do constitute an ancient asexual clade* Lisbon, Portugal: COST 872 Workshop & MC Meeting on NEMAGENICS Exploiting genomics to understand plant-nematode interactions, 24-27.05.2010

Maboreke, H.; **Rybarczyk-Mydłowska, K.**; Helder, J. (2011) *Cellulase diversity in lesion nematodes* Wageningen, The Netherlands: Arbeitskreise Nematologie und Freilebende Nematoden, 15.03.2011

Rybarczyk-Mydłowska, K.; Megen, H. van; Elsen, S. van den; Mooijman, P.; Helder, J. (2011) *Use of RNA polymerase II sequences to elucidate the evolutionary origin of root-knot nematodes* Gent, Belgium: 63rd International Symposium on Crop Protection, 24.05.2011

Rybarczyk-Mydłowska, K.; Megen, H. van; Elsen, S. van den; Mooijman, P.; Smant, G.; Bakker, J.; Helder, J. (2011) *Phylogenetic relationships among agronomically important representatives of the order Tylenchida based on multiple phylogenetic markers* Corvallis, OR, USA: The Society of Nematologists 50th Anniversary Meeting 17-20.07.2011

Vervoort, M.; Mooijman, P.; Megen, H. van; Elsen, S. van den; **Rybarczyk-Mydłowska, K.**; Vonk, J.; Mulder, C.; Ruiter, P. de; Bakker, J.; Helder, J. (2011) *DNA Barcode-based tool for the monitoring of nematode assemblages* Corvallis, OR, USA: The Society of Nematologists 50th Anniversary Meeting 17-20.07.2011

PE&RC PhD Training Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (5 ECTS)

- Interactions between terrestrial nematodes and soil bacteria (2007); presentation during nematode identification course: Nematodes in environmental studies (2008)
- β-1,4-endoglucanase as a non-neutral (pathogenicity related) phylogenetic marker in nematode evolution; presentation during EEDG meeting (2010)

Post-graduate courses (3.6 ECTS)

- Molecular phylogenies: reconstruction & interpretation; EPS (2010)
- Basic statistics; PE&RC (2010)
- Bayesian statistics; PE&RC (2011)

Deficiency, refresh, brush-up courses (3 ECTS)

- Microbial ecology; WUR (2007)
- Nematodes in environmental studies; identification course; Laboratory of Nematology, WUR (2008)

Competence strengthening / skills courses (4.5 ECTS)

- Academic writing II; CENTA (2007)
- PhD Competence assessment; WGS (2008)
- Entrepreneurial boot camp; DAFNE, Wageningen / University of Wisconsin, Madison, USA (2008)
- PhD Scientific writing; CENTA (2010)
- Mobilising your scientific network; WGS (2011)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.4 ECTS)

- PE&RC Weekend (2008)
- PE&RC Days (2007-2011)

Discussion groups / local seminars / other scientific meetings (7 ECTS)

- Experimental Evolution Discussion Group (EEDG) (2007-2011)
- Farewell symposium of PAA Loof and AMT Bongers (2007)
- Current Themes in ECOLOGY: Plants-insects-microbes: An ecological dance of three (2008)
- Ecogenomic Discussion Group (2009)
- Dies Natalis 91st Celebration Wageningen University & Research Centre; Darwin's Legacy: Biodiversity as Natural Capital (2009)
- 2nd National Ecogenomics Day; Netherlands Ecogenomics Research Organisation (NERO) (2009)

International symposia, workshops and conferences (8.8 ECTS)

- ARB Workshop; Ribocon GmbH, Bremen Germany (2008)
- 3rd Annual Meeting, Nemagenics: exploiting genomics to understand plant-nematode interactions; poster presentation; Toledo, Spain (2009)
- Evolutionary Biology of Caenorhabditis and Other Nematodes; poster presentation; Cambridge, England (2010)
- 63rd International Symposium on Crop protection (ISCP) and Nemagenics: exploiting genomics to understand plant-nematode interactions; poster presentation; Gent, Belgium (2011)
- The Society of Nematologists 50th Anniversary Meeting; oral presentation; Corvallis, Oregon, USA (2011)

Lecturing / supervision of practical's / tutorials; (1 ECTS)

- Biology and management of plant pathogens insects and weeds I (2009/2010)

Supervision of 1 MSc student (3 ECTS)

- Cellulase diversity in lesion nematodes

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