Phylogenetic relationships within the phylum Nematoda as revealed by ribosomal DNA, and their biological implications

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Chapter 1

Introduction

Introduction

Nematodes

Nematodes – members of the phylum Nematoda – can be considered as a success story within the Metazoa: they are speciose and – probably - the most numerous group of multicellular animals on our planet. Nematodes are present in virtually all terrestrial, freshwater and marine habitats (Bongers and Ferris 1999). Usually, these worm-shaped animals are about 0.5-1.5 mm long, although animal parasitic nematodes are often longer (the current record being 8 meter for *Placentonema gigantissima*, a parasite recovered from the placenta of a sperm whale; Gubanov 1951). Nematodes are trophically diverse; they may feed on bacteria, fungi/oomycetes, algae and protozoa, other nematodes or on a combination of these (omnivores), or live as facultative or obligatory parasites of plants or animals. As they are abundant, ubiquitous and occupy several trophic levels, they play an important role in the soil food web (De Ruiter, Neutel, and Moore 1998). Nematode parasites of humans affect billions of individuals; just the four most prevalent soil-transmitted species alone (*Ascaris lumbricoides*, *Trichuris trichura* and the hookworms, *Necator americanus* and *Ancylostoma ceylanicum*) infect nearly 3 billion people (WHO 2006). Plant parasites such as cyst, root knot and lesion nematodes cause losses estimated at US$ 80 billion annually (Agrios 2005).

Nematode systematics

Keeping the medical, ecological and economical importance of this animal phylum in mind, it is remarkable to see that nematode systematics is far from established. It has a long history of constant revision, and there may be as many classifications as there are nematode taxonomists (≈ a few dozen). One of the first phylum wide classifications was proposed by Chitwood and Chitwood (1933). They divided the phylum into two classes, the Phasmidia and

Aphasmidia, later renamed to Secernentea and Adenophorea respectively. This was based mainly on the fact that the Secernentea share several characters, including the presence of phasmids, small sensory organs on the tail. Although it was already recognized at the time that the Adenophorea did not form a natural group, the division of the Nematoda into these two groups persisted for a long time (Maggenti 1963; De Coninck 1965; Siddiqi 1983; Maggenti 1983). The first to propose a tripartite system was Andrássy (1976), who divided the Adenophorea into the Penetrantia and the Torquentia (names referring to the amphid structure). However, for various valid reasons, few people adhered to this system. The first person to apply cladistic principles to nematode systematics was Lorenzen (1981). He also recognized that the Adenophorea were not a monophyletic group, but could not provide an alternative. Also at lower taxonomic levels (order, family and genus level), systematics were far from stable (De Ley and Blaxter 2002). The main reason for this is that - although nematodes are ecologically and physiologically very diverse - their conserved morphology and small size resulted in a paucity of observable, phylogenetically informative characters. Furthermore many characters display a convergent evolution. In recent years DNA sequence data have brought a revival to the field of systematics (see *e.g.* Blaxter *et al.* 1998; Mullin, Harris, and Powers 2005; Subbotin *et al.* 2006; Meldal *et al.* 2007). The first major classification to incorporate both morphological and molecular phylogenetic information is that of De Ley and Blaxter (2002).

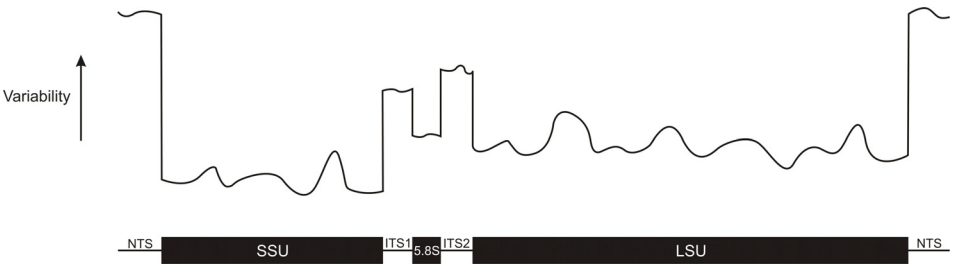

Figure 1.1. A fictive view on sequence variation in the rDNA cistron.

Molecular phylogeny and the small subunit ribosomal DNA gene

With the advent of DNA technology new information has become available for nematode phylogenetics. The sequence of a single gene usually spans hundreds if not thousands of nucleotides, and in principle all those characters can be used for phylogenetic analysis (although not all characters will be informative). Not every DNA sequence offers useful information however. If a gene evolves too fast, phylogenetic signals once present will have been obscured by subsequent mutations. On the other hand, if a gene evolves at a very slow rate, not enough mutations may have accumulated to trace the steps of evolution. This implies that a relatively conserved gene is useful for the reconstruction of deep phylogenetic relationships but could be too conserved to recover phylogenetic relationships at a species level and *vice versa*.

Especially among invertebrates, the small subunit ribosomal DNA (SSU rDNA) gene – coding for SSU rRNA - is frequently used to deduce deep phylogenetic relationships (Aguinaldo *et al.* 1997; Blaxter *et al.* 1998; Kjer 2004). Because of their vital role in the assembly of proteins in the ribosomes, there is a strong selection on the ribosomal DNA genes. As a consequence these genes – or at least parts thereof – are very conserved. Among the ribosomal RNA encoding genes the SSU rDNA is most conserved (Figure 1.1; Dorris, De Ley, and Blaxter 1999). Ribosomal DNA genes are usually present in multiple copies (the *Caenorhabditis elegans* genome harbours 55 copies; Ellis, Sulston, and Coulson 1986) and this implies that a relatively small quantity of starting material (*e.g.* a single nematode ≈ 0.2 ng DNA) is sufficient for a polymerase chain reaction (PCR)-based amplification. Normally it would be inadvisable to use a multicopy gene in phylogenetics because there is a substantial risk of comparing paralogs instead of orthologous gene copies. However, intrachromosomal homogenization ensures that when a mutation takes place in a copy of the SSU rDNA gene, it is either removed or copied into the other copies of the SSU rDNA gene (Dover *et al.* 1993; Liao *et al.* 1997).

In most organisms the SSU rDNA is very conserved and as such mainly suitable for the unravelling of deep phylogenetic relationships. For some time, SSU rDNA in nematodes was suggested to be 2-3 times more variable than in most other Metazoa, and in their study on the phylogenetic position of the

Table 1.1. P-values for the relative rate test performed with RRTree (Robinson-Rechavi and Huchon 2000) comparing the evolutionary rates within the Ecdysozoa using slowly evolving nematode sequences. The tree and sequences displayed in Fig. 1.3 were used, *Antedon serrata* (Deuterostomia) was used as outgroup. Numbers in bold marked with an * are significant (P ≤ 0.0024 using a Bonferroni correction).

	Nematoda	Nemato-morpha	Priapulida	Kino-rhyncha	Tardigrada	Arthro-poda
Nematomorpha	0.679460					
Priapulida	**0.000120***	**0.001151***				
Kinorhyncha	0.726136	0.475024	**5.30E-05***			
Tardigrada	0.028549	0.019499	**1.00E-07***	0.144630		
Arthropoda	0.003604	0.027101	0.216917	0.005719	**8.61E-07***	
Lophotrochozoa	**3.87E-05***	**0.000706***	0.883598	**5.12E-05***	**1.00E-07***	0.151677

arthropods Aguinaldo and co-workers carefully selected a nematode species with a relatively low SSU rDNA substitution rate (Aguinaldo *et al.* 1997; Table 1.1). The SSU rDNA substitution rate within the phylum Nematoda appears to be relatively variable, and for taxa with a high substitution rate this gene could be suitable for the resolution of lower phylogenetic relationships as well.

The Ecdysozoa

The phylum Nematoda is thought to be a member of the superphylum Ecdysozoa, a group of animals comprising all moulting animals (the Arthropoda, Nematomorpha, Tardigrada, Priapulida, Kinorhyncha, Onychophora and the Loricifera; (Halanych 2004; Fig.1.2-4). The superphylum Ecdysozoa was first proposed by Aguinaldo et al. (1997). Before 1997, nematodes were considered to be members of the introverta, a group of animals characterized by an incomplete body cavity, as opposed to the Coelomata, which possess a true body cavity (Nielsen 2001). According to Aguinaldo's study, the pseudocoelom was simply a reduction of a true coelom and this reduction took place multiple times. Although most researchers now regard the Ecdysozoa as a valid group, the Ecdysozoa have been controversial ever since its proposal, a controversy that continues to this day (Zheng *et al.* 2007). In general, phylogenetic studies supporting the Ecdysozoa use data (often ribosomal DNA) from many different taxonomic groups (Ruiz-Trillo *et al.* 1999;

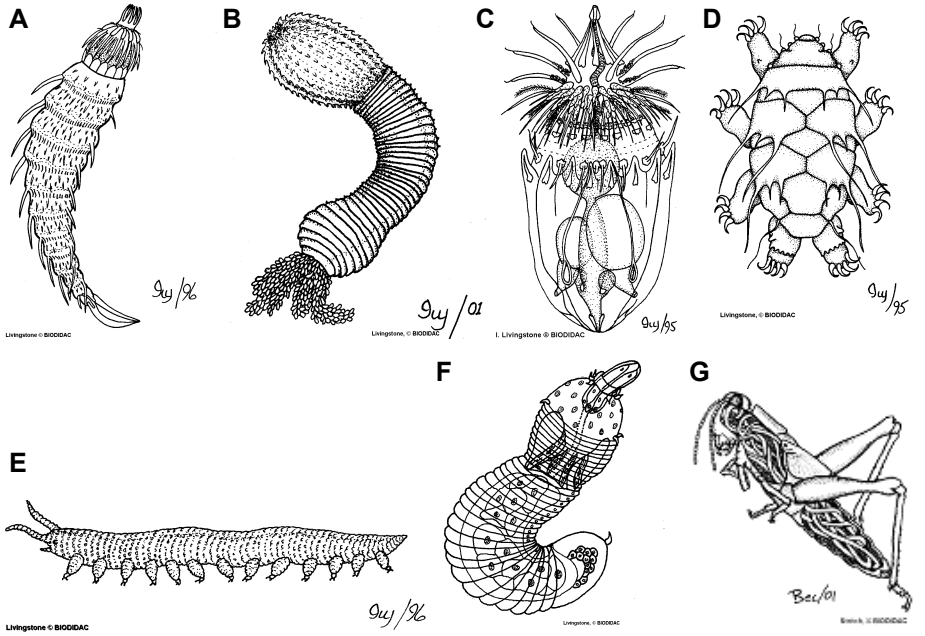

Figure 1.2. Ecdysozoans. A: Kinorhyncha (mud dragons) B: Priapulida (penis worms) C: Loricefera D: Tardigrada (water bears) E: Onychophora (velvet worms) F: Nematomorpha larvae (gordian or horsehair worms) G: adult nematomorph parasitizing a grasshopper (Arthropoda) – images taken from the BIODIDAC project: (http://biodidac.bio.uottawa.ca/thumbnails/catquery.htm?kingdom=Animalia).

Ruiz-Trillo *et al.* 2002; Giribet 2003; Philippe, Lartillot, and Brinkmann 2005; Longhorn, Foster, and Vogler 2007). On the other hand, studies rejecting the Ecdysozoa generally combine data from many different genes, thereby limiting themselves to a few species which have been (almost) fully sequenced, such as *Caenorhabiditis elegans* and *Drosophila melagonaster* (Hausdorf 2000; Blair *et al.* 2002; Wolf, Rogozin, and Koonin 2004; Philip, Creevey, and McInerney 2005). Both approaches have their merits as both increased taxa sampling as well as using data from more genes tend to improve the reliability of a phylogenetic tree. However, the species which are used for multi-gene analyses are often fast evolving model species and may not be the best representatives for their phylum. The resulting long branches combined with a very sparse taxon sampling (generally only a few Nematoda, Arthropoda and Chordata) means there is a very high potential for long branch attraction (LBA) artefacts. Until

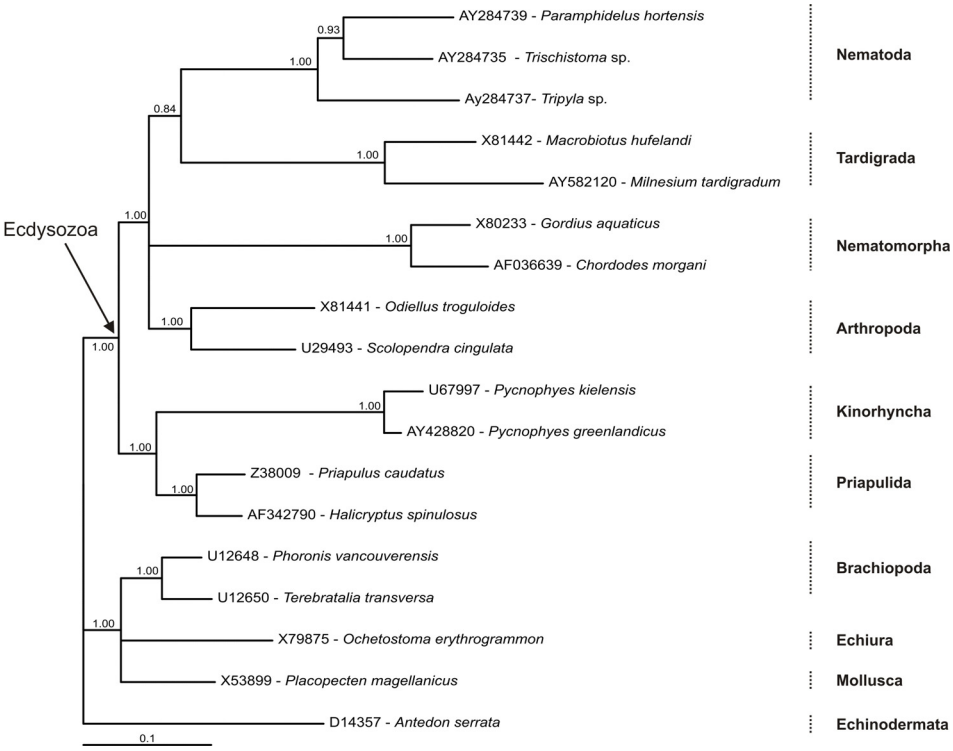

Figure 1.3. Bayesian phylogenetic reconstruction of the Ecdysozoa using SSU rDNA sequences. In case multiple SSU rDNA sequences were available within a given phylum, the most basal representatives were selected (for Nematoda members of Clade 1; Holterman *et al.* 2006). The tree was run in MrBayes 3.0 (Ronquist and Huelsenbeck 2003) using the general time-reversible (GTR) model of evolution with invariable sites, a gamma parameter and the covarion model. On the basis of secondary structure information of SSU rRNA, a stem and a loop partition was made. For the stem partition, doublets (basepairs) were used as the unit of substitution. The default flat priors were used as the starting value for the parameters and parameters were unlinked between partitions. The tree was run for 2,000,000 generations using 4 Markov chains. The first 200,000 generations were discarded as burnin. Numbers near the nodes are posterior probabilities. GenBank accessions are given.

both approaches - many taxa and many genes - are combined, the Ecdysozoa hypothesis probably will continue to be controversial.

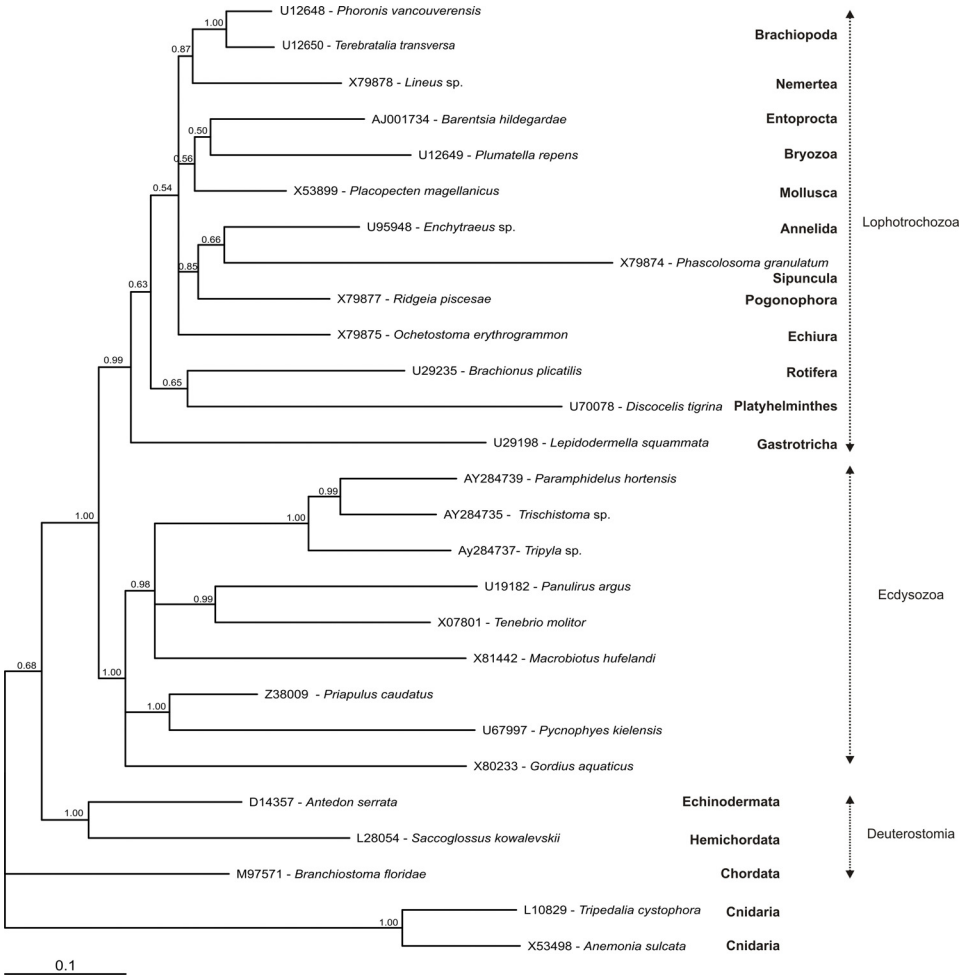

Figure 1.4. Bayesian phylogenetic reconstruction of the Protostomia, a group subdivided into two 'superphyla': Ecdysozoa and Lophotrochozoa, using SSU rDNA sequences. The tree was run using the same settings as described for Fig. 1.3. The tree was run for 3,000,000 generations using 4 Markov chains. The first 600,000 generations were discarded as burnin. Numbers near the nodes are posterior probabilities. GenBank accessions are given.

The evolution of plant parasitism in nematodes

The Nematoda contain three major groups of plant parasites: the Tylenchomorpha, some members of the Dorylaimidae and the Trichodoridae. Total losses caused by plant parasitic nematodes are estimated at $80 billion

annually (Agrios 2005), and most of the important plant parasites belong to the Tylenchomorpha, such as the cyst (Heteroderidae), lesion (*Pratylenchus* spp.) and root knot (*Meloidogyne* spp.) nematodes. The long standing and generally accepted hypothesis is that plant parasites evolved from fungal feeding ancestors (Maggenti 1971). Plant parasitism is assumed to have gradually evolved from simple forms of plant parasitism, such as epidermal and root hair feeding, into more complex forms of plant parasitism, eventually culminating in the evolution of sedentary endoparasites such as the cyst (Heteroderidae) and rootknot nematodes (*Meloidogyne*; Luc *et al.* 1987). The discovery of cell wall degrading enzymes in some species and their homology to bacterial versions has led to speculation that the genes involved in their production may have been acquired from bacteria by means of horizontal gene transfer (Keen and Roberts 1998; Yan *et al.* 1998).

Nematodes as environmental indicators

Among nematodes by far most attention is paid to the model organism *Caenorhabditis elegans* and to a number of notorious parasites of humans, animals and plants. However, *Caenorhabditis elegans* plays a negligible role in the soil food web (it is only occasionally found in compost heaps), and parasitic nematodes mostly constitute a (small) minority within the nematode community. The freeliving nematodes display a strong variation in their sensitivity towards environmental disturbances (Bongers and Ferris 1999). Given their high abundance and their importance in the soil food web, this makes nematodes very useful for detecting and monitoring environmental disturbances such as pollution. This led Bongers to coin the Maturity Index (Bongers 1990). The maturity index assigns a so-called *cp*-value to nematode families whose representative occur in terrestrial and freshwater habitats in North-Western Europe (*cp* standing for colonizer-persisters) on a scale of 1 to 5. Families on the lower end of the scale (*cp*-value 1-2) are so-called *colonizers*, roughly corresponding to *r*-strategists, and are relatively insensitive to disturbance. These nematodes are often small, present in high numbers, bacterial feeding, have a short generation time and can produce a lot of offspring (Bongers 1999). Families at the higher end of the scale (*cp*-value 4-5)

are so-called persisters, roughly corresponding to K-strategists, and are very sensitive to disturbance. They are generally relatively large nematodes, present in low numbers, are often predators or omnivorous, have long generation times and produce relatively little offspring. The assignment of families to these classes is based mainly on expert knowledge and there are no hard objective guidelines to determine to which *cp*-class a family should belong. Nevertheless, the maturity index has proven useful in environmental studies but the large scale application is hampered by the fact that the identification of nematode samples is time consuming and requires expert knowledge. A DNA barcode-based identification system which could be used by anybody with basic knowledge about molecular biology could be a substantial stimulus for the exploitation of non-parasitic nematodes as environmental indicators for soil and sediment health.

Outline of this thesis

The aim of the research described in this thesis was to provide a greater insight into the relationships of different nematodes and to study how certain traits, such as plant parasitism and the colonization of terrestrial habitats developed. Furthermore a first attempt was made to study which traits are involved in the tolerance towards stress while taking the confounding effects of phylogeny into account.

In **Chapter 2** the phylogenetic relationships between all groups of nematodes were studied using the SSU rDNA gene. On the basis of a series of bifurcations in the SSU rDNA tree twelve clades were defined, and the most basal clade was dominated by representatives of the Enoplida and the Triplonchida. It was observed that the SSU rDNA substitution rate was significantly different among the clades and this appeared to be correlated to short generation times and parasitic lifestyles. Furthermore it was observed that plant parasitic and hyphal feeding nematodes clustered together in the tree.

In **Chapter 3** the relationships within the subclass Dorylaimia were further studied. SSU rDNA provided no resolution within the order Dorylaimida and therefore the more variable large subunit (LSU) rDNA gene was used to study relationships within this order. A large discrepancy between

classical taxonomy and the molecular phylogeny was observed. It was further observed that plant parasitism in the Dorylaimida had arisen at least twice and not necessarily from fungal feeding ancestors. Furthermore, a proof of principle was delivered that single nucleotide polymorphisms (SNP's) in the ribosomal DNA of nematodes can be used to identify and quantify groups of nematodes using specific PCR primers.

In **Chapter 4** the relationships in the subclass Chromadoria (orders Chromadorida, Desmodorida, Monhysterida, Araeolaimida and Plectida; Rhabditida were not included) were studied using the SSU rDNA gene. In addition the transitions of nematodes from a marine to a limnoterrestrial habitat (and *vice versa*) were studied. It was found that these transitions have occurred frequently within the Chromadoria. The requirements for such a transition were discussed.

In **Chapter 5** the relationships within the order Tylenchida *sensu* Siddiqi (Siddiqi 2000) were studied using the SSU rDNA. The development of plant parasitism was studied using ancestral state reconstruction. It was confirmed that plant parasitism gradually evolved from simple to more complex forms, but the ancestors of the Tylenchida could not be confirmed as being fungal feeding.

In **Chapter 6** an approach is laid out for finding the corelations between nematode traits and tolerance towards environmental disturbances. This approach is a first step towards defining a more objective, less expert knowledge-based Maturity Index. The correlations between several traits (body size, reproductive potential, feeding type, survival stage, asexual reproduction and cuticle permeability) and tolerance to copper and pH stress were investigated. This was done using a generalized least squares (GLS) method to allow for the phylogenetic dependence of the data. The relevance of using this method was discussed.

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Chapter 2

Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution towards crown clades

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Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution towards crown clades

Inference of evolutionary relationships between nematodes is severely hampered by their conserved morphology, the high frequency of homoplasy, and the scarcity of phylum-wide molecular data. To study the origin of nematode radiation and to unravel the phylogenetic relationships between distantly related species, 339 nearly full length small subunit (SSU) ribosomal DNA sequences were analyzed from a diverse range of nematodes. Bayesian inference revealed a backbone comprising twelve consecutive dichotomies that subdivided the phylum Nematoda into twelve clades. The most basal clade is dominated by the subclass Enoplia, and members of the order Triplonchida occupy positions most close to the common ancestor of the nematodes. Crown clades 8-12 - a group formerly indicated as 'Secernentea' that includes *Caenorhabditis elegans* and virtually all major plant and animal parasites - show significantly higher nucleotide substitution rates than the more basal clades 1-7. Accelerated substitution rates are associated with parasitic lifestyles (clade 8 and 12) or short generation times (clade 9-11). The relatively high substitution rates in the distal clades resulted in numerous autapomorphies that allow in most cases DNA barcode-based species identification. *Teratocephalus*, a genus comprising terrestrial bacterivores, was shown to be most close to the starting point of 'Secernentean' radiation. Notably, fungal feeding nematodes were exclusively found basal to or as sister taxon next to the three groups of plant parasitic nematodes, namely Trichodoridae, Longidoridae and Tylenchomorpha. The exclusive common presence of fungivorous and plant parasitic nematodes supports a longstanding hypothesis saying that plant parasitic nematodes arose from fungivorous ancestors.

Introduction

Nematodes constitute one of the largest and most widely distributed groups of animals in marine, freshwater and terrestrial habitats. Their numerical dominance, exceeding often more than one million individuals per square meter and accounting for about 80% of all individual animals on earth (Platt 1994), their diversity in lifestyles and their presence at various trophic levels point at an important role in many ecosystems. Its most well-known representative is *Caenorhabditis elegans*; the first animal whose genome was completely sequenced (Herman 2004). Apart from the bacterivorous nematodes such as *C. elegans*, a wide range of trophic ecologies are displayed, such as fungal feeding, predation, and parasitism of plants, invertebrates, higher animals and humans. Among plant parasitic nematodes, the cyst (*Globodera* and *Heterodera* spp.) and root knot nematodes (*Meloidogyne* spp.) are most notorious, causing major damage to crops such as soybean, potato and sugar beet. Human parasitic nematodes include among others the pinworm *Enterobius vermicularis*, a worldwide intestinal parasite of mainly children, the causal agents of elephantiasis - *Wuchereria bancrofti* and *Brugia malayi* - and *Onchocerca volvulus* that - in combination with its endosymbiont *Wolbachia* - causes river blindness (onchocerciasis; Saint Andre *et al.* 2002). Non-parasitic nematodes are valuable indicators for the biological condition of soils as this ecologically highly diverse group shows much variation in sensitivity towards environmental stresses, and occupy key positions in the soil food web (Bongers and Ferris 1999).

One of the earliest and most influential classifications of the Nematoda was proposed by (Chitwood and Chitwood 1933) and (Chitwood 1937). They introduced a division of the phylum into the Aphasmidia and Phasmidia, later renamed Adenophorea ('*gland bearers*') and Secernentea ('*secretors*') respectively (Chitwood 1958). This division was based on the fact that the Secernentea share several characteristics including the presence of phasmids, a pair of sensory organs located in the lateral posterior region. This division was adhered to in many later classifications even though it was realized that the Adenophorea were not a uniform group (Maggenti 1963; De Coninck 1965). On the basis of an unweighted count of shared morphological features, (Andrássy 1976) proposed a tripartite system by subdividing the former Adenophorea into the Torquentia

and Penetrantia. The first taxonomic system based on cladistic principles was introduced by (Lorenzen 1981). His analysis made clear that there was no support for the Adenophorea as a monophyletic group. Moreover, he showed that the number of informative morphological characters was too low to come up with a plausible alternative.

Only two SSU rDNA based trees have been constructed so far that attempted to span the entire phylum (Aleshin *et al.* 1998a; Blaxter *et al.* 1998) to provide a template for phylogenetic studies. (Blaxter *et al.* 1998) defined five major clades and confirmed the paraphyly of the Adenophorea. Interestingly, the authors clearly showed that trophic ecologies such as animal and plant parasitism arose several times independently. However, mainly due to the relatively small data set used, *viz.* 53 taxa, the relationships among the major clades remained unresolved. Comparable results were acquired by Aleshin *et al.* (1998a) based on 19 nematode sequences. Here, we present a phylogenetic reconstruction of 339 nematode taxa throughout the entire phylum Nematoda, inferred from nearly full length SSU rDNA sequences. Our results revealed a subdivision of the phylum Nematoda into twelve major clades, where the most basal clade (Clade 1) was dominated by representatives of the subclass Enoplia *sensu* De Ley and Blaxter (2002; 2004). Clade 7 comprised only a single family, the Teratocephalidae, and members of the genus *Teratocephalus* were shown to be most close to the origin of Secernentean (Clade 8-12) radiation. The remarkable and significant acceleration of SSU rDNA substitution rates in the more distal clades that include most major plant and animal parasites gave - in most cases - rise to resolution till species level. This unforeseen resolution implies that SSU rDNA-base sequence signatures can be defined at species level for a wide range of parasitic and non-parasitic nematodes.

Materials and Methods

Taxon sampling

Nematodes were collected from various soil habitats, and extracted using standard techniques. Prior to DNA extraction, individual nematodes were identified using a light microscope (Zeiss Axioscope) equipped with differential interference contrast (DIC) optics. A CCD camera (CoolSnap, RS Photometrics)

was used to take a series of digital images from each nematode to retain the possibility to re-evaluate the identity of individual specimen. Series of digital images from individual nematodes are available upon request (contact the corresponding author). For classification at family level and below, the nomenclatural system of the Fauna Europaea was used (*http://www.faunaeur.org/*). For the classification above family level we conformed to De Ley and Blaxter (2002; 2004).

DNA extraction, SSU rDNA amplification and sequencing

Single nematodes were transferred to a 0.2 ml 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 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. SSU rDNA was amplified as two partially overlapping fragments using three universal and one nematode-specific primer (1912R). The latter was included to avoid amplification of non-target eukaryotic SSU rDNA, e.g., from fungal spores attached to the nematode cuticle. For the first fragment, either primer 988F (5'-ctcaaagattaagccatgc-3') or 1096F (5'-ggtaattctggagctaatac-3') was used in combination with primer 1912R (5'-tttacggtcagaactaggg-3'). The second fragment was amplified with primers 1813F (5'-ctgcgtgagaggtgaaat-3') and 2646R (5'-gctaccttgttacgactttt-3'). PCR was performed in a final volume of 25 µL and contained 3 µL of a 100 times diluted crude DNA extract, 0.1 µM of each PCR primer and a Ready-To-Go PCR bead (Amersham). The following PCR profile was used: 94°C for 5 min.; 5 x (94°C, 30 sec.; 45°C, 30 sec.; 72°C, 70 sec.) followed by 35 x (94°C, 30 sec.; 54°C, 30 sec.; 72°C, 70 sec.) and 72°C, 5 min. Gel-purified (Marligen Bioscience, Ijamsville, MD) amplification products were cloned into a TOPO TA vector (Invitrogen) and sequenced using standard procedures. Newly generated SSU rDNA sequences were deposited at GenBank under the following accession numbers: AY284581-AY284841 and AY593880 (for corresponding species names see Appendix A).

To distinguish between the two closely-related potato cyst nematode species *Globodera rostochiensis* and *G. pallida* on the basis of a single nucleotide

difference in the SSU-rDNA sequences, real time PCR was performed on a Bio-Rad MyiQ thermal cycler (Bio-Rad, Hercules, CA). In a total reaction volume of 25 µl, 3 µl template (10 times diluted nematode lysate prepared as described above) was mixed with a *G. rostochiensis*-specific primer GrosR1-650R (5'-ggccaacgccggggaa-3') and a general SSU rDNA primer 988F (5'-ctcaaagattaagccatgc-3') (end concentrations for both primers 200 nM) and 12.5 µl iQ SYBR Green supermix (Bio-Rad, Hercules, CA). After 3 amplification cycles with an annealing temperature of 60°C, the specificity was increased by raising the annealing temperature to 89.5°C.

Sequence alignment

Nematode SSU rDNA sequences were supplemented with publicly available sequences (accession numbers given in Appendix A). The choice of outgroup sequences was based on Aleshin *et al.* (1998b) and consisted of arthropods (3x), priapulids (2x), a kinorhynch (1x), nematomorphs (2x) and tardigrades (2x): *Dilta littoralis* (AF005457), *Podura aquatica* (AF005452), *Polydesmus coriaceus* (AF005449); *Priapulus caudatus* (Z38009), *Tubiluchus corallicola* (AF119086); *Pycnophyes kielensis* (U67997); *Chordodes morgani* (AF036639), *Gordius aquaticus* (X80233); *Macrobiotus hufelandi* (X81442) and *Thulinia stephaniae* (AF056023) respectively. Nearly full length SSU rDNA sequences were aligned using the ClustalW algorithm as implemented in BioEdit 5.0.9 (Hall 1999) and manually improved using arthropod secondary structure information (*http://www.psb.ugent.be/rRNA/secmodel /index.html*, in accordance with Wuyts *et al.*, 2000). The final alignment included 349 nearly full length SSU rDNA sequences and contained 2,471 aligned positions including gaps.

Phylogenetic analyses

Bayesian inference (BI) and Maximum parsimony (MP) were used to reconstruct the phylogeny within the phylum Nematoda. Modeltest 3.06 (Posada and Crandall 1998) identified the general time reversible (GTR) model with invariable sites and a Γ-shaped distribution of substitution rates as the best substitution model. The Bayesian tree was constructed using the program MrBayes 3.0 (Ronquist and Huelsenbeck 2003). The alignment was divided into a stem and a loop partition according to SSU rDNA secondary structure. For

both partitions, the GTR model with invariable sites was used with the default flat priors unlinked between partitions. A gamma parameter could not be included due to computing memory limitations. The program was run on the TERAS computer cluster (SARA Computing and Networking Services, Amsterdam, The Netherlands). Each chain was run on a separate processor. Four independent computations with random starting trees and four Markov chains each were run for 8,000,000 generations with a sampling frequency of 200 generations. The burnins of 1, 3, 3.5 and 1 million generations respectively were discarded. Sampled trees were combined in a 50% majority-rule consensus tree. Nodes with a posterior probability (pp) lower than 95 (Erixon *et al.* 2003) or a bootstrap support lower than 65% were considered unresolved (Hillis and Bull 1993).

The maximum parsimony tree was constructed using PAUP* 4.0b10 (Swofford 1998). Default parameters were used with gaps treated as a fifth character state. 16,887 equally parsimonious trees were saved and a 50% majority rule consensus tree was bootstrapped 1,000 times, not saving multiple trees during branch-swapping. The Neighbor-joining tree was constructed using PAUP* applying the model (GTR + I + Γ) and parameter values determined by Modeltest. The resulting tree was bootstrapped 1,000 times.

The program RRTree (Robinson-Rechavi and Huchon 2000) was used to compare SSU rDNA substitution rates between clades. Significance of relative rate differences was tested using a Bonferroni-correction.

Results & Discussion

Representative and balanced taxon sampling is a prerequisite for the reconstruction to of phylogenetic relationships within the widespread and speciose phylum Nematoda (Moreira and Philippe 2000). So far, Rhabditidae, relatives of the bacterivorous model organism *C. elegans,* the suborder Spirurina, which consists exclusively of zooparasites, and the Tylenchina, a suborder that includes numerous plant parasites are relatively over-represented (Blaxter, Dorris, and De Ley 2000; Sudhaus and Fitch 2001, and Baldwin, Nadler, and Adams 2004). Molecular information is scarce for the majority of bacterivorous, fungivorous, carnivorous and omnivorous nematodes. Here, we

present 260 newly generated full length SSU rDNA sequences mainly from representatives of basal clades, and non-parasitic representatives throughout the phylum, and use these data to derive deep phylogenetic relationships, to deduce the evolution of feeding types and to define its potential for DNA sequence signature-based community analysis.

While *C. elegans* and a number of other bacterivorous Rhabditidae can be grown on growth medium agar plates seeded with bacteria to obtain numerous individuals from the same species, the majority of nematodes appear to be non-culturable. Therefore single nematodes were used as starting material. After taking a series of high resolution images, individual nematodes were lysed and their SSU rDNA sequences were determined. By doing so, a data base was built that robustly links morphological and molecular data. Newly generated data were combined with 176 publicly available sequences. Consensus sequences were generated in case species were represented by multiple sequences. The final alignment consisted of 339 nematode taxa, and 10 outgroup sequences.

Phylogenetic analysis

In four independent runs with nearly identical results (only the first run is used here) Bayesian analysis of 349 taxa yielded a phylogenetic tree with a backbone consisting of twelve consecutive dichotomies from the tree root onwards (Fig. 2.1). Eight dichotomies are strongly supported (posterior probability (PP) of 1.0), one dichotomy is quite robust (PP of 0.95), whereas three nodes are weakly supported with PP values between 0.64 and 0.81. Maximum Parsimony (MP)-

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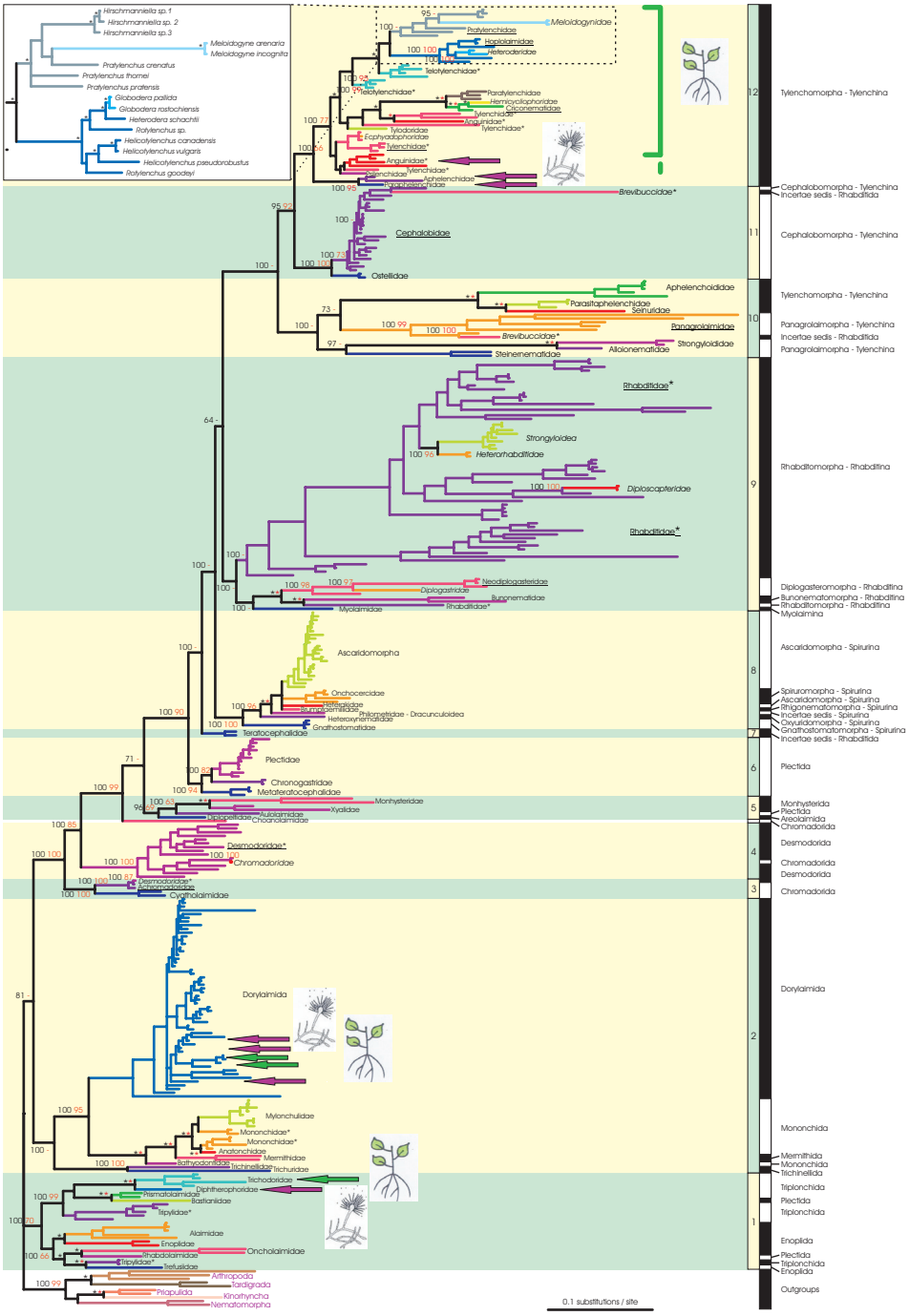
Fig. 2.1. Bayesian tree of the phylum Nematoda. Alternating yellow and green backgrounds define the subdivision of the phylum Nematoda into twelve clades. Within each clade, nematode families have separate colors. Support values are indicated at the deep nodes: The first number (black) is the Bayesian posterior probability, the second number (orange) is the maximum parsimony (MP) bootstrap value. "–" indicates that the node was part of a polytomy in MP. Other nodes down to family-level are marked with a black asterisk if the support from the BI tree is significant (p.p. ≥ 95) and an orange asterisk if the support from the MP tree is significant (bootstrap ≥ 65). Underlined family names are paraphyletic, family names marked with an asterisk are polyphyletic and family names in italics are embedded in another family. The black and white bars indicate (sub-, infra-) orders as defined by De Ley and Blaxter (2002; 2004). Plant parasitic and fungivorous taxa are indicated by a pictogram and a purple (fungivores) or green (plant parasites) arrow or bar. The insert shows the most distal part of the tree in more detail.


Hirschmanniella sp.1
Hirschmanniella sp. 2
Hirschmanniella sp.3
Meloidogyne arenaria
Meloidogyne incognita
Pratylenchus crenatus
Pratylenchus thornei
Pratylenchus pratensis
Globodera pallida
Globodera rostochiensis
Heterodera schachtii
Rotylenchus sp.
Helicotylenchus canadensis
Helicotylenchus vulgaris
Helicotylenchus pseudorobustus
Rotylenchus goodeyi
Meloidogynidae
Pratylenchidae
Hoplolaimidae
Heteroderidae
Telotylenchidae*
Paratylenchidae
Hemicycliophoridae
Criconematidae
Tylenchidae
Anguinidae*
Tylenchidae*
Tylodoridae
Ecphyadophoridae
Tylenchidae*
Anguinidae*
Tylenchidae*
Aphelenchidae
Aphelenchoididae
Brevibuccoidae*
Cephalobidae
Ostellidae
Aphelenchoididae
Parasitaphelenchidae
Seinuridae
Panagrolaimidae
Brevibuccoidae*
Strongyloididae
Allolonematidae
Steinernematidae
Rhabditidae*
Strongyloidea
Heterorhabditidae
Diplopeltidae
Rhabditidae*
Neodiplogasteridae
Diplogastridae
Bunonematidae
Rhabditidae*
Myolaimidae
Ascaridomorpha
Onchocercidae
Philometridae
Philometridae - Dracunculoidea
Heteroxynematidae
Gnathostomatidae
Teratocephalidae
Plectidae
Chronogastridae
Metateratocephalidae
Monhysteridae
Xyalidae
Xiphinemidae
Aulolaimidae
Choanolaimidae
Desmodoridae*
Chromadoridae
Desmodoridae*
Desmodoridae
Cyatholaimidae
Dorylaimida
Mylonchulidae
Mononchidae*
Mononchidae*
Anatonchidae
Mesrithidae
Bathyodontidae
Trichinellidae
Trichuridae
Trichodoridae
Diphtherophoridae
Prismatolaimidae
Bastianiidae
Tripylidae*
Alaimidae
Enoplidae
Rhabdolaimidae
Tripylidae*
Trefusiidae
Onchulaimidae
Arthropoda
Tardigrada
Priapulida
Kinorhyncha
Nematomorpha
0.1 substitutions / site
12
11
10
9
8
7
6
5
4
3
2
1
Tylenchomorpha - Tylenchina
Cephalobomorpha - Tylenchina
Incertae sedis - Rhabditida
Cephalobomorpha - Tylenchina
Tylenchomorpha - Tylenchina
Panagrolaimorpha - Tylenchina
Incertae sedis - Rhabditida
Panagrolaimorpha - Tylenchina
Rhabditomorpha - Rhabditina
Diplogasteromorpha - Rhabditina
Bunonematomorpha - Rhabditina
Rhabditomorpha - Rhabditina
Myolaimina
Ascaridomorpha - Spirurina
Spiruromorpha - Spirurina
Ascaridomorpha - Spirurina
Rhigonematomorpha - Spirurina
Incertae sedis - Spirurina
Oxyuridomorpha - Spirurina
Gnathostomatomorpha - Spirurina
Incertae sedis - Rhabditida
Plectida
Monhysterida
Plectida
Araeolaimida
Chromadorida
Desmodorida
Chromadorida
Desmodorida
Chromadorida
Dorylaimida
Mononchida
Mermithida
Mononchida
Trichinellida
Triplonchida
Plectida
Triplonchida
Enoplida
Plectida
Triplonchida
Enoplida
Outgroups

based data analysis revealed a similar tree topology, although the number of resolved nodes was lower. Neighbor-joining analysis (NJ) resulted in a tree topology comparable to the MP tree, although the resolution within the clades was lower. Fig. 2.2 shows the overall topologies of the Bayesian inference (BI), the MP and the NJ trees. Detailed representations including full taxon names are presented as supplementary material in the appendices (Appendices B, C and D). The lower resolution of MP can be explained by 1) saturation - a consequence of the large number of sequences analyzed - and by 2) a relatively high variation in branch lengths - inherent to the analysis of a phylum-wide data set (Felsenstein 1978; Philippe, Germot, and Moreira 2000). The BI criterion is less susceptible to both methodological problems since it includes a mutation model (Moreira and Philippe 2000). In addition, the BI is more sensitive in detecting phylogenetic signal when taxa differ in few characters (Alfaro, Zoller, and Lutzoni 2003). The relatively poor resolution of the NJ tree can be explained by the fact that distance methods, such as NJ, are in fact not suitable for the inference of more distant phylogenetic relationships, especially when the molecular clock assumption is not valid, as is the case with our data (Holder and Lewis 2003).

The use of full length SSU rDNA sequences (≈ 1,700 bp each) and extensive additional taxon sampling gives a detailed insight in the deep

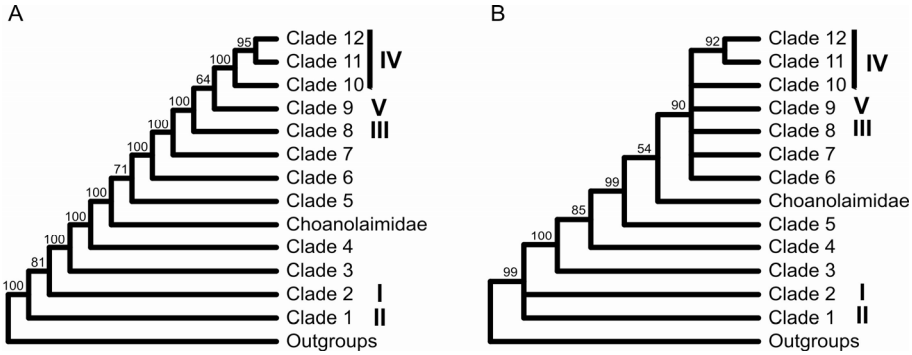


Fig. 2.2. Schematic representations of the division of the phylum Nematoda into twelve clades, according to (**A**) Bayesian inference, (**B**) maximum parsimony and (**C**) neighbor joining. Branches with bootstrap support <50% in MP and NJ are shown as unresolved. The five clades defined by Blaxter *et al.* (1998) are indicated in Roman numerals behind the corresponding clades in our clade division.

phylogenetic relationships between all major taxa within the phylum Nematoda for the first time. On the basis of this analysis we propose a revision of the current clade division (Blaxter *et al.* 1998; Fig. 2.2). Instead of a division into five clades with many families not placed in a clade at all, we propose a division into twelve clades that - except for the Choanolaimidae - include all sampled nematode families (Table 2.1 – NJ clades are omitted as NJ is not suitable for pinpointing distant relationship). Bayesian analysis suggested the family Choanolaimidae to be placed between Clades 4 and 5, whereas in the parsimony tree it was positioned between Clades 5 and 6 (Fig. 2.2). Therefore we refrained from assigning a clade to this family.

The origin of nematode radiation

Clade 1 is presumably the most basal clade in the BI tree (Fig. 2.2). However, it is not possible to make a strong statement on the basis of the currently presented SSU-rDNA sequence data as the node joining Clades 2-12 in the Bayesian inference-based tree is supported by a relatively low PP value (0.81). Bayesian inference and maximum likelihood analysis of a limited number of representatives with short branch lengths did not result in a more robustly topology at the base of the tree (data not shown). Hence, it was investigated whether the hypothesis of Clade 1 being the most basal clade was supported by other, independent data.

Clade 1 includes representatives of the Enoplia and two Plectida families, the Rhabdolaimidae and the Bastianiidae. This subclass Enoplia comprises only two orders, Enoplida and Triplonchida, and representatives of the latter, *Trischistoma* sp. and *Tripyla* sp. 4, occupy the positions closest to the base of the phylum Nematoda. The basal position of the Enoplia in this SSU rDNA-based phylogenetic tree is supported by patchy embryological and morphological data. Embryo development within this subclass deviates from the standard pattern observed for nematodes as there is no asymmetrically dividing germ line and no bilateral symmetry during early embryogenesis (Malakhov 1994; Voronov, Panchin, and Spiridonov 1998; Schierenberg 2005) and they have only a weakly centralized nervous system (Malakhov 1994). In these aspects of embryo development Enoplia resemble other animals and thus

Table 2.1. Clade topology as reconstructed with Bayesian inference (Fig. 2.1) and compared to MP analysis and (Blaxter *et al.* 1998).

BI clades (Fig. 2.1)	MP subclades	Clade taxa	Blaxter *et al.* (1998)	Bayesian p.p.	bootstrap MP
1	100	Enoplida, Triplonchida, Bastianiidae* and Rhabdolaimidae* (Plectida)	**II**	100	70
2		Trichinellida, Mononchida, Mermithida, Dorylaimida	**I**	100	-
	2a	Trichinellida		100	100
	2b	Mononchida, Mermithida, Dorylaimida		100	95
3		Chromadorida, *Prodesmodora* (Desmodoridae, Desmodorida)	-	100	100
4		Desmodorida, Chromadoridae (Chromadorida)	-	100	100
		Choanolaimidae (Chromadorida)	-	-	-
5		Monhysterida, Areolaimida, Aulolaimidae (Plectida)	-	96	69
6		Plectida	-	100	94
7		Teratocephalidae* (Rhabditida, *incertae sedis*)	-	100	100
8		Spirurina	**III**	100	100
9		Myolaimina, Rhabditina	**V**	100	-
	9a	*Myolaimus* sp. (Myolaimina)		-	-
	9b	Diplogasteromorpha, Bunonematomorpha & *Rhabditoides inermis* (Rhabditomorpha)		100	66
	9c	*Rhabditoides inermiformis* (Rhabditomorpha)		-	-
	9d	*Poikilolaimus* spp. (Rhabditomorpha)		100	100
	9e	Rhabditomorpha		100	91
10		Tylenchina, Brevibuccidae (Rhabditida, *incertae sedis*)	**IV**	100	-
	10a	*Steinernema* (Panagrolaimorpha) & *Brevibucca* (Rhabditida, *incertae sedis*)		-	57
	10b	Panagrolaimidae (Panagrolaimorpha) & *Plectonchus* (Rhabditida, *incertae sedis*)		100	99
	10c	Panagrolaimidae (Panagrolaimorpha) & *Plectonchus* (Rhabditida, *incertae sedis*)		-	69
11		Cephalobomorpha	**IV**	100	100
12		Tylenchomorpha	**IV**	100	66

BI = bayesian inference, MP = maximum parsimony, - = not applicable, pp = posterior probability, * discussed in text.

they can be considered as plesiomorphies (Schierenberg 2005). The basal position of the Enoplia is further supported by the retention of the nuclear envelope in the mature spermatozoa, an ancestral character (Baccetti *et al.* 1983). Spermatozoa from other nematodes outside the Enoplia always lack such an envelope (Justine 2002; Yushin 2003). Taking these additional morphological and embryological data into account, we suggest that Clade 1 as defined in Fig. 2.1 is indeed the most basal clade within the phylum Nematoda.

Within Clade 1 members of the family Tripylidae (order Triplonchida) have the shortest branch lengths. In contrast to the (limited number of) nematode taxa investigated so far (including the Enoplida), *Tobrilus diversipapillatus*, a representative of the Triplonchida, was shown to form a prominent coeloblastula, and gastrulation followed a pattern that is common within the animal kingdom but highly unusual among nematodes (Schierenberg 2005). Hence, currently available embryological data apparently support the very basal position of the Triplonchida in the SSU rDNA-based phylogenetic tree.

At first sight, the firm placement of the Bastianiidae and the Rhabdolaimidae (order Plectida according to De Ley and Blaxter, 2002) within the basal orders Triplonchida and Enoplida is remarkable as the Plectida were previously suggested as the origin of Secernentean radiation (Blaxter *et al.* 1998). However, the order Plectida suggested to be a mixture of paraphyletic and misplaced families (De Ley and Blaxter 2002). The Bastianiidae strongly resemble the Prismatolaimidae (Triplonchida; Coomans and Raski 1988; Lorenzen 1994), and this supports its newly established phylogenetic position. Morphological support for the Rhabdolaimidae as a member of Enoplida comes in the shape of the amphids, a pair of sensory organs located on the head of a nematode. These are non-spiral and pocket shaped (Lorenzen 1994), a feature which is characteristic for the Enoplia *sensu* Lorenzen (newly proposed clades 1 and 2).

Acceleration of SSU rDNA substitution rates

Based on a limited number of SSU rDNA sequences, nematodes were suggested to have a substitution rate 2-3 times greater than those of most other Metazoa (Aguinaldo *et al.* 1997). The large branch lengths of *e.g.* crown taxa belonging to

Clade 9 (including *Caenorhabditis elegans*) and 10 (including *e.g. Strongyloides stercoralis*; Fig. 2.1) seemed to support this statement and a relative rate test (Li and Bousquet 1992) was performed to compare substitution rates of SSU rDNA among the clades. This test compares the weighted distances of the taxa of two clades to an outgroup (Robinson *et al.* 1998) and, in general, basal clades (Clade 1-7; formerly indicated as Adenophorea) evolve significantly slower than distal clades (Clade 8-12; formerly indicated as Secernentea; Table 1). Within Clade 1, sequences of *Tripyla* sp. (family Tripylidae), *Paramphidelus hortensis* (family Alaimidae) and *Trischistoma* sp. (Tripylidae) were closest to the most basal node within the phylogenetic tree.

Acceleration of nucleotide substitutions could be attributed to (a combination of) two causes: an elevated production of free radicals due to *e.g.* increased metabolic rates (usually associated with small body size) or an accumulation of DNA replication errors due to shorter generation times (*e.g.* (Gillooly *et al.* 2005). Both in plant and animals parasitism infective nematodes are exposed to free radicals (mostly reactive oxygen species) released by hosts as part of their defense response. Clades 8 and 12 are dominated by respectively animal and plant parasites. We hypothesize that the release of free radicals by plant or animals has contributed to an accelerated evolution of these parasitic nematodes.

Clades 9 and 10 are dominated by bacterial feeding nematode families and contain only a few animal parasitic (Strongyloidea in Clade 9, Strongyloididae in Clade 10) and entomopathogenic nematodes (Heterorhabditidae in Clade 9, Steinernematidae in Clade 10). Clade 11 solely comprises bacterial feeding families. Hence, in Clades 9-11 intimate interactions with other organisms do not explain the observed accelerated substitution rates. Generalized life history traits, including generation time, are one of the major components that were used by (Bongers 1990) to develop an ecological scale for non-parasitic nematode families. Colonizers (*c*) and persisters (*p*) are extremes on a scale from 1 to 5, and *c-p* values of 1 are used to characterize stress-tolerant nematodes. Nematode families with a *c-p* value 1 have very short life cycles, produce large numbers of small eggs, have voluminous gonads and are often viviparous. These families show high fluctuations in population densities, and - if present - they are present in huge numbers. Non-parasitic nematode families

Table 2.2. Pairwise differences among clades in relative evolutionary rates as calculated by RRTREE. The outgroup containing other metazoan species is used as outgroup in all pairwise comparisons.

Clade	1	2	3	4	5	6	7	8	9	10	11	12
1	-	1.249	0.932	1.065	1.133	1.177	1.171	1.274	1.399	1.847	1.381	1.464
2	**0.034***	-	0.786	0.891	0.913	0.905	1.050	1.072	1.096	1.554	1.107	1.261
3	-0.010	**-0.039***	-	1.135	1.199	1.202	1.309	1.371	1.513	1.948	1.499	1.573
4	0.010	-0.019	0.022	-	1.088	1.092	1.167	1.245	1.333	1.737	1.370	1.402
5	0.020	-0.015	**0.028***	0.014	-	1.040	1.031	1.121	1.181	1.604	1.268	1.330
6	0.027	-0.016	0.031	0.015	0.007	-	1.000	1.100	1.150	1.582	1.209	1.296
7	0.026	0.009	**0.053***	0.032	0.005	0.000	-	1.072	1.173	1.490	1.223	1.260
8	**0.042***	0.013	**0.059***	**0.042***	0.021	0.018	0.015	-	1.072	1.276	1.259	1.353
9	**0.042***	0.012	**0.047***	**0.034***	0.021	**0.018***	**0.021***	0.010	-	1.282	1.203	1.432
10	**0.108***	**0.086***	**0.126***	**0.109***	**0.090***	**0.088***	**0.086***	**0.056***	**0.038***	-	0.784	0.825
11	**0.056***	0.019	**0.074***	**0.060***	**0.046***	**0.038***	**0.041***	**0.048***	**0.028***	**-0.053***	-	1.049
12	**0.062***	**0.042***	**0.082***	**0.064***	**0.049***	**0.046***	**0.047***	**0.064***	**0.053***	**-0.042***	0.010	-

NOTE. - Below diagonal: pairwise differences in number of substitutions per site (clade in column – clade in row). Significance level after Bonferroni correction for multiple tests is P< 0.001, significant results are in bold and indicated with an asterisk. Above diagonal: relative differences in evolutionary rate calculated as distance to outgroup from clade in column divided by distance to outgroup from clade in row.

in Clade 9 have exceptionally low *c-p* values: Rhabditidae (*c-p* value 1), Diploscapteridae (1), Neodiplogastridae (1), Diplogastridae (1), Bunonematidae (1) and Myolaimidae (2). Essentially the same holds for the non-parasitic families in Clade 10: Panagrolaimidae (1), Brevibuccidae (1) and Alloionematidae (1). Notably, Monhysteridae (Clade 5, relatively long branches) is the only family with a *c-p* value of 1 that is not residing in Clade 9 or 10. Clade 11 consists of Osstellidae and Cephalobidae (the positioning of *Brevibucca* sp. in this clade is probably an LBA artifact) and both families have a *c-p* value of 2. Hence, the relatively high SSU rDNA substitution rates in Clade 9 and 10 (Clade 11 to some extent) are associated with extremely low *c-p* values and, by extension, with short generation times.

Nematode barcoding

The relatively high substitution rates of the SSU rDNA gene in nematodes in Clades 8-12 resulted in a level of sequence diversity that allows – in most cases

– nematode identification at species level. Autapomorphies, mostly single nucleotide polymorphisms (SNP), were found for, *e.g.*, the morphologically highly similar potato cyst nematode species *Globodera rostochiensis* and *G. pallida* and between the various *Helicotylenchus* species (see insert Fig. 2.1). In Fig. 2.3, it is shown how a single SNP can be used to (quantitatively) detect *G. rostochiensis*, whereas equal DNA concentrations of its sibling species *G. pallida* hardly give rise to any amplification product ($\Delta C_T \approx 19$ (10 juveniles) and $\Delta C_T \approx 21$ (100 juveniles); C_T: threshold cycle). These potato cyst nematode species were chosen to illustrate the potential of SSU rDNA polymorphisms for detection because these two species are morphologically nearly indistinguishable (*e.g.* Jones *et al.* 1970).

The current phylum wide data set allows for the identification of individual nematode species within a pool of non-target taxa, as for instance in case of soil samples (Helder *et al.* 2004). For nematode biodiversity studies it has been proposed to define molecular operational taxonomic units (MOTU's) on the basis of a defined number of SSU rDNA sequence differences instead of classical species concepts (Floyd *et al.* 2002). However, different rates of evolution among nematode clades (Table 2.2) implicate that a defined number of nucleotide differences can not always be linked unequivocally to meaningful biological differences.

<u>The origin of the 'Secernentean' radiation</u>

The SSU rDNA sequence data presented here provides detailed insight in the relationship between the 'Adenophorea' (Fig. 2.1; Clade 1 - Clade 7) and the 'Secernentea' (Fig. 2.1; Clade 8 - Clade 12), a partition of the phylum Nematoda that has dominated nematode systematics since it was proposed by Chitwood and Chitwood in 1933 (Chitwood and Chitwood 1933). The 'Secernentea' (equivalent to the order Rhabditida with the exception of the Teratocephalidae) – a group that includes virtually all major animal and plant parasites - arose from the Adenophorea (Blaxter *et al.* 1998), and the current SSU rDNA data set suggests that members of the genus *Teratocephalus* (Clade 7) are the closest living representatives of the common ancestor of the Secernentea. The genus *Teratocephalus*, the only genus within the family Teratocephalidae, exhibits a mixture of morphological characters of Secernentea and Adenophorea (*e.g.*

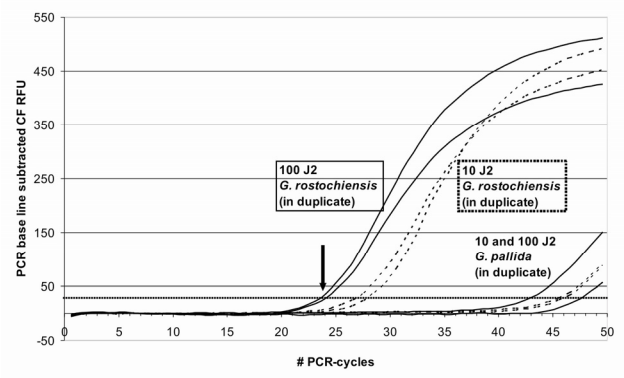


Fig. 2.3. Real time PCR amplification curves (in duplicate) showing that single nucleotide differences in SSU rDNA sequences can be used to (quantitatively) detect second stage juveniles (J2) of the potato cyst nematode species *Globodera rostchiensis*, whereas equal DNA concentrations of its sibling species *G. pallida* hardly give rise to a product (ΔC_T around 20). C_T - cycle number at which the fluorescent signal exceeds the threshold value as indicated by the dotted horizontal line (for most left curve indicated by an arrow).

Zhang and Baldwin, 2001) and the taxonomic position of the family Teratocephalidae is still unclear (De Ley and Blaxter 2002). The family Metateratocephalidae (genera *Euteratocephalus* and *Metateratocephalus*) was included in the Teratocephalidae in the past (Lorenzen 1983), but the current dataset point at a position in a separate clade, Clade 6, that also includes the Plectidae and Chronogastridae. This observation corresponds with the results from a detailed morphological (SEM) study on teratocephalids by Boström (1989). Boström listed eight morphological characters that all call for a taxonomic separation of *Teratocephalus* and *Metateratocephalus*, but he found no phasmids – small organs in the tail region that are characteristic for most Secernentea (in some Secernentean taxa they are secondarily lacking) - in any of the *Teratocephalus* species under investigation. On the basis of SSU rDNA sequence data, members of the genus *Teratocephalus* can be considered as the immediate sister group of the Secernentea.

Did plant parasites evolve from fungivorous ancestors?

A longstanding hypothesis on the evolution of feeding types among nematodes suggests that plant parasitic nematodes arose from fungivorous ancestors (Maggenti 1971). Analysis of full length SSU rDNA data reveals the presence of

fungivorous nematodes (Yeates *et al.* 1993) in three clades. In Clade 1, representatives of the genus Diphtherophora, Clade 2, members of the Tylencholaimidae (Dorylaimida), and Clade 12, various representatives of the Tylenchomorpha. The tree suggests the presence of a fourth group of fungivorous nematode in Clade 10 (not marked in Fig. 2.1 as fungivorous), namely representatives of the Aphelenchoididae (various *Aphelenchoides* species) and Parasitaphelenchidae (*i.e. Bursaphelenchus* spp.). However, the position of the Aphelenchoididae, Parasitaphelenchidae and Seinuridae (Tylenchomorpha) within Clade 10 is most likely an LBA artifact as their GC-content (≈ 46%) and that of the Panagrolaimorpha (≈ 43%) is relatively low and they exhibit long branch lengths as compared to the Aphelenchidae and Paraphelenchidae (GC% ≈ 48%) with whom they are normally associated. Similar concerns were raised by De Ley and Blaxter (2002). It is noted that an additional analysis of Clade 10, 11 and 12 excluding the Panagrolaimorpha did not unite the Paraphelenchidae and Aphelenchidae with the Aphelenchoididae, Parasitaphelenchidae and Seinuridae (*results not shown*). Remarkably, fungivorous nematodes are only observed in clades that contain plant parasitic nematodes as well. In Clades 1 and 12, SSU rDNA shows sufficient variation to determine the phylogenetic relationships between fungivorous and plant parasitic nematodes. The SSU rDNA of the Dorylaimida within Clade 2 is remarkably conserved (possibly an indication for rapid speciation), and consequently the position of fungivores within this clade is unresolved. In Fig. 2.1 we show the SSU rDNA-based phylogenetic relationships between fungivorous and plant parasitic nematodes. In Clade 12 fungivorous nematodes occupy a basal position as compared to their plant parasitic relatives. This finding implies a first molecular support for a hypothesis stating that plant parasitic nematodes arose from fungivorous ancestors. In Clade 1 the fungivores and plant parasites are sister groups and it is impossible to predict the feeding type of their last common ancestor.

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Appendix A

GenBank accession numbers for the SSU rDNA sequences used in this study.

Sequenced for this paper

species	NCBI accession	species	NCBI accession
Achromadora sp.	AY284718	Meloidogyne incognita	AY284621
Achromodora sp.	AY284717	Merlinius brevidens	AY284597
Acrobeles complexus	AY284671	Mermithidae	AY284743
Acrobeloides apiculatus	AY284673	Mesocriconema xenoplax	AY284625
Acrobeloides nanus	AY284672	Mesocriconema xenoplax	AY284626
Alaimus parvus	AY284738	Mesocriconema xenoplax	AY284627
Allodorylaimus andrassyi	AY284801	Mesodorylaimus centrocercus	AY284799
Anaplectus grandepapillatus	AY284697	Mesodorylaimus sp.	AY284780
Anaplectus grandepapillatus	AY284698	Mesorhabditis sp.	AY284660
Anaplectus porosus	AY284696	Metateratocephalus crassidens	AY284686
Anatonchus tridentatus	AY284768	Metateratocephalus crassidens	AY284687
Anatonchus tridentatus	AY284769	Microdorylaimus miser	AY284804
Aphelenchoides bicaudatus	AY284643	Microdorylaimus modestus	AY284805
Aphelenchoides blastophtorus	AY284644	Microdorylaimus modestus	AY284806
Aphelenchoides fragariae	AY284645	Mononchus aquaticus	AY284764
Aphelenchoides sp.	AY284646	Mononchus aquaticus	AY284765
Aphelenchoides sp.	AY284647	Mononchus truncatus	AY284762
Aphelenchus avenae	AY284639	Mylonchulus brachyuris	AY284752
Aphelenchus avenae	AY284640	Mylonchulus brachyuris	AY284753
Aphelenchus sp.	AY284641	Mylonchulus brachyuris	AY284754
Aporcelaimellus cf.. paraobtusicaudatus	AY284812	Mylonchulus rotundicaudatus	AY284751
		Mylonchulus sigmaturus	AY284755
Aporcelaimellus obtusicaudatus	AY284811	Mylonchulus sigmaturus	AY284756
Aporcelaimellus sp.	AY284813	Mylonchulus sigmaturus	AY284757
Aulolaimus oxycephalus	AY284724	Mylonchulus sp.	AY284758
Axonchium propinquum	AY284820	Mylonchulus sp.	AY284759
Bastiana gracilis	AY284725	Mylonchulus sp.	AY284760
Bastiana gracilis	AY284726	Mylonchulus sp.	AY284761
Bathyodontus mirus	AY284744	Neodiplogasteridae	AY284689
Bitylenchus dubius	AY284601	Neodolichorhynchus lamelliferus	AY284598
Bunonema reticulatum	AY284661		
Bursaphelenchus mucronatus	AY284648	Neopsilenchus magnidens	AY284585
Bursaphelenchus sp.	AY284649	Nygolaimus cf. brachyuris	AY284770
Bursaphelenchus sp.	AY284650	Nygolaimus cf. brachyuris	AY284771

Caenorhabditis elegans	AY284652	Opisthodorylaimus sylphoides	AY284785
Carcharodiscus banaticus	AY284827	Opisthodorylaimus sylphoides	AY284786
Cephalenchus hexalineatus	AY284594	Ottolenchus discrepans	AY284590
Cephalobus persegnis	AY284662	Oxydirus oxycephaloides	AY284823
Cephalobus persegnis	AY284663	Oxydirus oxycephalus	AY284824
Ceratoplectus armatus	AY284706	Oxydirus oxycephalus	AY284825
Cervidellus sp.	AY284674	Panagrolaimus subelongatus	AY284681
cf. Tylencholaimus	AY284832	Paractinolaimus macrolaimus	AY284826
cf. Tylencholaimus	AY284833	Paramphidelus hortensis	AY284739
Chiloplacus propinquus	AY284677	Paramphidelus sp.	AY284740
Choanolaimus psammophilus	AY284714	Paramphidelus sp.	AY284741
Choanolaimus psammophilus	AY284715	Paramphidelus sp.	AY284742
Choanolaimus psammophilus	AY284716	Paraphelenchus sp.	AY284642
Chromadoridae	AY284713	Paratripyla sp.	AY284737
Chronogaster sp.	AY284708	Paratylenchus cf.	AY284634
Chronogaster sp.	AY284709	neoamblycephalus	
Chrysonema attenuatum	AY284779	Paratylenchus microdorus	AY284632
Clarkus papillatus	AY284748	Paratylenchus microdorus	AY284633
Clarkus papillatus	AY284749	Paratylenchus straeleni	AY284630
Clarkus papillatus	AY284750	Paratylenchus straeleni	AY284631
Clavicaudoides trophurus	AY284772	Paravulvus hartingii	AY284774
Clavicaudoides trophurus	AY284773	Paravulvus hartingii	AY284775
Coomansus parvus	AY284766	Paraxonchium laetificans	AY284808
Coomansus parvus	AY284767	Paraxonchium laetificans	AY284809
Coslenchus cf. franklinae	AY284582	Paraxonchium laetificans	AY284810
Coslenchus costatus	AY284581	Plectus aquatilis	AY284700
Coslenchus franklinae	AY284583	Plectus cf. cirratus	AY284701
Cruznema sp.	AY284655	Plectus cf. parietinus	AY284702
Cruznema sp.	AY284656	Plectus cf. parietinus	AY284703
Cruznema sp.	AY284657	Plectus cf. parvus	AY284699
Cruznema sp.	AY284658	Plectus cf. pusillus	AY284704
Diphterophora obesa	AY284838	Plectus cf. pusillus	AY284705
Diphterophora obesa	AY284839	Pratylenchus crenatus	AY284610
Discolaimus major	AY284828	Pratylenchus pratensis	AY284611
Ditylenchus brevicauda	AY284635	Pratylenchus thornei	AY284612
Ditylenchus dipsaci	AY284636	Pratylenchus thornei	AY284613
Ditylenchus sp.	AY284637	Prionchulus muscorum	AY284745
Dorylaimellus montenegricus	AY284821	Prionchulus punctatus	AY284746
Dorylaimoides micoletzkyi	AY284830	Prionchulus punctatus	AY284747
Dorylaimus stagnalis	AY284776	Prismatolaimus cf. dolichurus	AY284727
Dorylaimus stagnalis	AY284777	Prismatolaimus cf. dolichurus	AY284728
Drilocephalobus sp.	AY284678	Prismatolaimus intermedius	AY284729

Drilocephalobus sp.	AY284679	Pristionchus lheritieri	AY284690
Ecumenicus monohystera	AY284783	Prodesmodora circulata	AY284719
Ecumenicus monohystera	AY284784	Prodesmodora circulata	AY284720
Ecumenicus sp.	AY284781	Prodesmodora circulata	AY284721
Ecumenicus sp.	AY284782	Prodesmodora circulata	AY284722
Enchodelus macrodorus	AY284791	Prodorylaimus uliginosus	AY284778
Enchodelus sp.	AY284792	Pseudhalenchus minutus	AY284638
Enchodelus sp.	AY284793	Psilenchus cf. hilarulus	AY284593
Epidorylaimus lugdunensis	AY284802	Pungentus silvestris	AY284788
Epidorylaimus lugdunensis	AY284803	Rhabditella axei	AY284654
Eucephalobus cf. oxyuroides	AY284664	Rhabditis cf. terricola	AY284653
Eucephalobus oxyuroides	AY284665	Rhabdolaimus cf. terrestris	AY284710
Eucephalobus striatus	AY284666	Rhabdolaimus cf. terrestris	AY284711
Eucephalobus striatus	AY284667	Rhabdolaimus cf. terrestris	AY284712
Eudorylaimus sp.	AY284800	Rotylenchus goodeyi	AY284609
Eumonhystera cf. simplex	AY284692	Rotylenchus sp.	AY284608
Euteratocephalus palustris	AY284684	Sauertylenchus maximus	AY284602
Euteratocephalus sp.	AY284685	Sauertylenchus maximus	AY284603
Filenchus filiformis	AY284592	Sauertylenchus maximus	AY284604
Filenchus thornei	AY284591	Scutylenchus quadrifer	AY284599
Globodera pallida	AY284618	Sectonema barbatoides	AY284814
Globodera pallida	AY284620	Sectonema sp.	AY284815
Helicotylenchus canadensis	AY284605	Seinura sp.	AY284651
Helicotylenchus pseudorobustus	AY284606	Steinernema glaseri	AY284682
Helicotylenchus vulgaris	AY284607	Teratocephalus terrestris	AY284683
Hemicriconemoides	AY284622	Theristus agilis	AY284693
pseudobrachyurus		Theristus agilis	AY284694
Hemicriconemoides	AY284623	Theristus agilis	AY284695
pseudobrachyurus		Thonus circulifer	AY284795
Hemicriconemoides	AY284624	Thonus minutus	AY284794
pseudobrachyurus		Thonus sp.	AY284796
Heterocephalobus elongatus	AY284668	Thonus sp.	AY284797
Heterocephalobus elongatus	AY284669	Thonus sp.	AY284798
Heterocephalobus elongatus	AY284670	Thornia steatopyga	AY284787
Heterodera schachtii	AY284617	Trichodorus variopapillatus	AY284841
Hirschmanniella sp.1	AY284614	Tripyla cf. filicaudata	AY284730
Hirschmanniella sp.2	AY284615	Tripyla cf. filicaudata	AY284731
Hirschmanniella sp.3	AY284616	Tripyla sp.	AY284732
Labronema vulvapapillatum	AY284807	Trischistoma sp.	AY284735
Lelenchus leptosoma	AY284584	Trischistoma sp.	AY284736
Leptonchus granulosus	AY284831	Trypila sp.	AY284733
Longidorella sp.	AY284789	Trypila sp.	AY284734

Longidorella sp.	AY284790	Tylencholaimellus striatus	AY284837
Longidorus cf. intermedius	AY284816	Tylencholaimus mirabilis	AY284835
Longidorus dunensis	AY284817	Tylencholaimus mirabilis	AY284836
Longidorus dunensis	AY284818	Tylencholaimus sp.	AY284834
Longidorus dunensis	AY284819	Tylenchus davainei	AY284588
Loofia thienemanni	AY284628	Tylenchus sp.	AY284589
Loofia thienemanni	AY284629	Tylocephalus auriculatus	AY284707
Macrotrophurus arbusticola	AY284595	Zeldia sp.	AY284675
Macrotrophurus arbusticola	AY284596	Zeldia sp.	AY284676
Malenchus andrassyi	AY284587		

only used for tree with basal clades:

Granonchulus sp.	AY593953	Mononchus tunbridgensis	AY593954
Diphterophora communis	AY593955	Prismatolaimus dolichurus	AY593956
Mononchus tunbridgensis	AY284763	Prismatolaimus dolichurus	AY593957

sequences acquired from Genbank

species	**NCBI accession**	**species**	**NCBI accession**
Acanthopharynx micans	Y16911	Mesorhabditis sp.	U73452
Acrobeles ciliatus	AF202148	Mesorhabditis spiculigera	AF083016
Acrobeles complexus	U81577	Metachromadora sp.	AF036595
Acrobeles sp.	U81576	Mylonchulus arenicolus	AF036596
Acrobeloides bodenheimeri	AF202159	Myolaimus sp.	U81585
Acrobeloides bodenheimeri	AF202162	Nematodirus battus	U01230
Acrobeloides sp.	AF034391	Nippostrongylus brasiliensis	AF036597
Acrostichus halicti	U61759	Oscheius dolichuroides	AF082998
Adoncholaimus sp.	AF036642	Oscheius insectivora	AF083019
Anisakis sp.	U81575	Oscheius myriophila	U13936
Anisakis sp.	U94365	Oscheius myriophila	U81588
Aphelenchoides fragariae	AB067755	Oscheius sp.	AF082994
Aphelenchus avenae	AF036586	Oscheius sp.	AF082995
Ascaris lumbricoides	U94366	Oscheius sp.	U81587
Ascaris suum	U94367	Ostertagia ostertagi	AF036598
Baylisascaris procyonis	U94368	Otostrongylus sp.	U81589
Baylisascaris transfuga	U94369	Panagrellus redivivus	AF036599
Brevibucca sp.	AF202163	Panagrellus redivivus	AF083007
Brugia malayi	AF036588	Panagrobelus stammeri	AF202153
Brumptaemilius justini	AF036589	Panagrolaimus sp.	U81579
Bunonema sp.	U81582	Paracanthonchus caecus	AF047888

Bursaphelenchus mucronatus	AB067759	Parafilaroides sp.	U81590
Bursaphelenchus sp.	AF037369	Parascaris equorum	U94378
Bursaphelenchus xylophilus	AB067760	Parasitorhabditis sp.	AF083028
Caenorhabditis briggsae	U13929	Paraspidodera sp.	AF083005
Caenorhabditis drosophilae	AF083025	Paratrichodorus anemones	AF036600
Caenorhabditis elegans	X03680	Paratrichodorus pachydermus	AF036601
Caenorhabditis sonorae	AF083026	Pellioditis marina	AF083021
Caenorhabditis sp.	AF083006	Pellioditis mediterranea	AF083020
Caenorhabditis sp.	U13930	Pellioditis typica	U13933
Caenorhabditis vulgaris	U13931	Pelodera teres	AF083002
Catanema sp.	Y16912	Philonema sp.	U81574
Cephaloboides sp.	AF083027	Plectonchus hunti	AF202154
Cephalobus cubaensis	AF202161	Plectus acuminatus	AF037628
Cephalobus oryzae	AF034390	Plectus aquatilis	AF036602
Cephalobus sp.	AF202158	Plectus sp.	U61761
Cephalobus sp.	AF202160	Poikilolaimus oxycerca	AF083023
Cervidellus alutus	AF202152	Poikilolaimus regenfussi	AF083022
Choriorhabditis dudichi	AF083012	Poikilolaimus sp.	U81583
Chromadoropsis vivipara	AF047891	Pontonema vulgare	AF047890
Contracaecum multipapillatum	U94370	Porrocaecum depressum	U94379
Cruzia americana	U94371	Praeacanthonchus sp.	AF036612
Cruznema tripartita	U73449	Pratylenchoides magnicauda	AF202157
Cylindrolaimus sp.	AF202149	Prismatolaimus intermedius	AF036603
Daptonema procerum	AF047889	Pristionchus lheritieri	AF036640
Dentostomella sp.	AF036590	Pristionchus lheritieri	AF036643
Desmodora ovigera	Y16913	Pristionchus pacificus	AF083010
Diplolaimelloides meyli	AF036644	Pristionchus pacificus	U81584
Diploscapter sp.	AF083009	Protorhabditis sp.	AF083001
Diploscapter sp.	U81586	Protorhabditis sp.	AF083024
Dirofilaria immitis	AF036638	Pseudacrobeles variabilis	AF202150
Distolabrellus veechi	AF082999	Pseudoterranova decipiens	U94380
Distolabrellus veechi	AF083011	Rhabditella axei	U13934
Dolichorhabditis sp.	AF036591	Rhabditella sp.	AF083000
Enoplus brevis	U88336	Rhabditis blumi	U13935
Enoplus meridionalis	Y16914	Rhabditis sp.	AF083008
Eubostrichus dianae	Y16915	Rhabditoides inermiformis	AF083017
Eubostrichus parasitiferus	Y16916	Rhabditoides inermis	AF082996
Eubostrichus topiarius	Y16917	Rhabditoides regina	AF082997
Globodera pallida	AF036592	Rhabditophanes sp.	AF202151
Gnathostoma binucleatum	Z96946	Robbea hypermnestra	Y16921
Gnathostoma neoprocyonis	Z96947	Steinernema carpocapsae	AF036604
Gnathostoma turgidum	Z96948	Stilbonema majum	Y16922

Goezia pelagia	U94372	Strongyloides ratti	U81581
Haemonchus contortus	L04153	Strongyloides stercoralis	AF279916
Haemonchus similis	L04152	Strongyloides stercoralis	AJ417023
Halicephalobus gingivalis	AF202156	Strongyloides stercoralis	M84229
Heterakis sp.	AF083003	Subanguina radicicola	AF202164
Heterocheilus tunicatus	U94373	Syngamus trachea	AF036606
Heterorhabditis bacteriophora	AF036593	Teratocephalus lirellus	AF036607
Heterorhabditis hepialus	AF083004	Teratorhabditis palmarum	U13937
Hysterothylacium fortalezae	U94374	Teratorhabditis synpapillata	AF083015
Hysterothylacium pelagicum	U94375	Terranova caballeroi	U94381
Hysterothylacium reliquens	U94376	Toxascaris leonina	U94383
Iheringascaris inquies	U94377	Toxocara canis	U94382
Laxus cosmopolitus	Y16918	Trefusia zostericola	AF329937
Laxus oneistus	Y16919	Trichinella spiralis	U60231
Leptonemella sp.	Y16920	Trichodorus primitivus	AF036609
Litomosoides sigmodontis	AF227233	Trichuris muris	AF036637
Longidorus elongatus	AF036594	Turbatrix aceti	AF202165
Meloidogyne arenaria	U42342	Tylocephalus auriculatus	AF202155
Meloidogyne incognita	U81578	Wuchereria bancrofti	AF227234
Mermis nigrescens	AF036641	Xiphinema rivesi	AF036610
Mesorhabditis anisomorpha	AF083013	Xyzzors sp.	Y16923
Mesorhabditis scanica	AF083014	Zeldia punctata	U61760

only used for tree with basal clades:

Anoplostoma rectospiculum	AY590149	Miconchus cf. fasciatus	AY552973
Bathyodontus cylindricus	AY552964	Mononchus aquaticus	AY297821
Campydora demonstrans	AY552965	Mylonchulus sp.	AJ875156
Clarkus papillatus	AY552966	Soboliphyme baturini	AY277895
Diphtherophora obesa	AY552968	Trichinella britovi	AY851257
Ironus sp.	AY552970	Trichuris suis	AY851265
Isolaimium sp.	AY552971	Trichuris suis	AY856093

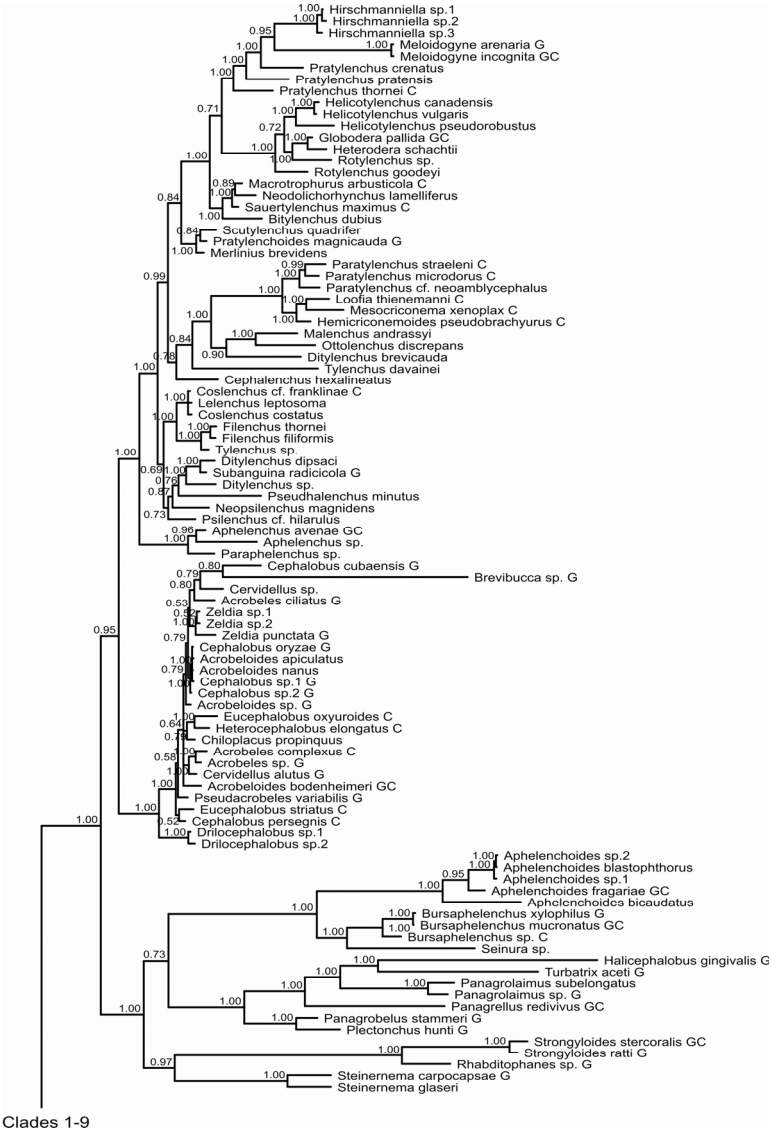
Outgroup sequences acquired from GenBank

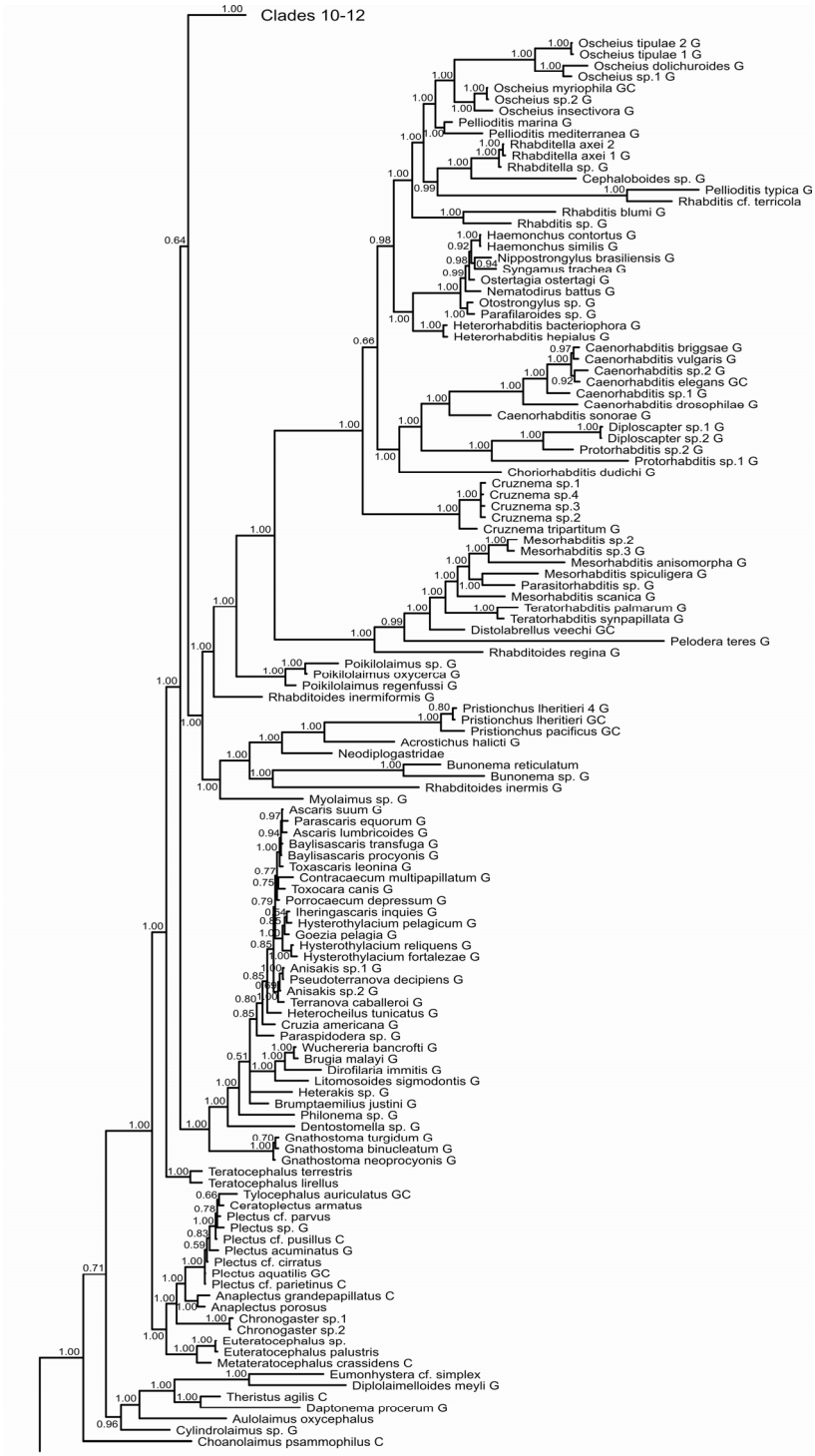
phylum	**species**	**NCBI accession**
Arthropoda	Dilta littoralis	AF005457
Arthropoda	Podura aquatica	AF005452
Arthropoda	Polydesmus coriaceus	AF005449
Kinorhyncha	Pycnophyes kielensis	U67997
Nematomorpha	Chordodes morgani	AF036639

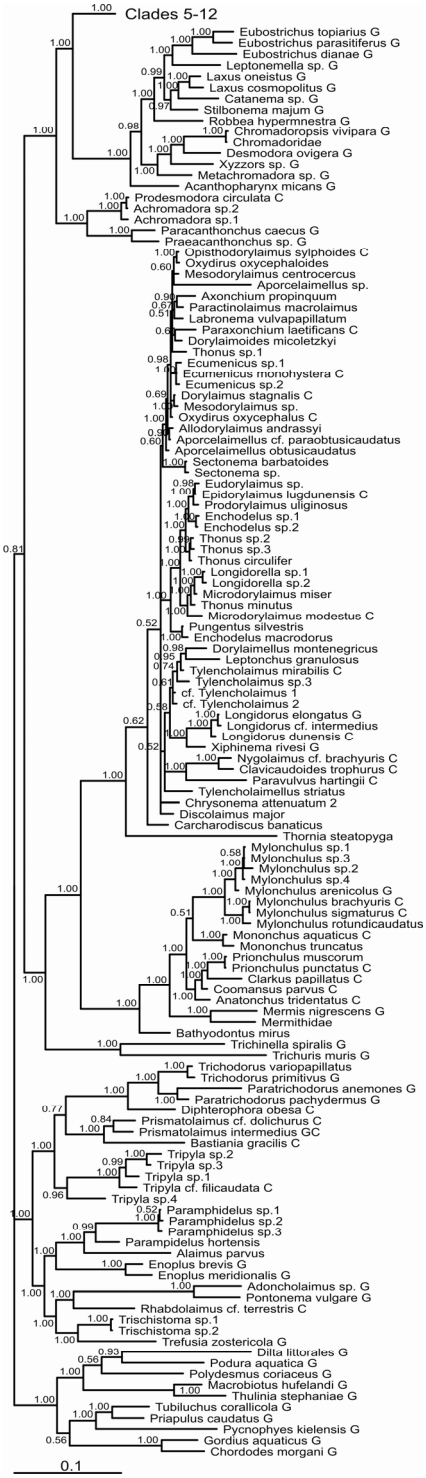
Nematomorpha	Gordius aquaticus	X80233
Priapulida	Priapulus caudatus	Z38009
Priapulida	Tubiluchus corallicola	AF119086
Tardigrada	Macrobiotus hufelandi	X81442
Tardigrada	Thulinia stephaniae	AF056023

Appendix B

Complete tree from the Bayesian phylogenetic reconstruction of the phylum Nematoda, depicted in shortened form in Fig. 2.1. Numbers indicate the posterior probability. A 'G' behind the name indicates the sequence was acquired from GenBank. A 'C' behind the name means that the sequence is a consensus of multiple sequences of the same species.

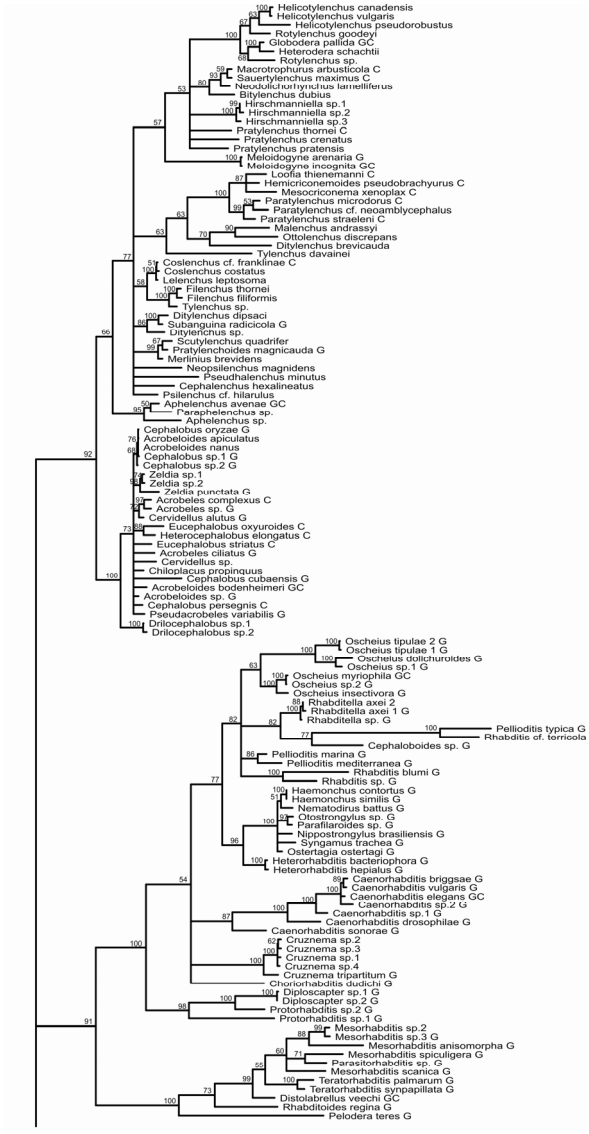



Clades 10-12
Oscheius tipulae 2 G
Oscheius tipulae 1 G
Oscheius dolichuroides G
Oscheius sp.1 G
Oscheius myriophila GC
Oscheius sp.2 G
Oscheius insectivora G
Pellioditis marina G
Pellioditis mediterranea G
Rhabditella axei 2
Rhabditella axei 1 G
Rhabditella sp. G
Cephaloboides sp. G
Pellioditis typica G
Rhabditis cf. terricola
Rhabditis blumi G
Rhabditis sp. G
Haemonchus contortus G
Haemonchus similis G
Nippostrongylus brasiliensis G
Syngamus trachea G
Ostertagia ostertagi G
Nematodirus battus G
Otostrongylus sp. G
Parafilaroides sp. G
Heterorhabditis bacteriophora G
Heterorhabditis hepialus G
Caenorhabditis briggsae G
Caenorhabditis vulgaris G
Caenorhabditis sp.2 G
Caenorhabditis elegans GC
Caenorhabditis sp.1 G
Caenorhabditis drosophilae G
Caenorhabditis sonorae G
Diploscapter sp.1 G
Diploscapter sp.2 G
Protorhabditis sp.2 G
Protorhabditis sp.1 G
Choriorhabditis dudichi G
Cruznema sp.1
Cruznema sp.4
Cruznema sp.3
Cruznema sp.2
Cruznema tripartitum G
Mesorhabditis sp.2
Mesorhabditis sp.3 G
Mesorhabditis anisomorpha G
Mesorhabditis spiculigera G
Parasitorhabditis sp. G
Mesorhabditis scanica G
Teratorhabditis palmarum G
Teratorhabditis synpapillata G
Distolabrellus veechi GC
Pelodera teres G
Rhabditoides regina G
Poikilolaimus sp. G
Poikilolaimus oxycerca G
Poikilolaimus regenfussi G
Rhabditoides inermiformis G
Pristionchus lheritieri 4 G
Pristionchus lheritieri GC
Pristionchus pacificus GC
Acrostichus halicti G
Neodiplogastridae
Bunonema reticulatum
Bunonema sp. G
Rhabditoides inermis G
Myolaimus sp. G
Ascaris suum G
Parascaris equorum G
Ascaris lumbricoides G
Baylisascaris transfuga G
Baylisascaris procyonis G
Toxascaris leonina G
Contracaecum multipapillatum G
Toxocara canis G
Porrocaecum depressum G
Iheringascaris inquies G
Hysterothylacium pelagicum G
Goezia pelagia G
Hysterothylacium reliquens G
Hysterothylacium fortalezae G
Anisakis sp.1 G
Pseudoterranova decipiens G
Anisakis sp.2 G
Terranova caballeroi G
Heterochelius tunicatus G
Cruzia americana G
Paraspidodera sp. G
Wuchereria bancrofti G
Brugia malayi G
Dirofilaria immitis G
Litomosoides sigmodontis G
Heterakis sp. G
Brumptaemilius justini G
Philonema sp. G
Dentostomella sp. G
Gnathostoma turgidum G
Gnathostoma binucleatum G
Gnathostoma neoprocyonis G
Teratocephalus terrestris
Teratocephalus lirellus
Tylocephalus auriculatus GC
Ceratoplectus armatus
Plectus cf. parvus
Plectus sp. G
Plectus cf. pusillus C
Plectus acuminatus G
Plectus cf. cirratus
Plectus aquatilis GC
Plectus cf. parietinus C
Anaplectus grandepapillatus C
Anaplectus porosus
Chronogaster sp.1
Chronogaster sp.2
Euteratocephalus sp.
Euteratocephalus palustris
Metateratocephalus crassidens C
Eumonhystera cf. simplex
Diplolaimelloides meyli G
Theristus agilis C
Daptonema procerum G
Aulolaimus oxycephalus
Cylindrolaimus sp. G
Choanolaimus psammophilus C
Clades 1-4

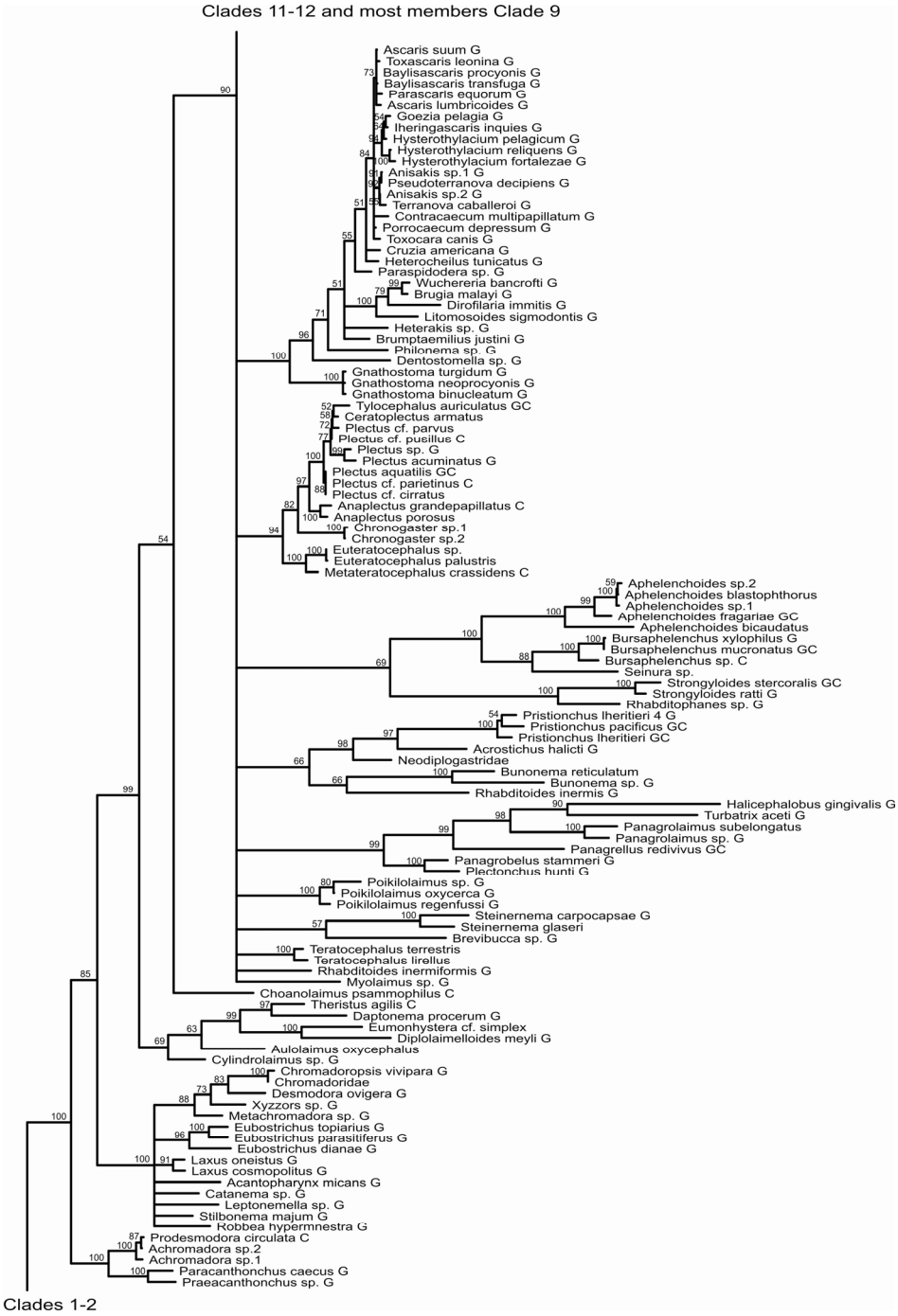

Clades 5-12
Eubostrichus topiarius G
Eubostrichus parasitiferus G
Eubostrichus dianae G
Leptonemella sp. G
Laxus oneistus G
Laxus cosmopolitus G
Catanema sp. G
Stilbonema majum G
Robbea hypermnestra G
Chromadoropsis vivipara G
Chromadoridae
Desmodora ovigera G
Xyzzors sp. G
Metachromadora sp. G
Acanthopharynx micans G
Prodesmodora circulata C
Achromadora sp.2
Achromadora sp.1
Paracanthonchus caecus G
Praeacanthonchus sp. G
Opisthodorylaimus sylphoides C
Oxydirus oxycephaloides
Mesodorylaimus centrocercus
Aporcelaimellus sp.
Axonchium propinquum
Paractinolaimus macrolaimus
Labronema vulvapapillatum
Paraxonchium laetificans C
Dorylaimoides micoletzkyi
Thonus sp.1
Ecumenicus sp.1
Ecumenicus monohystera C
Ecumenicus sp.2
Dorylaimus stagnalis C
Mesodorylaimus sp.
Oxydirus oxycephalus C
Allodorylaimus andrassyi
Aporcelaimellus cf. paraobtusicaudatus
Aporcelaimellus obtusicaudatus
Sectonema barbatoides
Sectonema sp.
Eudorylaimus sp.
Epidorylaimus lugdunensis C
Prodorylaimus uliginosus
Enchodelus sp.1
Enchodelus sp.2
Thonus sp.2
Thonus sp.3
Thonus circulifer
Longidorella sp.1
Longidorella sp.2
Microdorylaimus miser
Thonus minutus
Microdorylaimus modestus C
Pungentus silvestris
Enchodelus macrodorus
Dorylaimellus montenegricus
Leptonchus granulosus
Tylencholaimus mirabilis C
Tylencholaimus sp.3
cf. Tylencholaimus 1
cf. Tylencholaimus 2
Longidorus elongatus G
Longidorus cf. intermedius
Longidorus dunensis C
Xiphinema rivesi G
Nygolaimus cf. brachyuris C
Clavicaudoides trophurus C
Paravulvus hartingii C
Tylencholaimellus striatus
Chrysonema attenuatum 2
Discolaimus major
Carcharodiscus banaticus
Thornia steatopyga
Mylonchulus sp.1
Mylonchulus sp.3
Mylonchulus sp.2
Mylonchulus sp.4
Mylonchulus arenicolus G
Mylonchulus brachyuris C
Mylonchulus sigmaturus C
Mylonchulus rotundicaudatus
Mononchus aquaticus C
Mononchus truncatus
Prionchulus muscorum
Prionchulus punctatus C
Clarkus papillatus C
Coomansus parvus C
Anatonchus tridentatus C
Mermis nigrescens G
Mermithidae
Bathyodontus mirus
Trichinella spiralis G
Trichuris muris G
Trichodorus variopapillatus
Trichodorus primitivus G
Paratrichodorus anemones G
Paratrichodorus pachydermus G
Diphterophora obesa C
Prismatolaimus cf. dolichurus C
Prismatolaimus intermedius GC
Bastiania gracilis C
Tripyla sp.2
Tripyla sp.3
Tripyla sp.1
Tripyla cf. filicaudata C
Tripyla sp.4
Paramphidelus sp.1
Paramphidelus sp.2
Paramphidelus sp.3
Parampidelus hortensis
Alaimus parvus
Enoplus brevis G
Enoplus meridionalis G
Adoncholaimus sp. G
Pontonema vulgare G
Rhabdolaimus cf. terrestris C
Trischistoma sp.1
Trischistoma sp.2
Trefusia zostericola G
Dilta littorales G
Podura aquatica G
Polydesmus coriaceus G
Macrobiotus hufelandi G
Thulinia stephaniae G
Tubiluchus corallicola G
Priapulus caudatus G
Pycnophyes kielensis G
Gordius aquaticus G
Chordodes morgani G
0.1

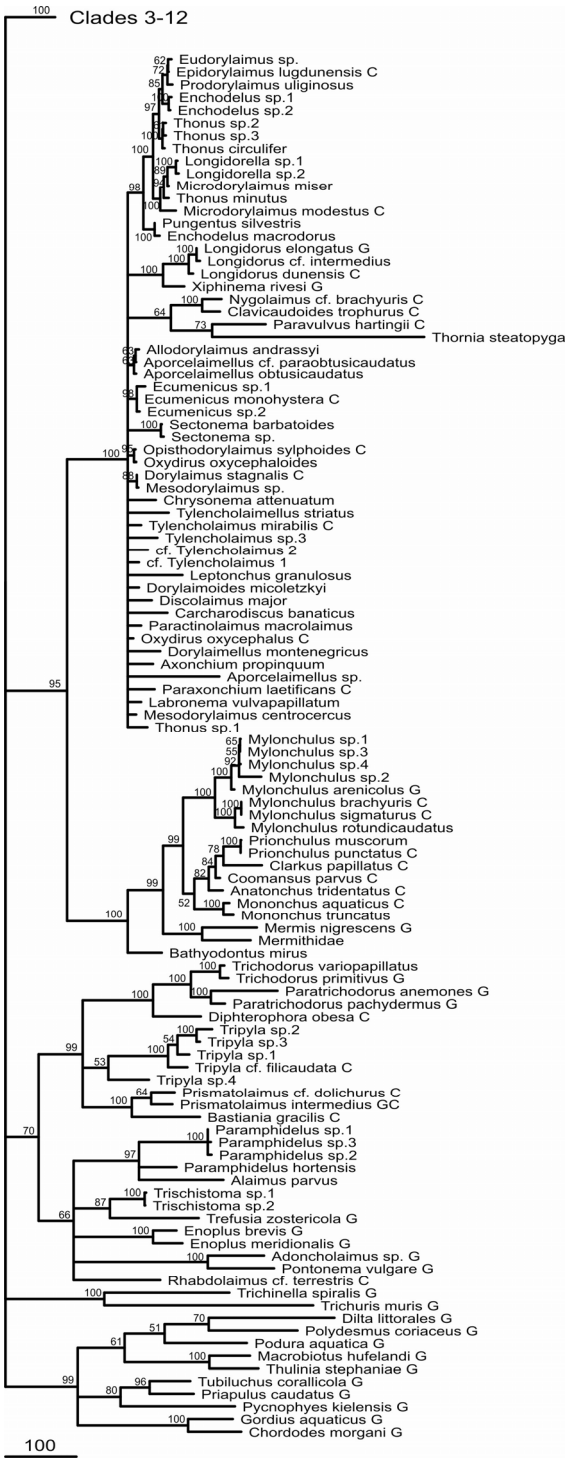
Appendix C

Complete maximum parsimony tree of the phylum Nematoda. Numbers near nodes indicate bootstrap values. A 'G' behind the name indicates the sequence was acquired from GenBank. A 'C' behind the name means that the sequence is a consensus of multiple sequences of the same species.



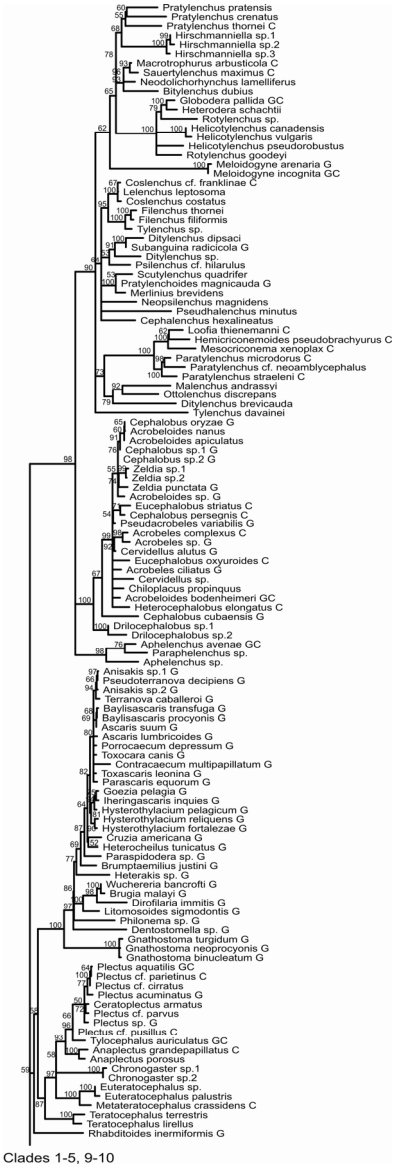
Clades 1-8, 10 and other members of Clade 9


Clades 11-12 and most members Clade 9
Ascaris suum G
Toxascaris leonina G
Baylisascaris procyonis G
Baylisascaris transfuga G
Parascaris equorum G
Ascaris lumbricoides G
Goezia pelagia G
Iheringascaris inquies G
Hysterothylacium pelagicum G
Hysterothylacium reliquens G
Hysterothylacium fortalezae G
Anisakis sp.1 G
Pseudoterranova decipiens G
Anisakis sp.2 G
Terranova caballeroi G
Contracaecum multipapillatum G
Porrocaecum depressum G
Toxocara canis G
Cruzia americana G
Heterocheilus tunicatus G
Paraspidodera sp. G
Wuchereria bancrofti G
Brugia malayi G
Dirofilaria immitis G
Litomosoides sigmodontis G
Heterakis sp. G
Brumptaemilius justini G
Philonema sp. G
Dentostomella sp. G
Gnathostoma turgidum G
Gnathostoma neoprocyonis G
Gnathostoma binucleatum G
Tylocephalus auriculatus GC
Ceratoplectus armatus
Plectus cf. parvus
Plectus cf. pusillus C
Plectus sp. G
Plectus acuminatus G
Plectus aquatilis GC
Plectus cf. parietinus C
Plectus cf. cirratus
Anaplectus grandepapillatus C
Anaplectus porosus
Chronogaster sp.1
Chronogaster sp.2
Euteratocephalus sp.
Euteratocephalus palustris
Metateratocephalus crassidens C
Aphelenchoides sp.2
Aphelenchoides blastophthorus
Aphelenchoides sp.1
Aphelenchoides fragariae GC
Aphelenchoides bicaudatus
Bursaphelenchus xylophilus G
Bursaphelenchus mucronatus GC
Bursaphelenchus sp. C
Seinura sp.
Strongyloides stercoralis GC
Strongyloides ratti G
Rhabditophanes sp. G
Pristionchus lheritieri 4 G
Pristionchus pacificus GC
Pristionchus lheritieri GC
Acrostichus halicti G
Neodiplogastridae
Bunonema reticulatum
Bunonema sp. G
Rhabditoides inermis G
Halicephalobus gingivalis G
Turbatrix aceti G
Panagrolaimus subelongatus
Panagrolaimus sp. G
Panagrellus redivivus GC
Panagrobelus stammeri G
Plectonchus hunti G
Poikilolaimus sp. G
Poikilolaimus oxycerca G
Poikilolaimus regenfussi G
Steinernema carpocapsae G
Steinernema glaseri
Brevibucca sp. G
Teratocephalus terrestris
Teratocephalus lirellus
Rhabditoides inermiformis G
Myolaimus sp. G
Choanolaimus psammophilus C
Theristus agilis C
Daptonema procerum G
Eumonhystera cf. simplex
Diplolaimelloides meyli G
Aulolaimus oxycephalus
Cylindrolaimus sp. G
Chromadoropsis vivipara G
Chromadoridae
Desmodora ovigera G
Xyzzors sp. G
Metachromadora sp. G
Eubostrichus topiarius G
Eubostrichus parasitiferus G
Eubostrichus dianae G
Laxus oneistus G
Laxus cosmopolitus G
Acantopharynx micans G
Catanema sp. G
Leptonemella sp. G
Stilbonema majum G
Kobbea hypermnestra G
Prodesmodora circulata C
Achromadora sp.2
Achromadora sp.1
Paracanthonchus caecus G
Praeacanthonchus sp. G
Clades 1-2

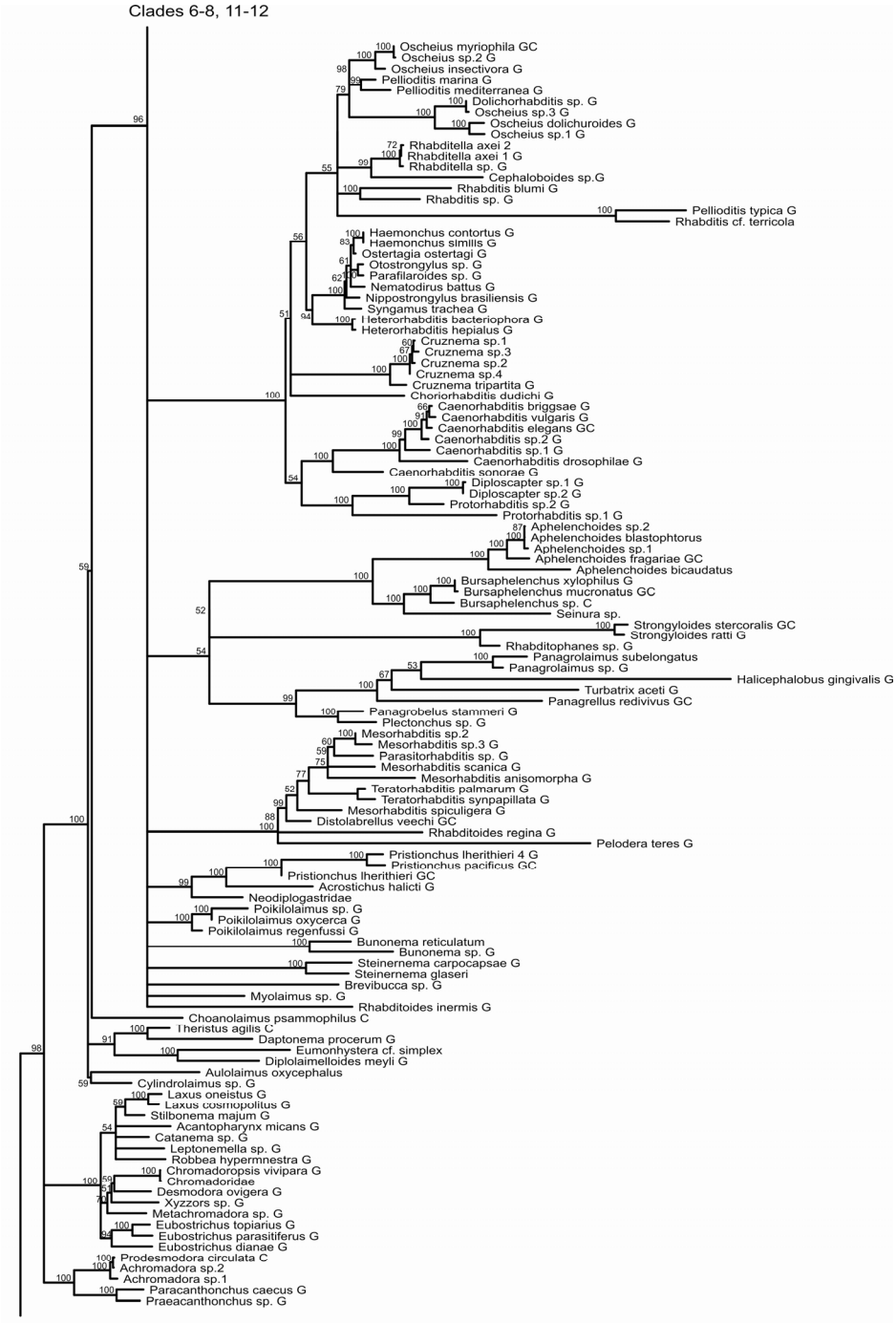

Clades 3-12
Eudorylaimus sp.
Epidorylaimus lugdunensis C
Prodorylaimus uliginosus
Enchodelus sp.1
Enchodelus sp.2
Thonus sp.2
Thonus sp.3
Thonus circulifer
Longidorella sp.1
Longidorella sp.2
Microdorylaimus miser
Thonus minutus
Microdorylaimus modestus C
Pungentus silvestris
Enchodelus macrodorus
Longidorus elongatus G
Longidorus cf. intermedius
Longidorus dunensis C
Xiphinema rivesi G
Nygolaimus cf. brachyuris C
Clavicaudoides trophurus C
Paravulvus hartingii C
Thornia steatopyga
Allodorylaimus andrassyi
Aporcelaimellus cf. paraobtusicaudatus
Aporcelaimellus obtusicaudatus
Ecumenicus sp.1
Ecumenicus monohystera C
Ecumenicus sp.2
Sectonema barbatoides
Sectonema sp.
Opisthodorylaimus sylphoides C
Oxydirus oxycephaloides
Dorylaimus stagnalis C
Mesodorylaimus sp.
Chrysonema attenuatum
Tylencholaimellus striatus
Tylencholaimus mirabilis C
Tylencholaimus sp.3
cf. Tylencholaimus 2
cf. Tylencholaimus 1
Leptonchus granulosus
Dorylaimoides micoletzkyi
Discolaimus major
Carcharodiscus banaticus
Paractinolaimus macrolaimus
Oxydirus oxycephalus C
Dorylaimellus montenegricus
Axonchium propinquum
Aporcelaimellus sp.
Paraxonchium laetificans C
Labronema vulvapapillatum
Mesodorylaimus centrocercus
Thonus sp.1
Mylonchulus sp.1
Mylonchulus sp.3
Mylonchulus sp.4
Mylonchulus sp.2
Mylonchulus arenicolus G
Mylonchulus brachyuris C
Mylonchulus sigmaturus C
Mylonchulus rotundicaudatus
Prionchulus muscorum
Prionchulus punctatus C
Clarkus papillatus C
Coomansus parvus C
Anatonchus tridentatus C
Mononchus aquaticus C
Mononchus truncatus
Mermis nigrescens G
Mermithidae
Bathyodontus mirus
Trichodorus variopapillatus
Trichodorus primitivus G
Paratrichodorus anemones G
Paratrichodorus pachydermus G
Diphterophora obesa C
Tripyla sp.2
Tripyla sp.3
Tripyla sp.1
Tripyla cf. filicaudata C
Tripyla sp.4
Prismatolaimus cf. dolichurus C
Prismatolaimus intermedius GC
Bastiania gracilis C
Paramphidelus sp.1
Paramphidelus sp.3
Paramphidelus sp.2
Paramphidelus hortensis
Alaimus parvus
Trischistoma sp.1
Trischistoma sp.2
Trefusia zostericola G
Enoplus brevis G
Enoplus meridionalis G
Adoncholaimus sp. G
Pontonema vulgare G
Rhabdolaimus cf. terrestris C
Trichinella spiralis G
Trichuris muris G
Dilta littorales G
Polydesmus coriaceus G
Podura aquatica G
Macrobiotus hufelandi G
Thulinia stephaniae G
Tubiluchus corallicola G
Priapulus caudatus G
Pycnophyes kielensis G
Gordius aquaticus G
Chordodes morgani G
100

Appendix D

Complete neighbor-joining tree of the phylum Nematoda. Numbers near nodes indicate bootstrap values. A 'G' behind the name indicates the sequence was acquired from GenBank. A 'C' behind the name means that the sequence is a consensus of multiple sequences of the same species.



Clades 1-5, 9-10


Clades 6-8, 11-12
Oscheius myriophila GC
Oscheius sp.2 G
Oscheius insectivora G
Pellioditis marina G
Pellioditis mediterranea G
Dolichorhabditis sp. G
Oscheius sp.3 G
Oscheius dolichuroides G
Oscheius sp.1 G
Rhabditella axei 2
Rhabditella axei 1 G
Rhabditella sp. G
Cephaloboides sp.G
Rhabditis blumi G
Rhabditis sp. G
Pellioditis typica G
Rhabditis cf. terricola
Haemonchus contortus G
Haemonchus similis G
Ostertagia ostertagi G
Otostrongylus sp. G
Parafilaroides sp. G
Nematodirus battus G
Nippostrongylus brasiliensis G
Syngamus trachea G
Heterorhabditis bacteriophora G
Heterorhabditis hepialus G
Cruznema sp.1
Cruznema sp.3
Cruznema sp.2
Cruznema sp.4
Cruznema tripartita G
Choriorhabditis dudichi G
Caenorhabditis briggsae G
Caenorhabditis vulgaris G
Caenorhabditis elegans GC
Caenorhabditis sp.2 G
Caenorhabditis sp.1 G
Caenorhabditis drosophilae G
Caenorhabditis sonorae G
Diploscapter sp.1 G
Diploscapter sp.2 G
Protorhabditis sp.2 G
Protorhabditis sp.1 G
Aphelenchoides sp.2
Aphelenchoides blastophtorus
Aphelenchoides sp.1
Aphelenchoides fragariae GC
Aphelenchoides bicaudatus
Bursaphelenchus xylophilus G
Bursaphelenchus mucronatus GC
Bursaphelenchus sp. C
Seinura sp.
Strongyloides stercoralis GC
Strongyloides ratti G
Rhabditophanes sp. G
Panagrolaimus subelongatus
Panagrolaimus sp. G
Halicephalobus gingivalis G
Turbatrix aceti G
Panagrellus redivivus GC
Panagrobelus stammeri G
Plectonchus sp. G
Mesorhabditis sp.2
Mesorhabditis sp.3 G
Parasitorhabditis sp. G
Mesorhabditis scanica G
Mesorhabditis anisomorpha G
Teratorhabditis palmarum G
Teratorhabditis synpapillata G
Mesorhabditis spiculigera G
Distolabrellus veechi GC
Rhabditoides regina G
Pelodera teres G
Pristionchus lheritieri 4 G
Pristionchus pacificus GC
Pristionchus lheritieri GC
Acrostichus halicti G
Neodiplogastridae
Poikilolaimus sp. G
Poikilolaimus oxycerca G
Poikilolaimus regenfussi G
Bunonema reticulatum
Bunonema sp. G
Steinernema carpocapsae G
Steinernema glaseri
Brevibucca sp. G
Myolaimus sp. G
Rhabditoides inermis G
Choanolaimus psammophilus C
Theristus agilis C
Daptonema procerum G
Eumonhystera cf. simplex
Diplolaimelloides meyli G
Aululaimus oxycephalus
Cylindrolaimus sp. G
Laxus oneistus G
Laxus cosmopolitus G
Stilbonema majum G
Acantopharynx micans G
Catanema sp. G
Leptonemella sp. G
Robbea hypermnestra G
Chromadoropsis vivipara G
Chromadoridae
Desmodora ovigera G
Xyzzors sp. G
Metachromadora sp. G
Eubostrichus topiarius G
Eubostrichus parasitiferus G
Eubostrichus dianae G
Prodesmodora circulata C
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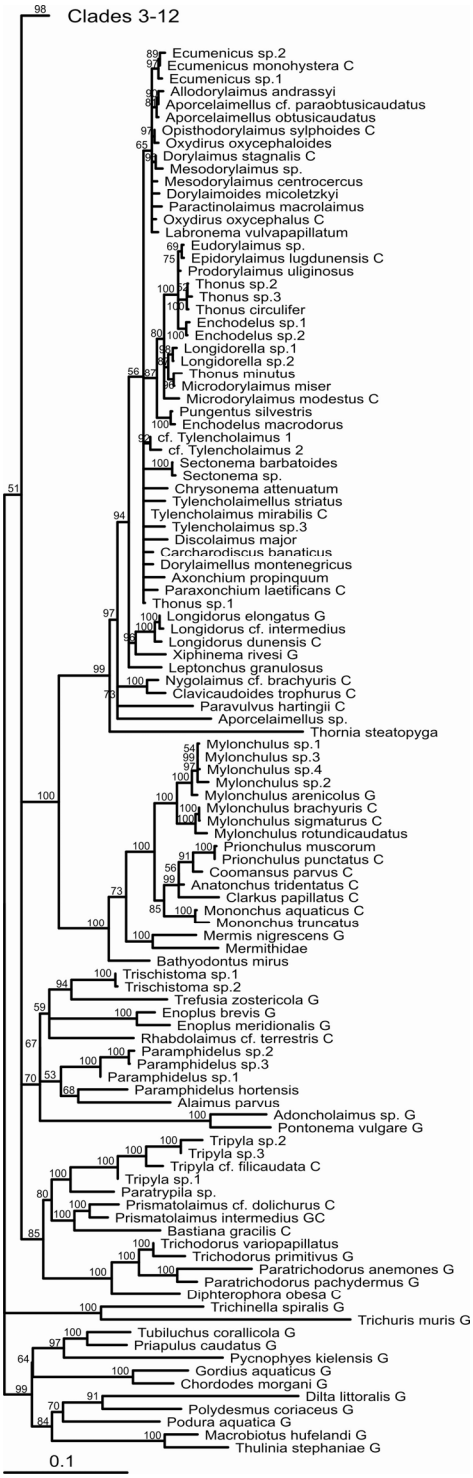
Paracanthonchus caecus G
Praeacanthonchus sp. G
Clades 1-2


Clades 3-12
Ecumenicus sp.2
Ecumenicus monohystera C
Ecumenicus sp.1
Allodorylaimus andrassyi
Aporcelaimellus cf. paraobtusicaudatus
Aporcelaimellus obtusicaudatus
Opisthodorylaimus sylphoides C
Oxydirus oxycephaloides
Dorylaimus stagnalis C
Mesodorylaimus sp.
Mesodorylaimus centrocercus
Dorylaimoides micoletzkyi
Paractinolaimus macrolaimus
Oxydirus oxycephalus C
Labronema vulvapapillatum
Eudorylaimus sp.
Epidorylaimus lugdunensis C
Prodorylaimus uliginosus
Thonus sp.2
Thonus sp.3
Thonus circulifer
Enchodelus sp.1
Enchodelus sp.2
Longidorella sp.1
Longidorella sp.2
Thonus minutus
Microdorylaimus miser
Microdorylaimus modestus C
Pungentus silvestris
Enchodelus macrodorus
cf. Tylencholaimus 1
cf. Tylencholaimus 2
Sectonema barbatoides
Sectonema sp.
Chrysonema attenuatum
Tylencholaimellus striatus
Tylencholaimus mirabilis C
Tylencholaimus sp.3
Discolaimus major
Carcharodiscus banaticus
Dorylaimellus montenegricus
Axonchium propinquum
Paraxonchium laetificans C
Thonus sp.1
Longidorus elongatus G
Longidorus cf. intermedius
Longidorus dunensis C
Xiphinema rivesi G
Leptonchus granulosus
Nygolaimus cf. brachyuris C
Clavicaudoides trophurus C
Paravulvus hartingii C
Aporcelaimellus sp.
Thornia steatopyga
Mylonchulus sp.1
Mylonchulus sp.3
Mylonchulus sp.4
Mylonchulus sp.2
Mylonchulus arenicolus G
Mylonchulus brachyuris C
Mylonchulus sigmaturus C
Mylonchulus rotundicaudatus
Prionchulus muscorum
Prionchulus punctatus C
Coomansus parvus C
Anatonchus tridentatus C
Clarkus papillatus C
Mononchus aquaticus C
Mononchus truncatus
Mermis nigrescens G
Mermithidae
Bathyodontus mirus
Trischistoma sp.1
Trischistoma sp.2
Trefusia zostericola G
Enoplus brevis G
Enoplus meridionalis G
Rhabdolaimus cf. terrestris C
Paramphidelus sp.2
Paramphidelus sp.3
Paramphidelus sp.1
Paramphidelus hortensis
Alaimus parvus
Adoncholaimus sp. G
Pontonema vulgare G
Tripyla sp.2
Tripyla sp.3
Tripyla cf. filicaudata C
Tripyla sp.1
Paratrypila sp.
Prismatolaimus cf. dolichurus C
Prismatolaimus intermedius GC
Bastiana gracilis C
Trichodorus variopapillatus
Trichodorus primitivus G
Paratrichodorus anemones G
Paratrichodorus pachydermus G
Diphterophora obesa C
Trichinella spiralis G
Trichuris muris G
Tubiluchus corallicola G
Priapulus caudatus G
Pycnophyes kielensis G
Gordius aquaticus G
Chordodes morgani G
Dilta littoralis G
Polydesmus coriaceus G
Podura aquatica G
Macrobiotus hufelandi G
Thulinia stephaniae G
0.1

Chapter 3

A ribosomal DNA-based framework for the detection and quantification of stress-sensitive nematode families in terrestrial habitats

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A ribosomal DNA-based framework for the detection and quantification of stress-sensitive nematode families in terrestrial habitats

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 (Anderson 2001). 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 (Yeates *et al.* 1993) – render them a key position in the soil food web (De Ruiter, Neutel, and Moore 1998; Bongers and Ferris 1999). Furthermore, nematodes themselves serve as a food source for a wide range of soil inhabitants (Bongers and Ferris 1999). 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 and Ilieva-Makulec 2004).

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; Bongers 1999) - 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 (Ettema and Bongers 1993; Bongers 1999; Bongers, Ilieva-Makulec, and Ekschmitt 2001; Georgieva *et al.* 2002). As compared to Mononchida, Dorylaimida are highly speciose; Jairajpuri & Ahmad (1992) 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 (Hollis 1961; Premachandran *et al.* 1988). 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 Jairajpuri and Ahmad, 1992). 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 (Aleshin *et al.* 1998; Blaxter *et al.* 1998; Rusin *et al.* 2003; Holterman *et al.* 2006), and recently a subdivision of the phylum into twelve clades has been proposed (Holterman *et al.* 2006). 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 (Holterman *et al.* 2006).

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 (Holterman *et al.* 2006), 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 Appendices A and B.

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 100 x 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: 94°C, 5 min; 5X (94°C, 30 s; 45°C, 30 s; 72°C, 70 s) followed by 35X (94°C, 30 s; 54°C, 30 s; 72°C, 70 s) and 72°C, 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.

Sequence alignment

SSU rDNA sequences were supplemented with publicly available sequences (Appendix A) and aligned using the ClustalW algorithm as implemented in BioEdit 5.0.9 (Hall 1999) and manually improved using secondary structure information from arthropods (http://www.psb.ugent.be/rRNA/secmodel/index.html, in accordance with Wuyts *et al.*, 2000).

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.*, 1999)). 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.* (2006), 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. 3.1). The Dorylaimida SSU rDNA dataset was run for 1,800,000 generations and the burnin was 500,000 generations (Fig. 3.2).

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 (Posada and Crandall 1998) 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 (Swofford 1998) 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 (Ronquist and Huelsenbeck 2003). 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 (Rambaut and Drummond 2005) was used to check if all parameters had converged. The program MacClade v.4.0 (Maddison & Maddison, 2000) 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. 3.1 (M1-M5), subclade-specific SSU rDNA-based primers were designed using the software package ARB (Ludwig *et al.* 2004). An alignment of about 1,200 full length SSU rDNA

Table 3.1. Primer combinations used for the detection and quantification of Mononchida subclades as defined in Fig. 3.1. Real time PCR results are presented in Figure 3.4. Close non-target species belonging to the subclass Dorylaimia are given in italics.

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

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

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. 3.1, and for each of the subclades the closest non-targets are shown in Table 3.1. Primer combinations as presented in Table 3.1 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 to10 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 (Table 3.1). 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 (Table 3.1) for 1 min and 72°C for 30 sec.

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

Trophic ecology:
Black = omnivores (excluding higher plants)
Blue = predators
Gray = bacterial feeders
Pink = animal parasites

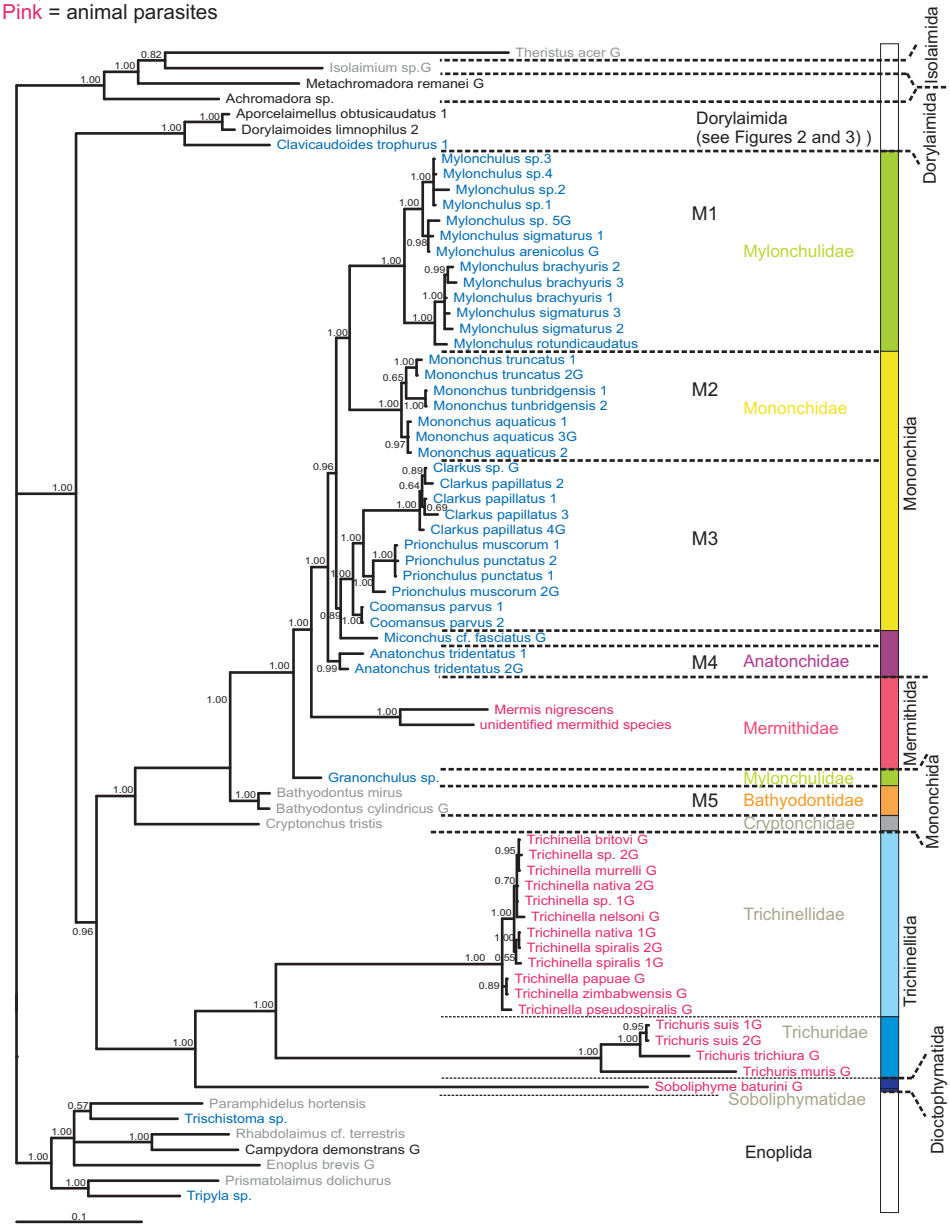

Figure 3.1. SSU rDNA-based Bayesian phylogeny of the subclass Dorylaimia (Clade 2 in Holterman *et al.* 2006). Numbers near nodes represent posterior probabilities. The colored bar indicates to which order, family, and subclade (M1-M5) a species belongs. The color of the species name indicates the feeding type (Yeates *et al.* 1993).

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. 3.1 and 3.2): 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. 3.1), whereas the phylogenetic resolution among representatives of the Dorylaimida was poor (Fig. 3.2). Addition of sequences from representatives of the Cryptonchidae and the Soboliphymatidae - both positioned basally in two major branches (see Fig. 3.1) - resulted in intra-clade relationships that differ from the relationships presented by Mullin *et al.* (2005) and Holterman *et al.* (2006). 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 (Mullin, Harris, and Powers 2005). 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; Bongers 1990).

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 (Yeates *et al.* 1993), 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 1987). The remarkable and firm placement of the Mermithidae (insect parasites) within the Mononchida confirms previous findings by Blaxter *et al.* (1998), Mullin *et al.* (2005) and Holterman *et al.* (2006). 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. 3.1). 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 (Zullini and Peneva 2006). 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 3.1). 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. 3.1) have only rudimentary tail glands and no spinneret (Zullini and Peneva 2006). 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 (Zullini and Peneva 2006). These characteristics support the results from our phylogenetic analysis.

The Anatonchidae appeared to be paraphyletic, too. Representatives of the genus Anatonchus (M4 in Fig. 3.1) are placed at the base of a subclade that includes most Mononchidae and Mylonchulidae. Our analysis suggest 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. 3.1) 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 (De Ley, Decraemer, and Abebe 2006). These are characterized by the nature of their stoma (Coomans 1964). The Dorylaimina are equipped with an axial odontostyle, whereas the Nygolaimina have a mural tooth. In the SSU rDNA-derived tree (Fig. 3.2), 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.

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. 3.3), parsimony tree (Appendix D) and neighbor-joining tree (Appendix E) 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

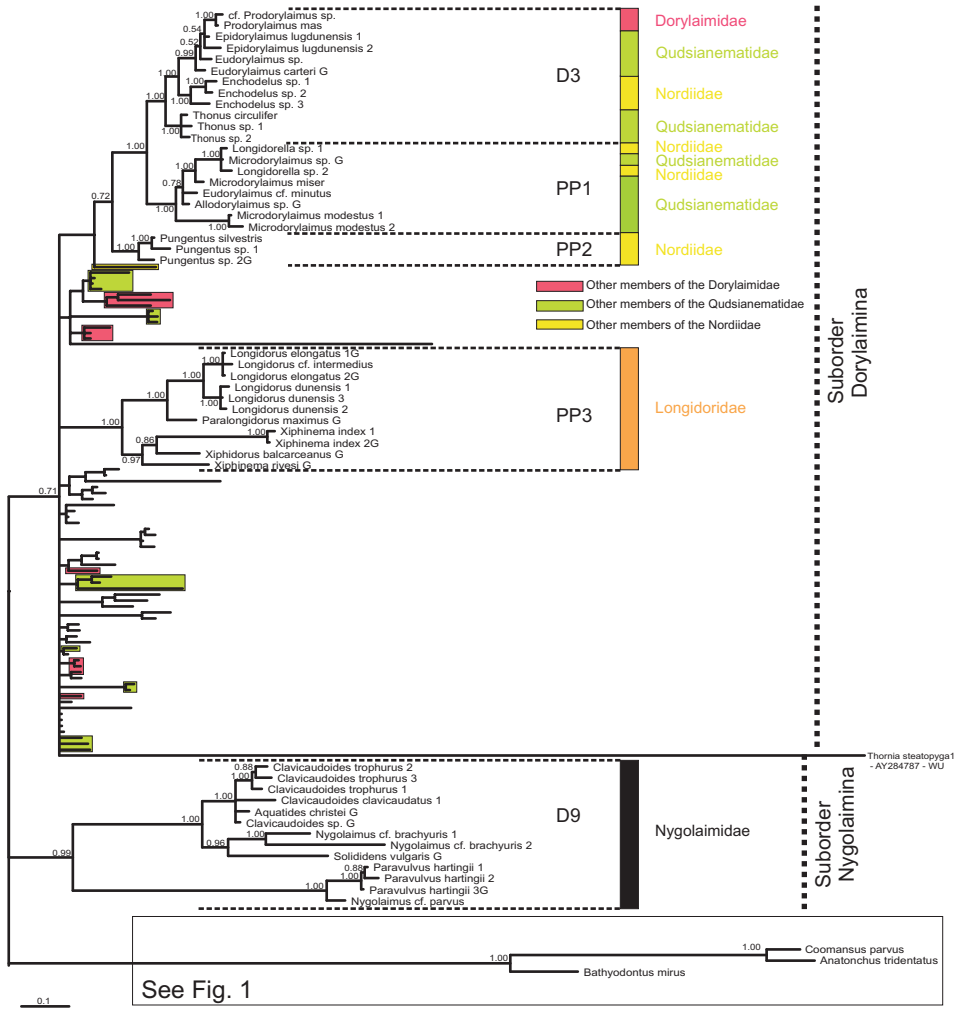

Figure 3.2. SSU rDNA-based Bayesian phylogeny of the order Dorylaimida. Numbers near nodes represent posterior probabilities. The colored bars indicate to which suborder, family and subclade a species belongs. Only species names belonging to well-resolved subclades are given (for detailed phylogenetic tree see Appendix C).

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 (Appendix F). (i) It groups several long-tailed taxa which currently are dispersed in separate

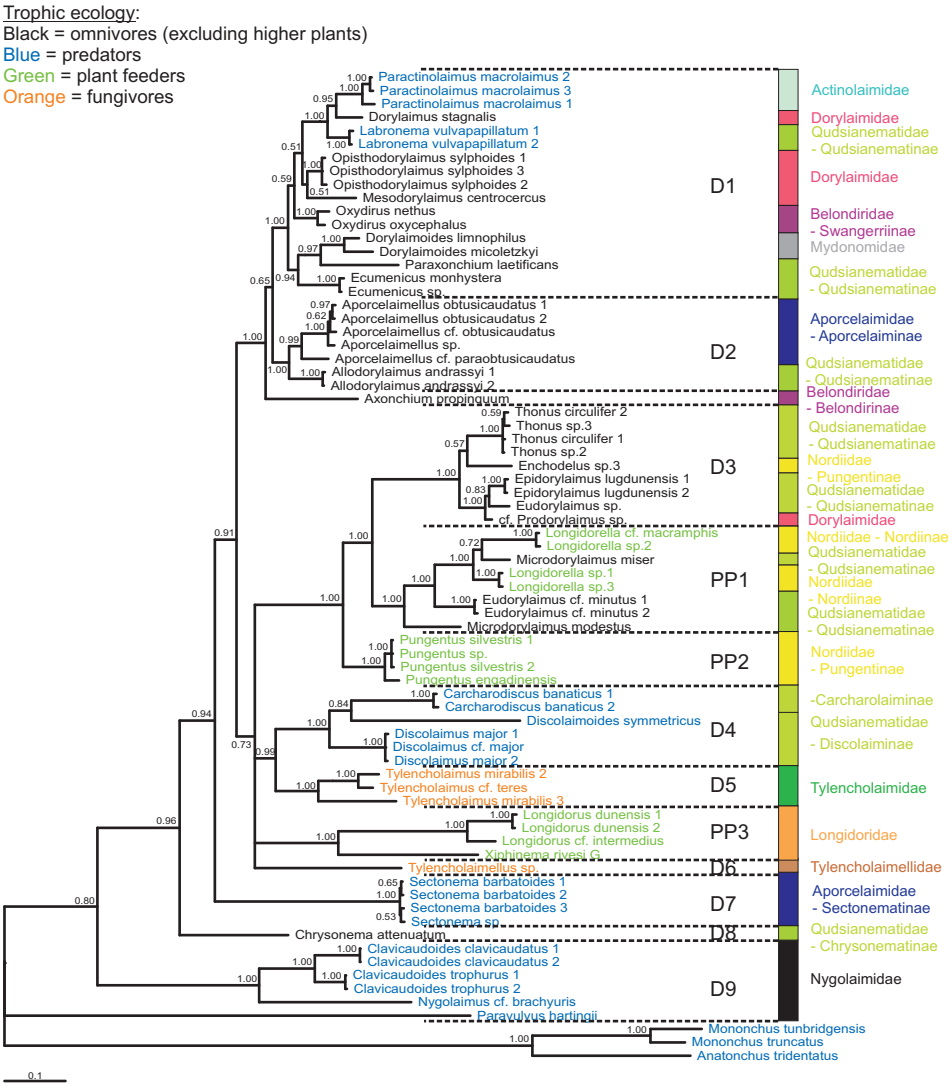

Figure 3.3. LSU rDNA-based Bayesian phylogeny of the order Dorylaimida. Numbers near nodes represent posterior probabilities. The colored bar indicates to which (sub-) family a species belongs. The Dorylaimida are divided into 12 subclades (D1-D9, PP1-PP3). The color of the species name indicates the feeding type (Yeates *et al.* 1993).

families (and even superfamilies): Dorylaimidae (Dorylaimoidea), Mydonomidae (Tylencholaimoidea) 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. 3.2). Although these taxa share several characters, none of these characters are unique for this subclade (Appendix F). 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.

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 (Siddiqi 1969; see also De Ley, Van Driessche, and Coomans, 2005) - 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. 3.2).

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 (Blaxter *et al.* 1998). For the latter case, Holterman *et al.* (2006) provided molecular support for a long-standing hypothesis stating that plant parasitic nematodes arose from fungivorous ancestors (Maggenti 1971). 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, Yeates *et al.* 1993; but also see Trudgill, 1976), 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. 3.1. On the basis of these signatures primers were designed that would work under similar annealing temperatures, and these were tested for their specificity (Fig. 3.4). It is noted that close non-targets as given in Table 3.1 do not necessarily belong to

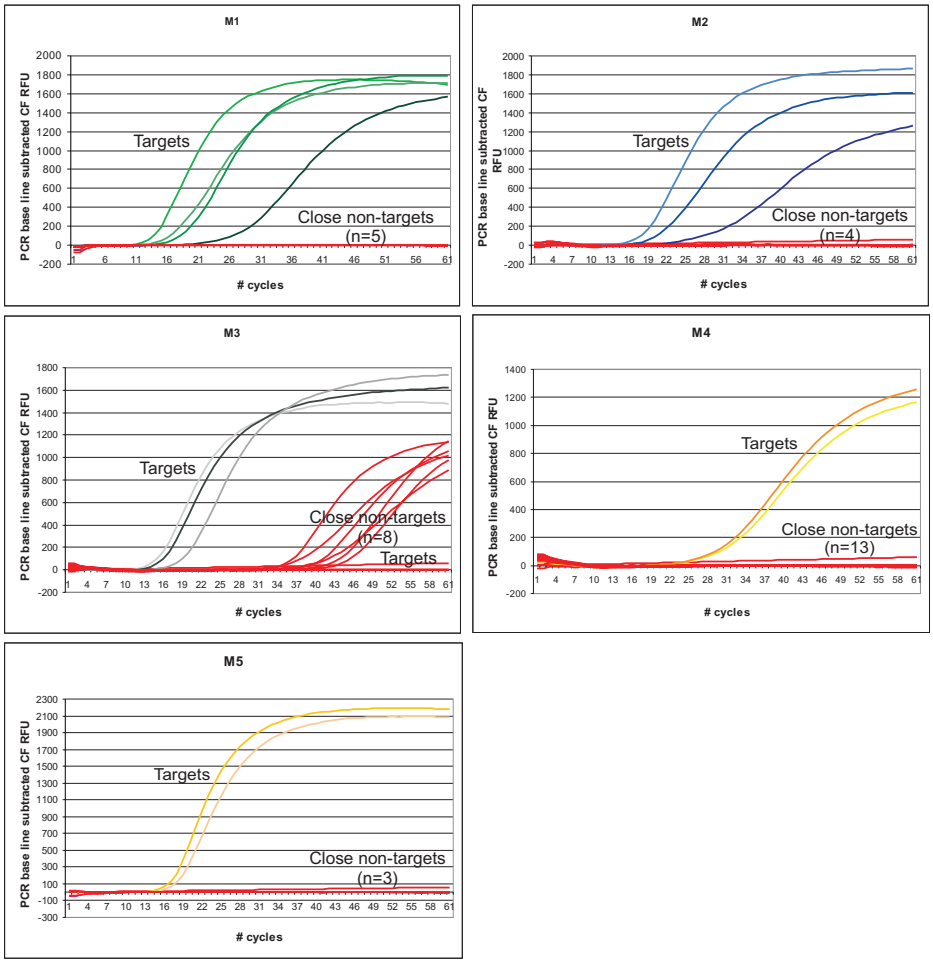

Figure 3.4. Real time PCR amplification curves showing the detection of trophically homogeneous subclades within the order Mononchida as defined in Fig. 3.1 on the basis of full length SSU rDNA sequences (M1-M5). Target species, closest non-targets, primers and annealing temperatures are given in Table 3.1.

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.

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. 3.2, 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 data base 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.

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, 1994), 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 and Ferris, 1999), 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.

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Appendix A

GenBank accession numbers for all SSU sequences used in this study. A "G" behind the name indicates the sequence was acquired from GenBank.

CLADE 2 - DORYLAIMIDA

species	NCBI accession	species	NCBI accession
Allodorylaimus andrassyi	AY284801	Longidorus dunensis 2	AY284818
Allodorylaimus sp. G	AJ966472	Longidorus dunensis 3	AY284819
Aporcelaimellus cf. paraobtusicaudatus	AY284812	Longidorus elongatus 1G	AF036594
		Longidorus elongatus 2G	AY687992
Aporcelaimellus obtusicaudatus 1	AY284811	Mesodorylaimus aberrans	AY593947
		Mesodorylaimus bastiani G	AJ966488
Aporcelaimellus obtusicaudatus 2G	DQ141212	Mesorylaimus centrocercus 1	AY284799
		Mesodorylaimus centrocercus 2	EF207248
Aporcelaimellus sp. 1	AY284813	Mesodorylaimus cf. nigritulus G	AJ966490
Aporcelaimellus sp. 2G	AJ875153		
Aporcelaimellus sp. 3G	AJ875155	Mesodorylaimus japonicus G	AJ966489
Aporcelaimellus sp. 4G	AJ875154	Mesodorylaimus sp.	AY284780
Aquatides christei G	AY552963	Metaporcelaimus simplex	AY593948
Axonchium propinquum	AY284820	Microdorylaimus miser	AY284804
Californidorus sp. G	AY283155	Microdorylaimus modestus 1	AY284805
Carcharodiscus banaticus	AY284827	Microdorylaimus modestus 2	AY284806
Chrysonema attenuatum 1	AY593945	Microdorylaimus sp. G	AJ966492
Chrysonema attenuatum 2	AY284779	Nygolaimus cf. brachyuris 1	AY284770
Chrysonema attenuatum 3	EF207245	Nygolaimus cf. brachyuris 2	AY284771
Clavicaudoides clavicaudatus	AY593944	Nygolaimus cf. parvus G	AY552974
Clavicaudoides sp. G	AY552967	Opisthodorylaimus sylphoides	AY284785
Clavicaudoides trophurus 1	AY284772	Oxydirus nethus	EF207251
Clavicaudoides trophurus 2	AY284773	Oxydirus oxycephaloides	AY284823
Clavicaudoides trophurus 3	AY593943	Oxydirus oxycephalus 1	AY284824
Discolaimus cf. major	EF207252	Oxydirus oxycephalus 2	AY284825
Discolaimus major	AY284828	Paractinolaimus macrolaimus 1	AY284826
Dorylaimellus montenegricus	AY284821	Paractinolaimus macrolaimus 2G	AY993978
Dorylaimellus virginianus G	AY552969		
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	Pungentus silvestris	AY284788
Epidorylaimus lugdunensis 1	AY284802	Pungentus sp. 1	AY284791
Epidorylaimus lugdunensis 2	AY284803	Pungentus 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	AY284798
Leptonchus granulosus	AY284831	Thonus sp. 2	AY284797
Longidorella sp. 1	AY284789	Thonus sp. 3	AY284796
Longidorella sp. 2	AY284790	Thornia steatopyga	AY284787
Longidorus cf. intermedius	AY284816	Tylencholaimellus affinis G	AY552978
Longidorus dunensis 1	AY284817	Tylencholaimellus striatus	AY284837
species	accession	species	accession
Tylencholaimus mirabilis 1	AY284835	cf. Tylencholaimus sp. 2	AY284833
Tylencholaimus mirabilis 2	AY284836	Xiphidorus balcarceanus G	AY297839
Tylencholaimus mirabilis 3	EF207253	Xiphinema index 1	EF207249
Tylencholaimus cf. teres	EF207254	Xiphinema index 2G	AY687997
Tylencholaimus sp. 1G	AJ966510	Xiphinema rivesi G	AF036610
Tylencholaimus sp. 3	AY284834	Xiphinema sp. 1	EF207250
cf. Tylencholaimus sp. 1	AY284832		

CLADE 2 - OTHER ORDERS

species	**NCBI accession**	**species**	**NCBI 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	**NCBI accession**	**species**	**NCBI accession**
Achromodora sp.	AY284717	Metachromadora remanei	AY854216
Isolaimium sp. G	AY552971	Theristus acer G	AJ966505

CLADE 1

species	**NCBI accession**	**species**	**NCBI 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		

Appendix B

GenBank accession numbers for all LSU sequences used in this study. A "G" behind the name indicates the sequence was acquired from GenBank.

DORYLAIMIDA

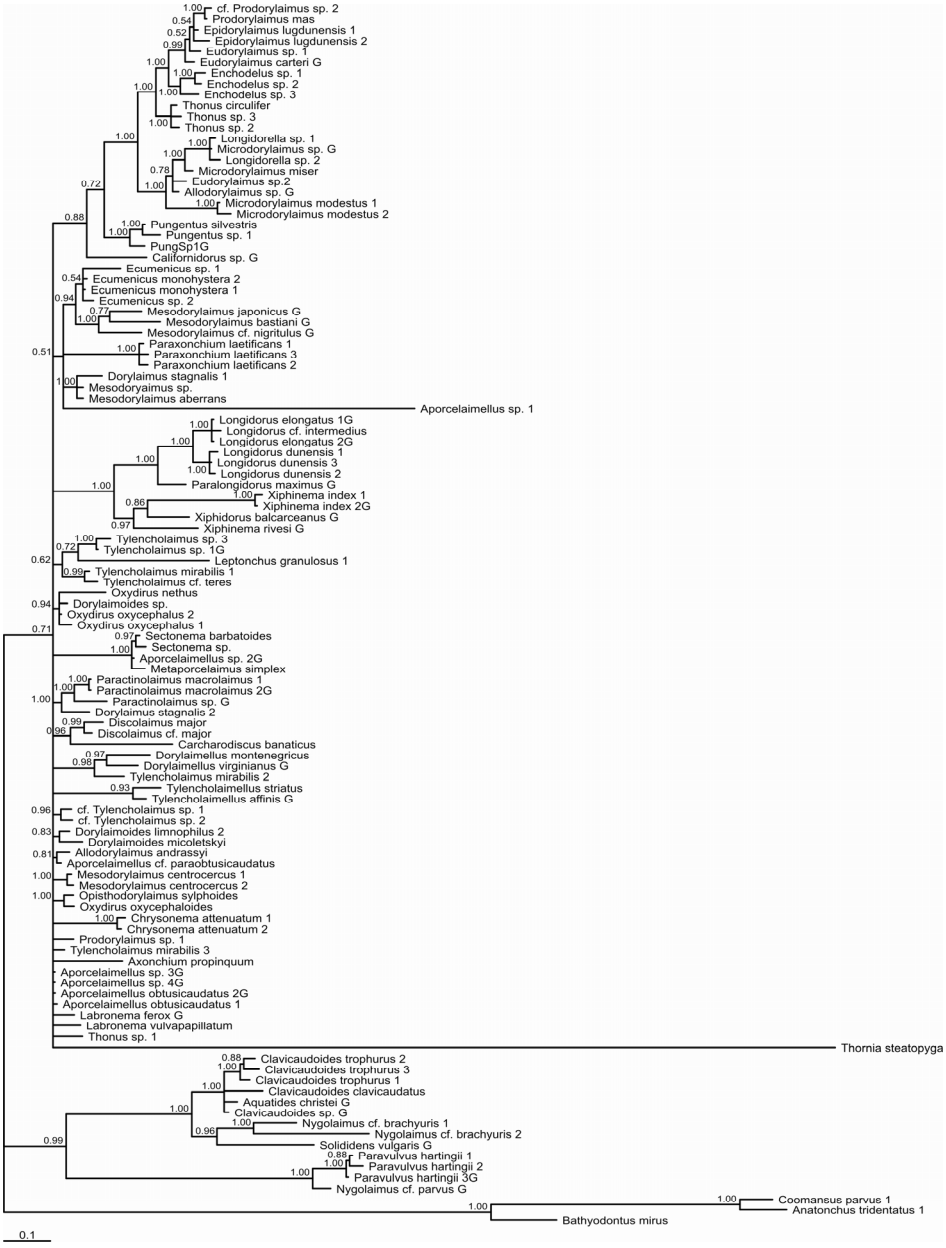
species	NCBI accession	species	NCBI accession
Allodorylaimus andrassyi 1	AY593015	Longidorus dunensis 1	AY593056
Allodorylaimus andrassyi 2	AY593016	Longidorus dunensis 2	AY593057
Aporcelaimellus cf..	AY593017	Mesodorylaimus sp.	AY593005
obtusicaudatus		Mesorylaimus centrocercus	AY593007
Aporcelaimellus cf.	AY593020	Microdorylaimus miser	AY593046
paraobtusicaudatus		Microdorylaimus modestus	AY593049
Aporcelaimellus	AY593018	Nygolaimus cf. brachyuris	AY593061
obtusicaudatus 1		Opisthodorylaimus	AY593008
Aporcelaimellus	AY593019	sylphoides 1	
obtusicaudatus 2		Opisthodorylaimus	AY593009
Aporcelaimellus sp.	AY593021	sylphoides 2	
Axonchium propinquum	AY593022	Opisthodorylaimus	AY593010
Carcharodiscus banaticus 1	AY593023	sylphoides 3	
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

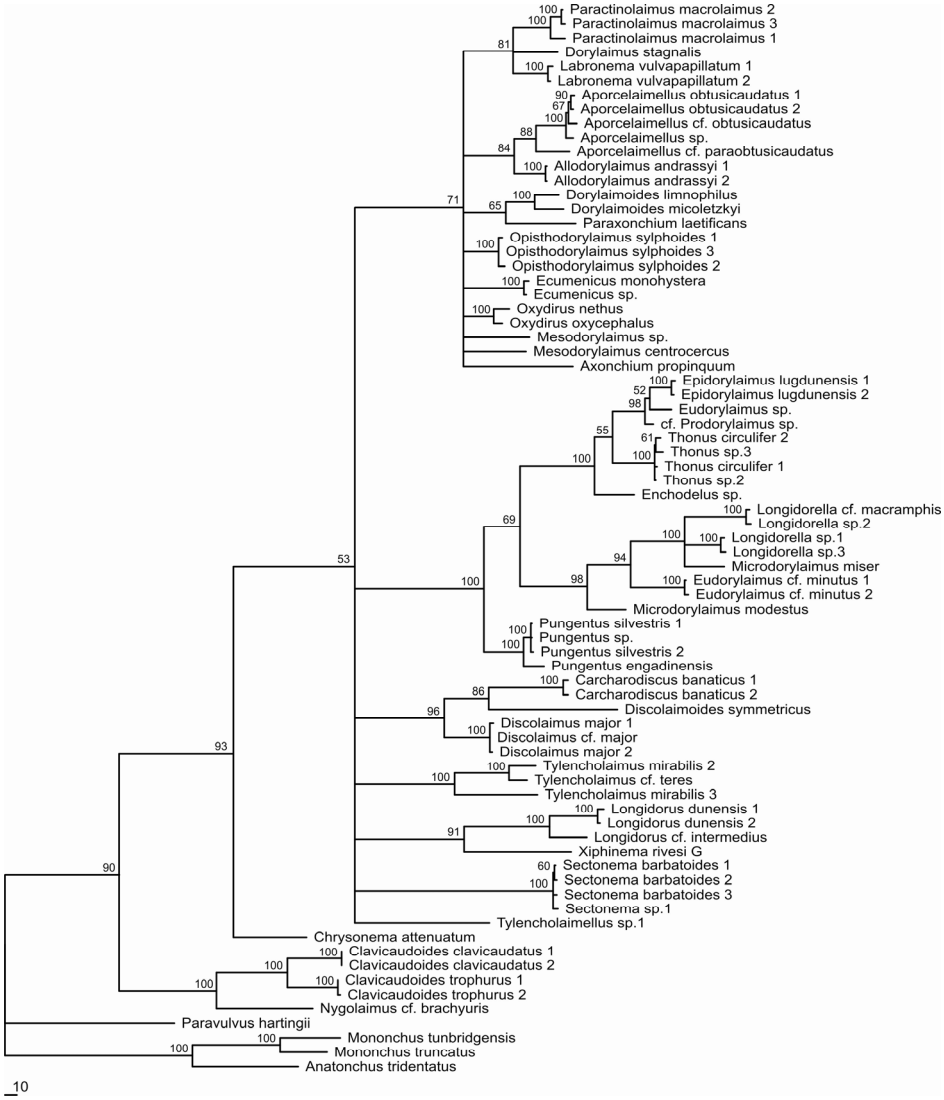
species	**NCBI accession**	**species**	**NCBI accession**
Anatonchus tridentatus	AY593065	Mononchus tunbridgensis	AY593063
Mononchus truncatus	AY593065		

Appendix C



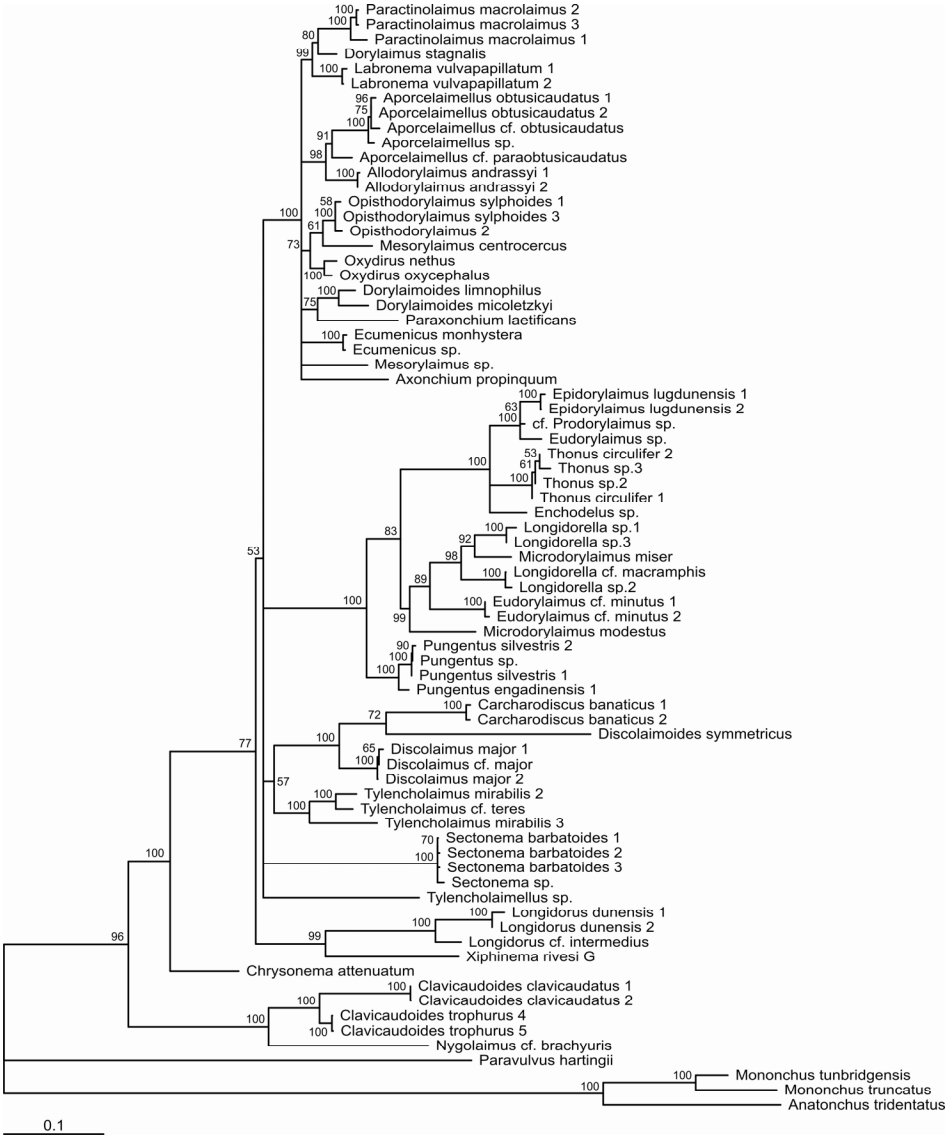
SSU Bayesian tree of the Dorylaimida. Numbers near nodes indicate bootstrap values. A "G" behind the name indicates the sequence was acquired from GenBank.

Appendix D



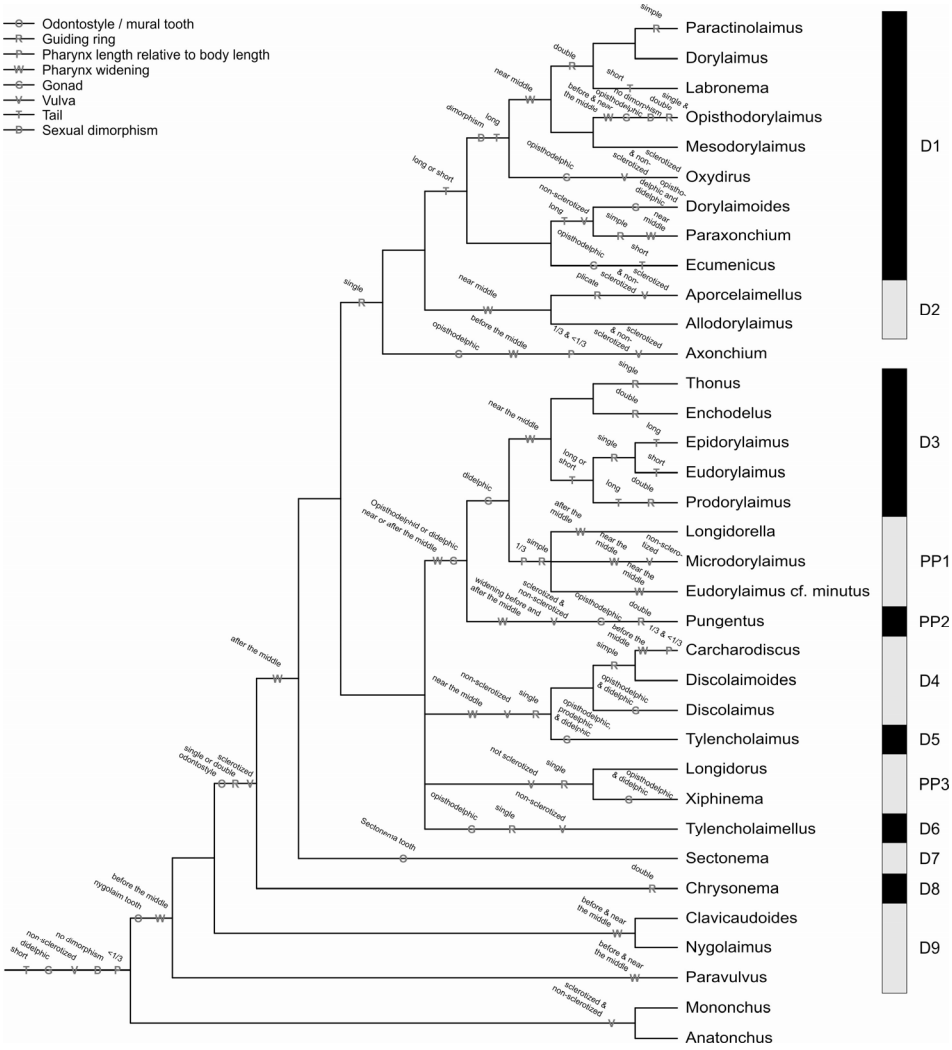
LSU maximum parsimony tree of the Dorylaimida. Numbers near nodes indicate bootstrap values. A "G" behind the name indicates the sequence was acquired from GenBank.

Appendix E



LSU neighbor-joining tree of the Dorylaimida. Numbers near nodes indicate bootstrap values. A "G" behind the name indicates the sequence was acquired from GenBank.

Appendix F



Simplified LSU Bayesian tree of the Dorylaimida. Letters indicate a change in the character with the new character state given.

Chapter 4

Small subunit ribosomal DNA-based phylogeny of basal Chromadoria (Nematoda) suggests that transitions from marine to terrestrial habitats (and *vice versa*) require relatively simple adaptations

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Small subunit ribosomal DNA-based phylogeny of basal Chromadoria (Nematoda) suggests that transitions from marine to terrestrial habitats (and *vice versa*) require relatively simple adaptations

Nematodes – presumably the most numerous multicellular animals on earth – live in terrestrial, freshwater, brackish and marine habitats. Here, we present the phylogenetic relationships of basal Chromadoria, a group with numerous representatives in all four major habitats. These were based on the small subunit ribosomal DNA gene. Members of the orders Plectida and Chromadorida were grouped into well-supported monophyletic clades. Within the mainly marine order Desmodorida, the two superfamilies Desmodoroidea and Microlaimoidea received respectively robust and moderate molecular support, and they ended up in completely separate positions. Our data imply that a revision of the orders Monhysterida and Araeolaimida is needed; one well-supported clade (Clade 5C) could at best be referred to as an Araeolaimida-dominated group, whereas another clade (Clade 5A) could be indicated as Monhysterida-dominated. Within this phylogenetic tree at least 16 major habitat changes could be pinpointed. Transitions took place in both directions and at various taxonomic levels (from species to subclass level). Our data suggest that within the phylum Nematoda major habitats transitions (thalassic to limnoterrestrial and *vice versa*) require relatively simple adaptations, and these adaptations are discussed.

Introduction

Five animal phyla are currently positioned at the base of the Ecdysozoa, a superphylum encompassing the moulting animals; Kinorhyncha (mud

dragons), Priapulida (penis worms), Loricifera, Nematoda (roundworms) and Nematomorpha (horsehair worms; Mallatt and Giribet 2006). Mud dragons, Loricifera and penis worms are species-poor phyla (respectively ≈ 180, ≈ 25, and ≈ 18 described species) whose representatives live in muddy or sandy sediments. The two other groups of basal Ecdysozoa are the Nematomorpha (≈ 300 species; (Schmidt-Rhaesa and Geraci 2006) and the Nematoda whose diversity is estimated to range between 0.1 and 100 million species (*e.g.* Coomans 2000). Hence, in terms of speciation nematodes can be characterized as a highly successful group.

Although nematodes have a relatively conserved morphology, there are a reasonable number of informative morphological characters. However, because of the many poor morphological descriptions, the phylogenetic relationships among major groups of nematodes have been unstable for decades. The phylum Nematoda is suggested to be around 1,000 Mya old, and this is one of the reasons why coding regions of ribosomal DNA (small (SSU) and large subunit (LSU) rDNA) are remarkably powerful in resolving the internal relationships within this phylum. Phylum-wide studies on the basis of SSU rDNA sequences suggested a subdivision of the Nematoda into several numbered clades (Blaxter *et al.* 1998; Holterman *et al.* 2006). Over the last five years we've seen a steep increase in the number of available sequences, and most recently Meldal *et al.* (2007) presented 46 new SSU rDNA sequences from marine taxa, a so far underrepresented group.

Within the phylum Nematoda a wide range of trophic ecologies is represented; nematodes feed on bacteria, fungi, protozoa, small invertebrates and lower plants, and parasitize higher plants and animals (*e.g.* Yeates *et al.* 1993). Molecular phylogenetic studies clearly show that each of these trophic groups arose multiple times independently. In parallel, nematodes can be found in virtually all terrestrial, freshwater and marine habitats, and it is interesting to investigate on the basis of neutral molecular data where and how frequent fundamental habitat transitions occurred. Furthermore, we hypothesize that the taxonomic levels at which the transitions take place (class, order, family or genus level) is indicative for the number of physiological changes needed to accomplish such a change. Nematode taxa within a family or genus are by definition morphologically similar, and - as compared to higher

taxonomic levels - they have diverged relatively recently. Hence, we hypothesize that habitat transitions within a family and especially within a genus points at a limited number of changes needed to settle in this new habitat.

For decades the hypothesis of Filipjev (1929; 1934) about the marine ancestry of nematodes has been widely accepted. Analysis of numerous SSU rDNA sequences from basal nematodes combined with embryological data led Holterman *et al.* (2006) to propose a most basal position for the subclass Enoplia, a group with representatives in terrestrial, freshwater and marine ecosystems. Molecular analysis of Ecdysozoa on the basis of both small and large subunit ribosomal DNA sequence information seems to support Filipjev's hypothesis (Mallatt and Giribet 2006). The Nematoda and Nematomorpha are positioned near the most ancestral Ecdysozoans in a sister relationship with the Panarthropoda. The most basal clade of the Ecdysozoa is formed by the Scalidophora, a group that includes three small, exclusively marine invertebrate phyla. The sistergroup of the Ecdysozoa, the Lophotrochozoa, also mainly consists of marine taxa. If these ribosomal DNA data reflect Ecdysozoa evolution correctly, this would imply that the last common ancestor of the Nematoda, Nematomorpha and Panarthropoda most likely lived in a marine habitat. Based on this information, we hypothesize that the nematode species which most closely resemble the common ancestor of all nematodes can be found among the Enoplia, and that this common ancestor presumably lived in a marine ecosystem.

Distribution of marine nematodes over the major nematode clades is strongly biased. In Clades 8-12 (Holterman *et al.* 2006) - a group formerly indicated as "Secernentea" - only a very few marine species can be found. In this paper we will concentrate on the orders Chromadorida, Desmodorida, Monhysterida, Araeolaimida and Plectida – orders in which marine nematodes are present in a high degree. We present 32 newly generated SSU rDNA data, and a total number of 128 nearly full length SSU rDNA sequences were analyzed. Apart from major taxonomic implications - *e.g.* the poor molecular support for the orders Monhysterida and Araeolaimida - detailed phylogenetic analysis of this relatively large data set reveals a surprisingly high number of

transitions from thalassic to limnoterrestrial habitats and *vice versa*, and the biological implications of this finding are discussed.

Materials and methods

Specimen collection

Terrestrial nematodes were collected from various habitats throughout The Netherlands, and extracted from the soil using standard techniques. The marine species were gathered at the shores of the Oosterschelde estuary. 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. For the nomenclature of taxonomic groups we essentially conformed to the systematics proposed by (De Ley, Decraemer, and Abebe 2006).

DNA extraction, amplification and sequencing

Single nematodes were transferred to a 0.2 ml 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 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. SSU rDNA was amplified as two partially overlapping fragments using three universal and one nematode-specific primer (1912R). The latter was included to avoid amplification of non-target eukaryotic SSU rDNA, *e.g.* from fungal spores attached to the nematode cuticle. For the first fragment, either primer 988F (5′-ctcaaagattaagccatgc-3′) or 1096F (5′-ggtaattctggagctaatac-3′) was used in combination with primer 1912R (5′-tttacggtcagaactaggg-3′). The second fragment was amplified with primers 1813F (5′-ctgcgtgagaggtgaaat-3′) and 2646R (5′-gctaccttgttacgactttt-3′). PCR was performed in a final volume of 25 µl and contained 3 µl of a 100 times diluted crude DNA extract, 0.1 µM of each PCR primer and a Ready-To-Go PCR bead (Amersham). The following PCR profile was used: 94°C for 5 min.; 5 x (94°C, 30 sec.; 45°C, 30 sec.; 72°C, 70 sec.) followed by 35 x (94°C, 30 sec.; 54°C, 30 sec.; 72°C, 70 sec.) and 72°C, 5 min. Gel-purified (Marligen Bioscience, Ijamsville,

MD) amplification products were cloned into a TOPO TA vector (Invitrogen) and sequenced using standard procedures. Newly generated SSU rDNA sequences were deposited at GenBank under the following accession numbers: AY593927, AY593931-AY593933, AY593938-AY593940, AY593942, EF591319-EF591342.

Sequence alignment

Newly generated nematode SSU rDNA sequences were supplemented with publicly available sequences (for a full list see Appendix A). The outgroup consisted of Dorylaimia. The SSU rDNA sequences were aligned using the ClustalW algorithm as implemented in BioEdit 5.0.9 (Hall 1999) and manually improved using secondary structure information from yeast (http://www.psb.ugent.be/rRNA/varmaps/Scer_lsu.html, in accordance with Ben Ali *et al.*, 1999). The final alignment consisted of 125 SSU rDNA sequences and contained 1,876 aligned positions (including gaps).

Phylogenetic analysis

The program Modeltest v.3.06 (Posada and Crandall, 1998) selected the GTR+I+Γ model as the best fitting model using both the likelihood ratio test and Akaike Information Criterion. The Bayesian tree was constructed using the program MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The alignment was divided into a stem and a loop partition according to the secondary structure. For both partitions the GTR model with invariable sites and gamma distribution was used. 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 5,000,000 generations with a sample frequency of 200 generations. The first 250,000 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 (Rambaut and Drummond 2005) was used to check if all parameters had converged.

A second phylogenetic tree was constructed with a fast maximum likelihood method. The SSU rDNA alignment was analyzed at a distant server (http://phylobench.vital-it.ch/raxml-bb/index.php) running the program,

RaxML-VI-HPC v.4.0.0 (Randomized Axelerated Maximum Likelihood for High Performance Computing; Stamatakis, 2006). A GTR model with invariable sites and gamma distribution was used, and the dataset was divided in a stem and loop partition. 100 bootstraps were performed.

Transition from marine to terrestrial habitats

The program MacClade v.4.0 (Maddison and Maddison 2000) was used to determine where the transitions from a thalassic to a limnoterrestrial lifestyle (or *vice versa*) had occurred. The ability to live in both thalassic and limnoterrestrial habitats was scored as a separate third character state, because MacClade does not reconstruct ancestral states as polymorphic. Nodes with a posterior probability lower than 0.95 were collapsed. For the purpose of this analysis the ancestor of the Nematoda was assumed to live in a thalassic habitat for the reasons given above.

Results and Discussion

For this phylogenetic analysis of basal Chromadoria 128 nearly full length SSU rDNA sequences were used. As compared to (Holterman *et al.* 2006) 76 new sequences were included: 32 sequences were collected by us and 44 were acquired from GenBank. Primary analysis of the data set (Appendix B) pointed at the presence of a few "rogue" taxa; species with a strong destabilizing effect on the tree (Sanderson and Shaffer 2002). Therefore, three species - *Halichoanolaimus sp.*, *Prodesmodora circulata* and *Desmoscolex sp.* - were not taken into consideration in subsequent analyses. The congruence of the resulting Bayesian and maximum likelihood phylogenies (Fig. 4.1, Appendix C) with the current nematode systematics is discussed for each order. Both trees have a highly similar topology, and robust nodes were nearly always supported by both methods (posterior probability ≥ 0.95; bootstrap ≥ 65). The phylogeny was subsequently used to reconstruct major habitat transitions. Finally the possible modes of adaptation to a limnoterrestrial environment are discussed.

Chromadorida

The order Chromadorida, represented here by 17 sequences from four major families, forms a monophyletic clade with high support (posterior probability = 1.00). The relationships within the Chromadorida are fairly well resolved. The families Cyatholaimidae and Achromadoridae are sister groups to each other, and are further grouped with the families Ethmolaimidae (represented here by a single species) and Chromadoridae.

Based on morphological characters, the genus *Choanolaimus* (family Selachinematidae) has always been placed in the Chromadorida. However, in this analysis, as well as in (Holterman *et al.* 2006), *Ch. psammophilus* (the genus harbors a single species) is placed outside the Chromadorida, although its exact phylogenetic position cannot be determined. Another member of this family – *Halichoanolaimus sp.* – was excluded from the final analysis (see above). Interestingly, this species always clustered close to the Chromadorida in the preliminary analyses (Appendix B), separate from *Choanolaimus*. To the best of our knowledge no morphological characters have been reported that either support a relationship of the Selachinematidae with another nematode order or that suggest the Selachinematidae are polyphyletic.

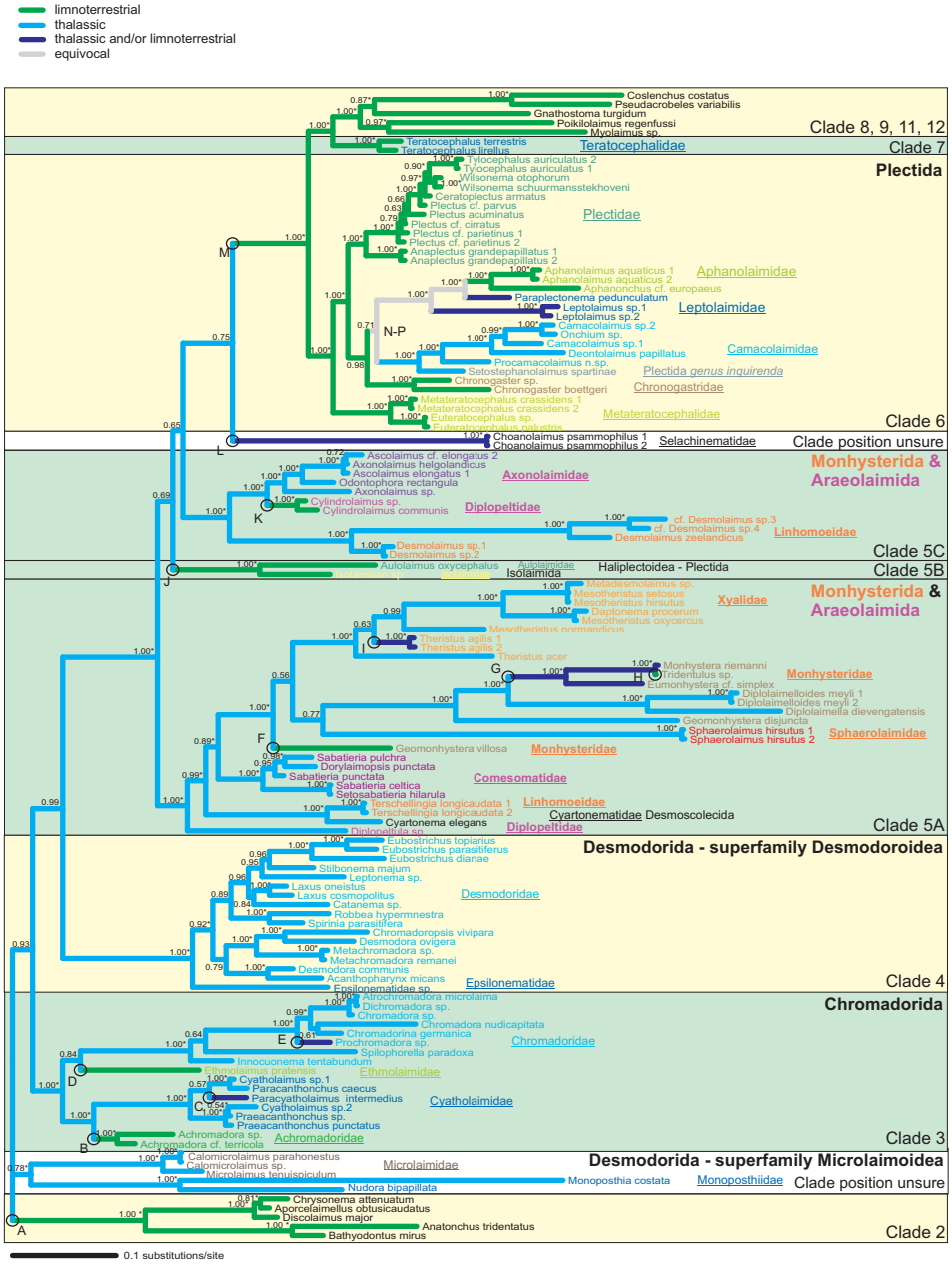
Desmodorida

For the mainly marine order Desmodorida the combined use of a stem - loop partition and a GTR+Γ+I model did not resolve this order as a monophyletic group. As such, our results confirm the recent findings of Meldal *et al.* (2007). The superfamily Microlaimoidea - here represented by the families

→

Fig. 4.1. A small subunit ribosomal DNA-based Bayesian tree of the basal Chromadoria (Nematoda). Support values (posterior probabilities) are given next to the nodes; nodes labelled with an asterisk are supported by maximum likelihood analysis (bootstrap ≥ 65%; Appendix C).. Alternating yellow and green backgrounds define clades. White backgrounds indicate an unsure clade position. For the nomenclature of taxonomic groups we essentially conformed to De Ley *et al*. (2006). Order names (and - if relevant – superfamily names) are given in black, except for the Monhysterida (orange) and the Araeolaimida (purple). Family names are underlined. Within Clade 5, families belonging to the Monhysterida are given in orange, families belonging to the Araeolaimida indicated in purple. Within each of the clades, members of a family have the same colour. The colours of the branches refer to the habitat. Habitat transitions are encircled, and capitals next to these circles refer to Tables 4.1 and 4.2.

Monoposthiidae and Microlaimidae (no sequences were available from the Aponchiidae) – is placed at the base of the class Chromadorea, separated from the Desmodoroidea.



Prodesmodora (Desmodoridae) is an exceptional genus within the order Desmodorida as it includes exclusively limnoterrestrial species in an otherwise completely thalassic order. Within our analyses *Prodesmodora* behaved as rogue taxon and as such they were excluded for the final phylogenetic analysis. However, in preliminary phylogenetic analysis *Prodesmodora* was placed outside the Desmodorida. Some sequences were placed within the Achromadoridae (see also Holterman *et al.*, 2006) while another sequence was placed in a basal polytomy of the Chromadorea (Appendix B). To the best of our knowledge, there are no morphological characters that support a positioning of *Prodesmodora* apart from the Desmodorida.

Monhysterida, Araeolaimida and Desmoscolecida

Neither the Monhysterida nor the Araeolaimida were found to be monophyletic in our analysis. According to De Ley *et al.* (2006) the order Monhysterida includes six families: Monhysteridae, Sphaerolaimidae, Xyalidae, Linhomoeidae, Fusivermidae and Siphonolaimidae. The latter two are not represented here. The Araeolaimida are covered by the Axonolaimidae, Comesomatidae and Diplopeltidae, but no representatives of the Coninckiidae are included. The Monhysterida-dominated clade (Clade 5A) also includes the Comesomatidae (Araeolaimida) and part of the Diplopeltidae (Araeolaimida). The Araeolaimida-dominated clade (Clade 5C) also includes a part of the Linhomoeidae (Monhysterida). The status of the Araeolaimida has always been uncertain, with various authors placing the Axonolaimidae and Comesomatidae in the Monhysterida (Chitwood and Chitwood 1950; Lorenzen 1981), in the Araeolaimida (Malakhov, Ryzhikov, and Sonin 1982; Inglis 1983; De Ley and Blaxter 2002) or even in the Chromadorida (Andrássy 1976; Hope and Zhang 1995). However, the Axonolaimidae and Comesomatidae were usually not separated from each other, except by Andrássy (1976).

The subdivision of the Linhomoeidae - according to Platt and Warwick (1988) a marine family that includes 11 genera - into two distinct groups is remarkable. *Desmolaimus* species ended up in Clade 5C in a sister relationship with the Axonolaimidae and some Diplopeltidae. Representatives of the genus *Terschellingia* were positioned close to *Cyartonema* (Cyartonematidae; Clade 5A). Our data suggest that the Linhomoeidae are not a monophyletic family. As we

sampled only two out of the eleven Linhomoeidae genera, there is no point in further discussing the systematics and/or positioning of this family. Another point of interest is the position of *Cyartonema* in Clade 5A. According to De Ley *et al.* (2006), the Cyartonematidae are part of the Desmoscolecida. However, *Desmoscolex*, one of the rogue taxa excluded for the final analysis, was positioned separate from *Cyartonema* in a basal position in the preliminary analyses (Appendix B). Until more sequences for the Desmoscolecida become available the position of the Desmoscolecida will remain uncertain.

Plectida and Isolaimida

Representatives of the order Plectida (with the exception of *Aulolaimus oxycephalus*) are grouped into a single, monophyletic clade (p.p. = 1.00) in a sister relationship with the Teratocephalidae (Clade 7) on the one hand and the order Rhabditida (Clade 8-12) on the other. SSU rDNA sequence analysis did not support the pharynx development-based hypothesis stating that the Rhabditida are nested within the family Plectidae (von Lieven 2003), nor did it support a direct sister relationship between the Plectidae and the Rhabditida.

Analysis of the rDNA sequences robustly identified the Metateratocephalidae as the most basal family within the order. Recently, Holovachov (2006) presented a morphology-based phylogeny of the Plectida, and the mainly marine families that were considered to be basal among the Plectida, Leptolaimidae and Camacolaimidae, appeared distally in the SSU rDNA-based tree. Moreover, the family Leptolaimidae was paraphyletic relative to the Aphanolaimidae. Hence, our molecular data suggest that both the common ancestor of the Plectida and the one that gave rise to the Plectida (Clade 6) and the Teratocephalidae (Clade 7) and Rhabditida (Clade 8-12) lived in a limnoterrestrial environment.

The only representative of the Plectida superfamily Haliplectoidea, *Aulolaimus oxycephalus*, clustered together with *Isolaimium* (Isolaimida) among the Monhysterida and Araeolaimida dominated Clades 5A and 5C. In the past *Isolaimium* was considered to be a member of the Dorylaimia. Although molecular data suggested this was probably not correct (Mullin, Harris, and Powers 2005; Meldal *et al.* 2007), we can now make a more precise statement about the correct placement of this monogenic order and its relationship to

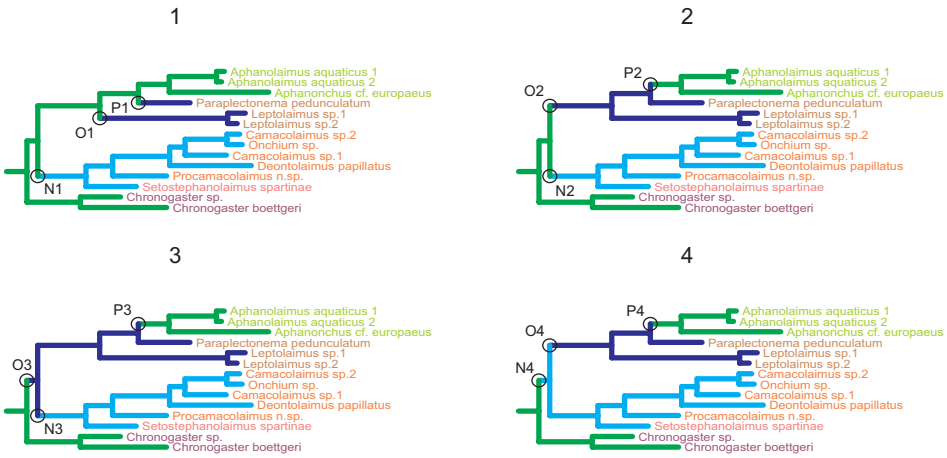


Fig. 4.2. Small subunit ribosomal DNA-based trees of four Plectida families giving five different equally parsimonious options for major habitat transitions. Habitat transitions are encircled, and capital-number combinations next to these circles refer to Tables 4.1 and 4.2.

other nematodes. The sister relationship between *Aulolaimus* and *Isolaimium* is supported by a number of morphological characters. The most obvious one is the strongly elongated cylindrical mouth cavity. *Cylindrolaimus* also has an elongated cylindrical mouth cavity but unlike *Aulolaimus* and *Isolaimium* this is partially (≈ 1/5) enveloped by the esophagus. Furthermore, *Aulolaimus* and *Isolaimium* posses inconspicuous pore-like amphids and reflexed ovaries whereas all nematodes in Clades 5A and 5C posses clearly visible circular or cryptospiral amphids and outstretched ovaries.

Validity of the current clade numbering system

Blaxter *et al.* (1998) divided the Nematoda into five numbered clades, excluding several orders they could not reliably position in the tree. This system was revised by Holterman *et al.* (2006) who defined twelve clades based on a series of mostly well-supported dichotomies. Here we offer a more detailed look into a previously underrepresented part of the Nematoda tree. The identities of Clades 3, 4, 6 and 7 were confirmed by this study. The monophyly of Clade 5 could not be confirmed. Three well-supported subclades could be distinguished: a Monhysterida-dominated clade (Clade 5A), *Aulolaimus* + *Isolaimium* (Clade 5B) and an Araeolaimida-dominated subclade (Clade 5C).

Table 4.1. Taxonomic groups wherein habitat transitions took place. (*): habitat of species involved further described in table 4.2.

Node	Habitat transition	Taxonomic level
A	thalassic → limnoterrestrial	Class
B	thalassic → limnoterrestrial	Superfamily
C	thalassic → thalassic and/or limnoterrestrial (*)	Family
D	thalassic → limnoterrestrial	Superfamily
E	thalassic → thalassic and/or limnoterrestrial (*)	Family
F	Thalassic → limnoterrestrial	Order
G	thalassic → thalassic and/or limnoterrestrial (*)	Family
H	thalassic and/or limnoterrestrial → limnoterrestrial	Family
I	thalassic → thalassic and/or limnoterrestrial (*)	Genus
J	thalassic → limnoterrestrial	Subclass
K	thallassic → limnoterrestrial	Superfamily
L	thalassic → thalassic and/or limnoterrestrial (*)	Subclass
M	thalassic → limnoterrestrial	Subclass
N1	limnoterrestrial → thalassic	Order
N2	limnoterrestrial → thalassic	Order
N3	thalassic and/or limnoterrestrial → thalassic	Order
N4	limnoterrestrial → thalassic	Order
O1	limnoterrestrial → thalassic and/or limnoterrestrial (*)	Family
O2	limnoterrestrial → thalassic and/or limnoterrestrial (*)	Superfamily
O3	limnoterrestrial → thalassic and/or limnoterrestrial (*)	Order
O4	thalassic → thalassic and/or limnoterrestrial (*)	Order
P1	limnoterrestrial → thalassic and/or limnoterrestrial (*)	Family
P2	thalassic and/or limnoterrestrial → limnoterrestrial	Superfamily
P3	thalassic and/or limnoterrestrial → limnoterrestrial	Superfamily
P4	thalassic and/or limnoterrestrial → limnoterrestrial	Superfamily

Because the backbone of Clade 5 is not supported it remains to be seen whether Clade 5 is mono- or paraphyletic. The position of the Selachinematidae and the Microlaimoidea is still uncertain.

The colonization of terrestrial/freshwater habitats by marine species

If the transition of nematodes from marine to terrestrial or freshwater habitats would imply major physiological and/or morphological adaptations, such changes would have been rare. However, this is not the case as at least 16 major habitat changes have occurred, mostly transitions from a marine to a limnoterrestrial lifestyle but there has been at least one reversal to a marine lifestyle (Camacolaimoidea; Figs. 4.1 and 4.2, Table 4.1). Furthermore, given that by definition species and genera within a family are relatively similar, habitat transitions at the genus level would be rare if major physiological

Table 4.2. Sampling locations and possible habitats for species which can occur in both thalassic and limnoterrestrial habitats or for which the actual habitat is uncertain because the nematode was not identified down to species level.

Node	Species	Sampling location	Habitat	Source
C	*Paracyatholaimus intermedius*	Limnoterrestrial habitat, Belgium	marine, brackish, freshwater, terrestrial	(Bongers 1994)
E	*Prochromadora sp.*	Adda river, Italy	most species thalassic *P. orleji*: marine, brackish and terrestrial	(Bongers 1994), (Hansson 1998)
G	*Monhystera riemanni*	Ditch, The Netherlands	brackish and freshwater	(Bongers 1994)
G	*Eumonhystera cf. simplex*	Freshwater pond, The Netherlands	*E. simplex*: brackish and freshwater most species: limnoterrestrial	(Bongers 1994), (Hansson 1998)
I	*Theristus agilis* 1, 2	Freshwater pond, The Netherlands	brackish, freshwater and terrestrial	(Bongers 1994)
L	*Choanolaimus psammophilus* 1, 2	Coastal dunes, The Netherlands	marine and terrestrial	(Bongers 1994), (Hansson 1998)
O	*Leptolaimus sp.*1, 2	Oosterschelde estuary, The Netherlands	most species thalassic *L. papilliger*: brackish, limnoterrestrial *L. setiger*: thalassic, freshwater *L. elegans*: thalassic, limnoterrestrial *L. acicula*: brackish, limnoterrestrial	(Bongers 1994), (Hansson 1998), Fauna Europaea: http://www.faunaeur.org
O-P	*Paraplectonema pedunculatum*	Adda river, Italy	brackish and freshwater	(Bongers 1994)

and/or morphological adaptations are required. Instead, of the 16 habitat transitions at least four took place within a family and one even within a genus (Table 4.1). These data suggest that the number and/or the complexity of the required changes is relatively low. This becomes even more obvious if one considers that the predominantly marine Chromadorida, Microlaimoidea, Monhysteridae and Axonolaimidae contain some more limnoterrestrial species that were not included in our analysis (Wu *et al.* 2000; Peters *et al.* 2005). In fact, the only clade that does not contain both thalassic and limnoterrestrial members is the strictly marine Desmodoroidea (hypothesizing that *Prodesmodora* does indeed not belong in this superfamily; Appendix B).

Probably the main physiological problem that needs to be solved while switching from a nearly iso-osmotic marine to a terrestrial habitat would be the large fluctuations in the external osmotic pressure (including possible desiccation) in the new environment. Nematode movement depends on the combined action of longitudinal muscles and a functional hydroskeleton.

Limnoterrestrial and intertidal nematodes are able to regulate osmotic pressure (Wright 2004). There are essentially two ways to cope with fluctuating conditions; osmoconformers conform their intracellular osmolarity to the extracellular osmotic pressure, while osmoregulators keep their internal osmotic value constant when the osmotic value of the environment is fluctuating. Osmoconformers are thought to be present among intertidal nematodes (Wright 2004). Osmoregulation has been demonstrated for terrestrial nematodes (Wright and Newall 1980). *Caenorhabditis elegans*, a bacterivorous terrestrial nematode, was shown to survive on growth media containing 21-500 mM of NaCl. To control the water balance, organic non-perturbing osmolytes are produced and broken down. In *C. elegans* glycerol was shown to play a major role in maintaining osmotic homeostasis. Hypertonic conditions result in the synthesis of glycerol, whereas hypotonic conditions are balanced by glycerol breakdown and/or excretion (Lamitina *et al.* 2004). For *C. elegans*, the role of aquaporins (AQP) – channel forming proteins that allow transport of water and small solutes (including glycerol) – in osmoregulation was investigated (Huang *et al.* 2007). AQP-2 (permeability to water), AQP-3 (to water and glycerol), AQP-8 (function unknown) are expressed in the excretory cell, and only AQP-2 is expressed in the hypodermis. AQP-1 (glycerol) and AQP-4 (water) are expressed in the intestine. Mutant analysis including *aqp-2*, *aqp-3*, *aqp-4* and *aqp-8* resulted in a phenotype only modestly more sensitive to hypotonic stress. These results could point at functional redundancy among aquaporins and their importance in osmoregulation remains unclear (Huang *et al.* 2007). Regarding overall permeability to water, terrestrial nematodes (*Aphelenchus avenae*, *Caenorhabditis briggsae*) were shown to be two orders of magnitude less penetrable to water than marine species such as *Enoplus* spp. (Wright 1998). On the basis of this information it would - as a first step - be relevant to investigate whether major habitat transitions as pinpointed in this paper correspond to changes in capability to synthesize and break down and/or excrete glycerol.

Alternatively, the excretory system could contribute to osmotic homeostasis. There are two types of excretory systems within the Nematoda, a tubular system within the Teratocephalidae + Rhabditida (Clades 7-12) and a glandular system within the former Adenophorea (Clades 1-5). The members of

the Plectida (Clade 6) harbor intermediate forms (Maggenti 1981). Probably the gut also plays an important role in the excretion of excess fluid (Wright 2004). The tubular system has been proven to play a role in the osmoregulation (Nelson and Riddle 1984; Wharton and Sommerville 1984). The glandular excretory system - if present at all - is more simple and non-canalicular, and it is questionable whether it plays a role in osmoregulation (Wright 1998). Within the order Plectida, Holovachov (2006) observed that only freshwater and terrestrial representatives had a long excretory duct, no ampulla and only for these nematodes the proximal part of the excretory duct was embedded in the renette cell. Holovachov (2006) suggested that these adaptations could be functional in maintaining the internal osmotic pressure. Their reversal to the ancestral state in the secondarily marine Camacolaimidae and Leptolaimidae supports the idea about their importance in limnoterrestrial habitats. In case of the absence of an excretory cell, basal Chromadoria often harbor numerous longitudinal hypodermal glands. It is not known whether these glands are functional in osmoregulation.

A further difference between limnoterrestrial and thalassic nematodes is that the former tend to have a higher turgor pressure. Possibly as a consequence of this higher turgor limnoterrestrial nematodes have a stronger cuticle. Radial striae and/or spiral fiber layers in the basal zone of the cuticle are important for delivering radial strength to the cuticle. Notably, the radial striae are always located in the basal cuticle zone in terrestrial nematodes, whereas in all marine nematodes they are located in the cortical cuticle zone. However, these striae are not homologous and the cortical zone plays a role in adding flexibility to the cuticle (Decraemer *et al.* 2003). Yet the strict positioning of radial striae according to the marine or limnoterrestrial habitat is striking and it is tempting to think that this adaptation is functional in the transition from marine to terrestrial habitats.

Conclusions

Detailed analysis of 128 nearly full length SSU rDNA sequences from basal Chromadoria by the combined use of a stem - loop partition and a GTR+Γ+I model revealed an overall topology that is largely in accordance with the clade

division proposed by Holterman *et al*. (2006). Among the orders considered as belonging to the basal Chromadoria - Chromadorida, Desmodorida, Desmoscolecida, Monhysterida, Araeolaimida and Plectida – only the Chromadorida appears to be robust and monophyletic. The order Plectida is fairly robust as well, with the exception of *Aulolaimus* which was placed together with *Isolaimium* in a separate clade. As only two sequences were available from the Desmoscolecida, no conclusions can be drawn about this order. Our data suggest a redefinition of the orders Monhysterida and Araeolaimida. As an alternative, we found robust support for two clades that can at best be labeled as Monhysterida- and Araeolaimida-dominated. For the Desmodorida we observed a subdivision corresponding to the two superfamilies defined within this order, the Desmodoroidea and the Microlaimoidea. The phylogenetic position of the latter could not be resolved. Our analysis revealed a remarkable high number of major habitat transitions in groups of various taxonomic levels (from class to genus level). The observation that families and genera whose monophyletic nature was confirmed by molecular data may comprise members living in essentially different habitats, point at a relatively small number of morphological and/or physiological adaptations that is needed to realize these transitions. In this respect basal Chromadoria show a remarkable ecological flexibility.

Acknowledgments

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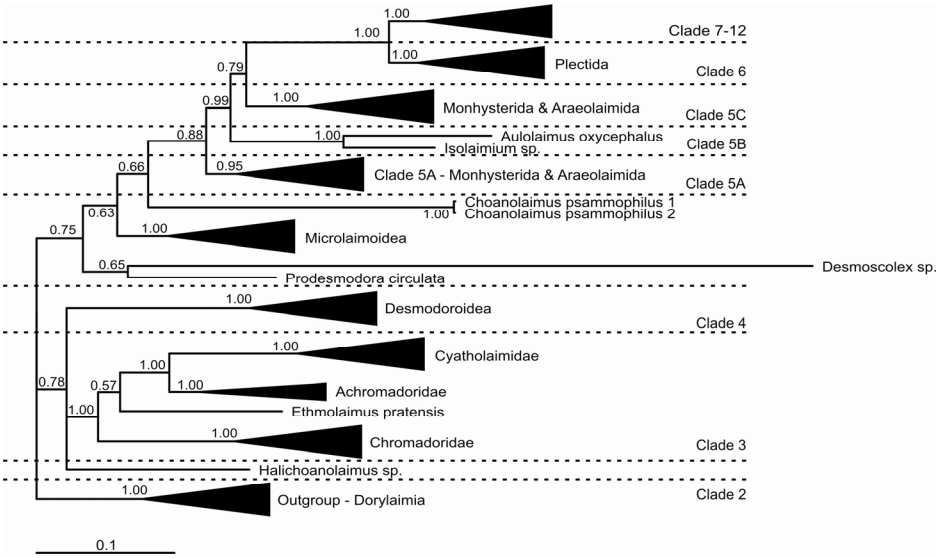
Appendix A

GenBank accession numbers for the SSU rDNA sequences used in this study.

Species	NCBI Accession	Species	NCBI Accession
Acanthopharynx micans	Y16911	Halichoanolaimus sp.	EF591338
Achromadora cf. terricola	AY593940	Innocuonema tentabundum	AY854208
Achromodora sp.	AY284717	Isolaimium sp.	AY552971
Anaplectus grandepapillatus 1	AY284697	Laxus cosmopolitus	Y16918
Anaplectus grandepapillatus 2	AY284698	Laxus oneistus	Y16919
Anatonchus tridentatus	AY284768	Leptolaimus sp.1	EF591323
Aphanolaimus aquaticus 1	AY593932	Leptolaimus sp.2	EF591324
Aphanolaimus aquaticus 2	AY593933	Leptonemella sp.	Y16920
Aphanonchus cf. europaeus	EF591319	Mesotheristus hirsutus	AY854223
Aporcelaimellus obtusicaudatus	AY284811	Mesotheristus normandicus	AY854224
Ascolaimus elongatus 1	AY854231	Mesotheristus oxycercus	AY854225
Ascolaimus cf. elongatus 2	EF591330	Mesotheristus setosus	AY854226
Atrochromadora microlaima	AY854204	Metachromadora remanei	AY854216
Aulolaimus oxycephalus	AY284724	Metachromadora sp.	EF591339
Axonolaimus helgolandicus	AY854232	Metadesmolaimus sp.	AJ966491
Axonolaimus sp.	EF591331	Metateratocephalus	AY284686
Bathyodontus mirus	AY284744	crassidens 1	
Calomicrolaimus parahonestus	AY854218	Metateratocephalus	AY284687
Calomicrolaimus sp.	AY854219	crassidens 2	
Camacolaimus sp.1	EF591325	Microlaimus tenuispiculum	AY854220
Camacolaimus sp.2	EF591327	Monhystera riemanni	AY593938
Catanema sp.	Y16912	Monoposthia costata	AY854221
Ceratoplectus armatus	AY284706	Myolaimus sp.	U81585
Choanolaimus psammophilus 1	AY284715	Nudora bipapillata	AY854222
Choanolaimus psammophilus 2	AY284716	Odontophora rectangula	AY854233
Chromadora nudicapitata	AY854205	Onchium sp.	EF591328
Chromadora sp.	AY854206	Paracanthonchus caecus	AF047888
Chromadorina germanica	AY854207	Paracyatholaimus intermedius	AJ966495
Chromadoropsis vivipara	AF047891	Paraplectonema pedunculatum	EF591320
Chronogaster boettgeri	AY593931	Plectus acuminatus	AF037628
Chronogaster sp.	AY284709	Plectus cf. cirratus	AY284701
Chrysonema attenuatum	AY284779	Plectus cf. parietinus 1	AY284702
Coslenchus costatus	AY284581	Plectus cf. parietinus 2	AY284703
Cyartonema elegans	AY854203	Plectus cf. parvus	AY284699
Cyatholaimus sp.1	AY854213	Poikilolaimus regenfussi	AF083022

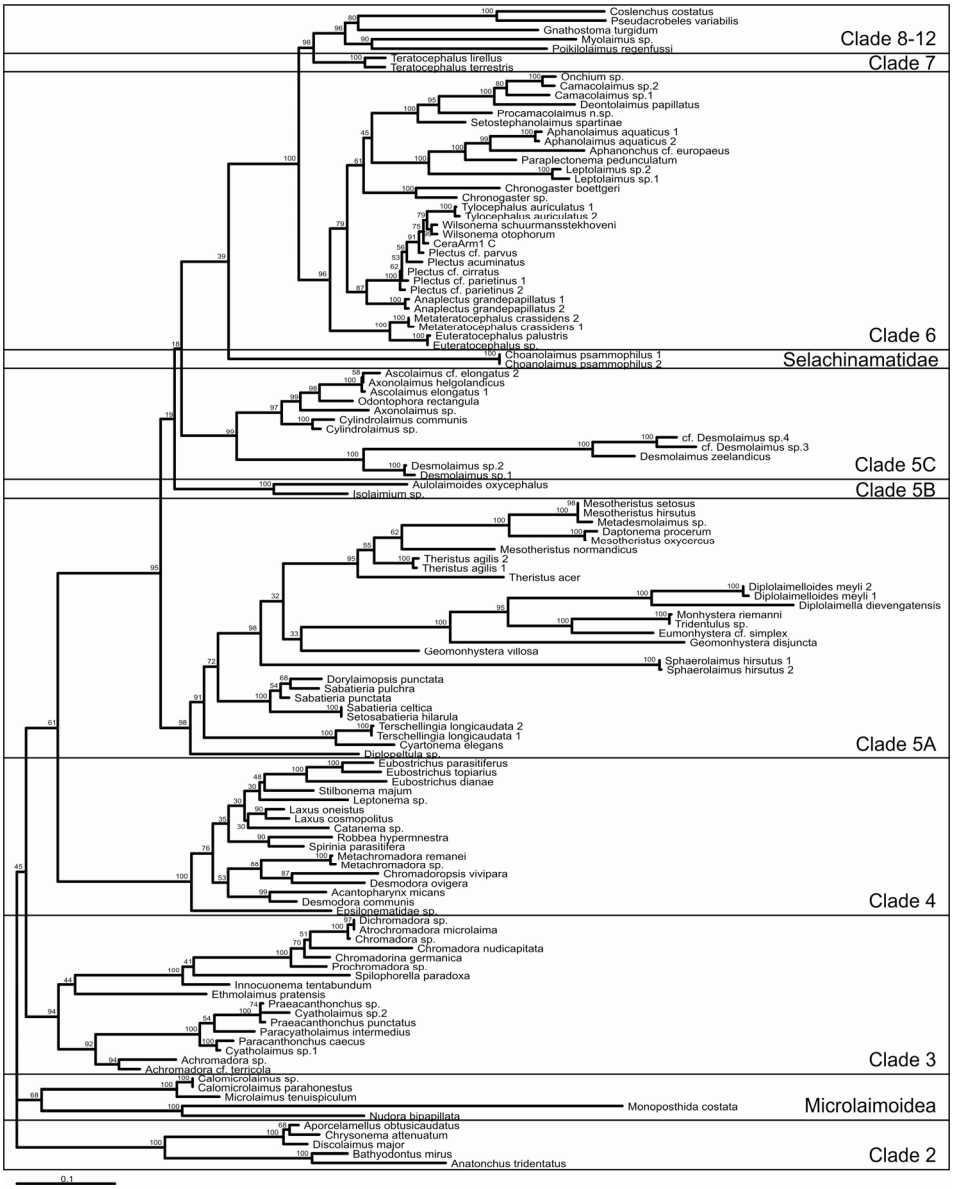
Cyatholaimus sp.2	AM234618	Praeacanthonchus punctatus	AY854214
Cylindrolaimus communis	AY593939	Praeacanthonchus sp.	AF036612
Cylindrolaimus sp.	AF202149	Procamacolaimus n.sp.	EF591326
Daptonema procerum	AF047889	Prochromadora sp.	EF591341
Deontolaimus papillatus	EF591322	Prodesmodora circulata	AY284722
Desmodora communis	AY854215	Pseudacrobeles variabilis	AF202150
Desmodora ovigera	Y16913	Robbea hypermnestra	Y16921
Desmolaimus sp.1	EF591332	Sabatieria celtica	AY854234
Desmolaimus sp.2	EF591333	Sabatieria pulchra	EF591335
cf. Desmolaimus sp.3	EF591336	Sabatieria punctata	AY854235
cf. Desmolaimus sp.4	EF591337	Setosabatieria hilarula	AY854240
Desmolaimus zeelandicus	AY854229	Setostephanolaimus spartinae	EF591321
Desmoscolex sp.	EF591342	Sphaerolaimus hirsutus 1	AY854228
Dichromadora sp.	AY854209	Sphaerolaimus hirsutus 2	AM234622
Diplolaimella dievengatensis	AJ966482	Spilophorella paradoxa	AY854211
Diplolaimelloides meyli 1	AF036644	Spirinia parasitifera	AY854217
Diplolaimelloides meyli 2	AF036611	Stilbonema majum	Y16922
Diplopeltula sp.	EF591329	Teratocephalus lirellus	AF036607
Discolaimus major	AY284828	Teratocephalus terrestris	AY284683
Dorylaimopsis punctata	AM234047	Terschellingia longicaudata 1	AY854230
Epsilonematidae sp.	EF591340	Terschellingia longicaudata 2	AM234716
Ethmolaimus pratensis	AY593942	Theristus acer	AJ966505
Eubostrichus dianae	Y16915	Theristus agilis 1	AY284693
Eubostrichus parasitiferus	Y16916	Theristus agilis 2	AY284695
Eubostrichus topiarius	Y16917	Tridentulus sp.	AJ966507
Eumonhystera cf. simplex	AY284692	Tylocephalus auriculatus 1	AF202155
Euteratocephalus palustris	AY284684	Tylocephalus auriculatus 2	AY284707
Euteratocephalus sp.	AY284685	Wilsonema otophorum	AY593927
Geomonhystera disjuncta	AJ966485	Wilsonema schuurmansstekhoveni	AJ966513
Geomonhystera villosa	EF591334		
Gnathostoma turgidum	Z96948		

Appendix B



Simplified tree from the preliminary Bayesian analysis including the rogue taxa Desmoscolex sp., Prodesmodora circulata and Halichoanolaimus sp. Numbers indicate posterior probabilities.

Appendix C



Tree with the best likelihood acquired by the maximum likelihood method. Bootstrap values are indicated at nodes (values > 65 are considered robust).

Chapter 5

SSU rDNA-based phylogeny of the Tylenchida sheds light on the evolution of plant feeding & establishes relationships among high impact plant parasitic nematodes

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SSU rDNA-based phylogeny of the Tylenchida sheds light on the evolution of plant feeding & establishes relationships among high impact plant parasitic nematodes

The nematode order Tylenchida predominantly harbors plant parasites, including cyst (Heteroderidae), root knot (*Meloidogyne* spp.) and lesion (*Pratylenchus* spp.) nematodes. Over decades a substantial number of (partially) conflicting hypotheses have been formulated about the phylogenetic relationships between and within these economically highly relevant groups, and their relatedness to other plant parasitic Tylenchida. Plant parasitism among nematodes is hypothesized to be the result of a gradual evolution from fungal feeding ancestors via simple forms of (facultative) ectoparasitism to complex forms of endoparasitism. We have constructed a molecular phylogeny of 116 Tylenchida taxa using the small subunit ribosomal DNA gene (SSU rDNA; ≈ 1,700 bp). A good resolution was achieved in distal clades that include cyst, root knot and lesion nematodes, and monophyly of most families was confirmed. However, our data suggest that *Meloidogyne* spp. are actually nested within the Pratylenchidae, and the burrowing nematode *Radopholus similis*, a notorious pest in citrus and banana, is positioned at the base of the Hoplolaimidae, and not within the Pratylenchidae. Reconstruction of the ancestral feeding types shows how plant parasitism developed often, but not always gradually from simple to complex forms. The evolution of plant parasitism is accompanied by the availability of specific tools, including cell wall-degrading enzymes. Cellulases are most widely studied, and the current analysis based on SSU rDNA - a gene unrelated to plant pathogenicity - allowed us to pinpoint basal groups within this order that will be highly informative in the reconstruction of the molecular evolution of plant parasitism and the putative role of horizontal genes transfer from soil bacteria therein.

Introduction

Nematodes are one of the most successful groups of animals; they are abundant, diverse, and live in virtually all soil, freshwater and marine habitats. These small vermiform organisms occupy several trophic levels and play an important role in the soil ecosystem (Bongers and Ferris 1999). Certain groups of nematodes also cause large economic losses as parasites of animals (including humans) or parasites of plants (Anderson 2000; Siddiqi 2000). Total losses caused by plant parasitic nematodes are estimated at $80 billion annually (Agrios 2005), and most of these – including the cyst (family Heteroderidae), lesion (*Pratylenchus* spp.) and root knot nematodes (*Meloidogyne* spp.) – belong to the order Tylenchida.

Because of the economic importance of this group, the Tylenchida have always received ample taxonomic attention. Nematodes have a relatively conserved morphology; generally spoken they are colorless, unsegmented worm-like animals, round in cross section. As such, nematodes show a paucity of clearly distinguishable, informative morphological characters, and - moreover - the polarity of morphological characters (ancestral or derived) is often hard to determine. This has led to several different classifications and constant taxonomic changes (Luc *et al*. 1987; Siddiqi 2000; De Ley and Blaxter 2002). Molecular data can provide a virtually unlimited number of characters, and therefore a phylogeny based on molecular data could help to create more clarity in the phylogenetic relationships within this group.

The Tylenchida contains mainly plant parasites, but it is hypothesized that this plant parasitism evolved from fungal feeding ancestors who acquired the ability to feed on algae, mosses or root hair and epidermal cells (Luc *et al*. 1987). These simple forms of plant parasitism are thought to have evolved gradually, via intermediary forms, towards more complex forms of plant parasitism that can be found among sedentary endoparasites such as cyst and root knot nematodes. The availability of a robust molecular phylogeny would allow us to test this hypothesis. Remarkably, the Tylenchida also harbor a number of insect parasites (members of the suborder Hexatylina). These represent the only case in the phylum Nematoda in which insect parasites are closely related to (and probably evolved from) fungal and plant feeding

nematodes.

In recent years much research has been done on the molecular interactions between plant parasitic nematodes (mainly endoparasites) and plants. One of the remarkable things discovered was that several endoparasitic species - *Globodera*, *Heterodera* ;Smant *et al*. 1998), *Meloidogyne* (Rosso *et al*. 1999), *Pratylenchus* (Uehara, Kushida, and Momota 2001) and recently *Bursaphelenchus* (Kikuchi, Shibuya, and Jones 2005) - produce and secrete cell wall-degrading enzymes, something which had never been recorded before in animals (Smant *et al*. 1998). Interestingly, the genes encoding for these enzymes show the highest homologies to bacterial equivalents, and this finding gave rise to the suggestion that they could have been acquired by horizontal gene transfer (Yan *et al*. 1998). Cell wall-degrading enzymes have now been found in a few other animals - sea snails (Suzuki, Ojima, and Nishita 2003; Wang *et al*. 2003), crayfish (Byrne *et al*. 1999), blue mussel (Xu, Janson, and Sellos 2001), beetle (Sugimura *et al*. 2003) - , but the origin of these genes in nematodes still remains much discussed. Are they indeed the result of horizontal gene transfer from bacteria, and - if so - were these genes or pathogenicity islands acquired once, or multiple times? The research presented here does not answer these questions, but it can help to select species that are the best candidates for further research on the origin of these genes. The phylogeny presented here is based on the small subunit ribosomal DNA (SSU rDNA) gene, which has proven to be useful for reconstructing nematode phylogeny. Accelerated evolution in this clade (Clade 12 in Holterman *et al*. 2006) - probably due to the parasitic nature of all its members - resulted in a high resolution among the Tylenchina in most cases even up to species level.

Material and methods

Taxon sampling and systematics

Nematodes were collected from various habitats throughout the Netherlands. They were extracted from the soil using standard techniques and identified under a light microscope. Prior to DNA extraction, pictures were taken using a CCD camera (CoolSnap, RS photometrics). SSU rDNA sequences from collected species were supplemented with sequences taken from GenBank. For a full

overview of the sequences used including GenBank accessions numbers, see appendix A. For the systematics throughout this paper we adhere to the classification of Siddiqi (2000).

DNA extraction and SSU rDNA sequencing

Single nematodes were transferred to 0.2 ml 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 Thermomixer (Eppendorf) at 65° C and 750 r.p.m. for 2 hours followed by a 5 minute incubation at 100° C. Lysate was immediately used or stored at -20° C. SSU rDNA was amplified as two partially overlapping fragment using three universal and one nematode-specific primer (1912R). For the first fragment, either the primer 988F (5′-ctcaaagattaagccatgc-3′) or the primer 1096F (5′-ggtaattctggagctaatac-3′) was used in combination with the primer 1912R (5′-tttacggtcagaactaggg-3′). The second fragment was amplified with primers 1813F (5′-ctgcgtgagaggtgaaat-3′) and 2646R (5′-gctaccttgttacgactttt-3′). PCR was performed in a final volume of 25 µl 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 program was used: 94° C for 5 min; 5 x (94° C, 30 s; 45° C, 30s; 72° C, 70s); 35 x (94° C, 30 s; 54° C, 30s; 72° C, 70s); 72° C for 5 min. Gel-purified amplification products (Marligen) were cloned into a TOPO-TA vecter (Invitrogen) and sent off for sequencing using standard procedures. Part of these sequences have been used in a previous study (Holterman *et al*. 2006), sequences newly generated for this study will be deposited at GenBank.

Sequence alignment and phylogenetic analysis

The sequences were aligned using the ClustalW algorithm as implemented in the program BioEdit 7.0.1 (Hall 1999). The aligment was then manually corrected using arthropod secondary structure information (http://bioinformatics.psb.ugent.be/webtools/rRNA/secmodel/index.html in accordance with (Wuyts *et al*. 2000)). The final Tylenchida alignment included 116 sequences with a total length of 1,916 bases including gaps.

The Tylenchida SSU rDNA tree was constructed using Bayesian

inference and a fast maximum likelihood model.. Modeltest 3.06 (Posada and Crandall 1998) selected the GTR model with invariable sites and a gamma substitution as the best fitting nucleotide substitution model using both the likelihood ratio test and the Akaike Information Criterion. The Bayesian phylogeny was constructed with the program MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) using the GTR model with invariable sites and a gamma-shaped distribution of substitution rates. On the basis of the secondary structure, the overall alignment was subdivided into stem and loop partitions. Parameters were unlinked between the partitions and the rateprior was set to variable. The default flat priors were used. Four independent runs were made with 4 Markov chains per run. The program was run for 5 million generations. Stabilization of the likelihood and parameters was checked with the program Tracer 1.2.1 (Rambaut and Drummond 2005) and the burnin was set at 700,000 generations.

The second phylogenetic tree was constructed with a fast maximum likelihood method. The SSU rDNA alignment was analyzed at a distant server (http://phylobench.vital-it.ch/raxml-bb/index.php) running the program, RaxML-VI-HPC v.4.0.0 (Randomized Axelerated Maximum Likelihood for High Performance Computing; Stamatakis, 2006). A GTR model with invariable sites and gamma distribution was used, and the dataset was divided in a stem and loop partition. 100 bootstraps were performed.

For the Pratylenchidae tree, 10 additional *Pratylenchus* and 5 additional *Hirschmanniella* sequences were included. The alignment consisted of 37 sequences with a total alignment length of 1,917 bases including gaps. A Bayesian tree was constructed using the same settings as used for the Tylenchida tree.

Reconstruction of ancestral feeding types

Feeding types of each species were according to Yeates *et al.* (1993). When more than one feeding type was listed for a genus, we referred to Siddiqi (2000) or Bongers (1994) to infer the correct feeding type for each species. In a few cases other literature had to be used (appendix B) and for one species, *Ditylenchus adasi*, the correct feeding type (hyphal feeding, migratory endoparasite or both) could not be determined.

Table 5.1. Stepmatrix used in parsimony reconstruction of ancestral feeding types. The different feeding types were put on a gradual scale ranging from bacterial feeding at the one end to sedentary endoparasitism on the other end. The change from bacterial feeding to hyphal feeding was considered to be greater, because of the morphological changes required. Insect parasitism did not fit in this gradual scale so every change to or from insect parasitism was considered equally likely.

	Bact.	**Hyph.**	**A.l.m.**	**R.h. epid.**	**Ectop.**	**Semi-endop.**	**Migr. endop.**	**Sed. endop.**	**Insect**
Bact.	x	3	4	5	6	7	8	9	3
Hyph.	3	x	1	2	3	4	5	6	3
A.l.m.	4	1	x	1	2	3	4	5	3
R.h. epid.	5	2	1	x	1	2	3	4	3
Ectop.	6	3	2	1	x	1	2	3	3
Semi-endop.	7	4	3	2	1	x	1	2	3
Migr. endop.	8	5	4	3	2	1	x	1	3
Sed. endop.	9	6	5	4	3	2	1	x	3
Insect	3	3	3	3	3	3	3	3	x

Bact.: bacterial feeding, Hyph.: hyphal feeding, A.l.m.: algal, lichen and moss feeding, R.h. epid.: root hair and epidermal feeding, Ectop.: ectoparasite, Semi-endop.: semi-endoparasite, Migr. endop.: migratory endoparasite, Sed. endop.: sedentary endoparasite, Insect: insect parasite.

Ancestral feeding types were reconstructed using the program Mesquite 1.12 (Maddison and Maddison 2006). Ancestral character states can be reconstructed in several different ways. We chose to reconstruct ancestral feeding types using both the parsimony and likelihood model. Both an unordered parsimony analysis and a parsimony analysis using a stepmatrix (Table 5.1) were performed. For the likelihood analysis no polymorphic character states could be included, so two character matrices were used, one including the most ancestral character state (usually hyphal feeding) for polymorphic species and the second matrix containing the most derived character states (appendix B). Branches in the tree with a posterior probability <0.95 were collapsed for the analyses.

Results and Discussion

Tylenchida phylogeny

Bayesian and a maximum likelihood trees were constructed for the nematode order Tylenchida on the basis of 116 full length small subunit ribosomal DNA sequences (≈ 1.700 bp each) The Bayesian tree (Figure 5.1) and maximum likelihood tree (Appendix C) were virtually indentical in terms of their resolution. In the top part of the tree, we observe a robust sister relationship between two major subclades (A and B). One predominantly consists of members of the Hoplolaimidae and the Heteroderidae (A), while the other subclade is dominated by members of the Meloidogynidae, Pratylenchidae and Telotylenchidae (B). Together with the genus *Pratylenchoides* and a few representatives of the Telotylenchidae, whose SSU rDNA sequences defined a separate subclade (C), these major subclades constitute the suborder Hoplolaimina. According to current taxonomy, *Psilenchus* belongs to the Hoplolaimina as well, but this is not supported by our tree and indeed several authors place *Psilenchus* in the Tylenchina (Geraert and Raski 1987; Brzeski 1989). In between the most basal and the most distal parts of the tree, a number of nodes are poorly supported and there are several polytomies. Although we can not define the relationship between the suborder Criconematina and other Tylenchida suborders, its members clearly constitute a separate and well-supported subclade (Fig. 5.1, D). On the other hand, SSU rDNA sequences from representatives of the suborders Tylenchina and Hexatylina do not cluster as monopheletic groups. The families Aphelenchidae (harbouring a single genus; *Aphelenchus*) and the Paraphelenchidae (harbouring a single genus; *Paraphelenchus*) are robustly positioned at the base of this phylogenetic tree.

At family level, SSU rDNA-based systematics corresponds closely to current morphology-based systematics. Only 6 out of the 22 families included are demonstrably para- (Hoplolaimidae, Criconematidae) or polyphyletic (Pratylenchidae, Telotylenchidae, Ecphyadophoridae, Neotylenchidae). For three families (Tylenchidae, Anguinidae and Neotylenchidae) no solid conclusion can be drawn due to the poor support, but they seem to be para- or polyphyletic as well. It is noteworthy that four of these para- or polyphyletic families - Telotylenchidae, Criconematidae, Tylenchidae and Ecphyadophori-

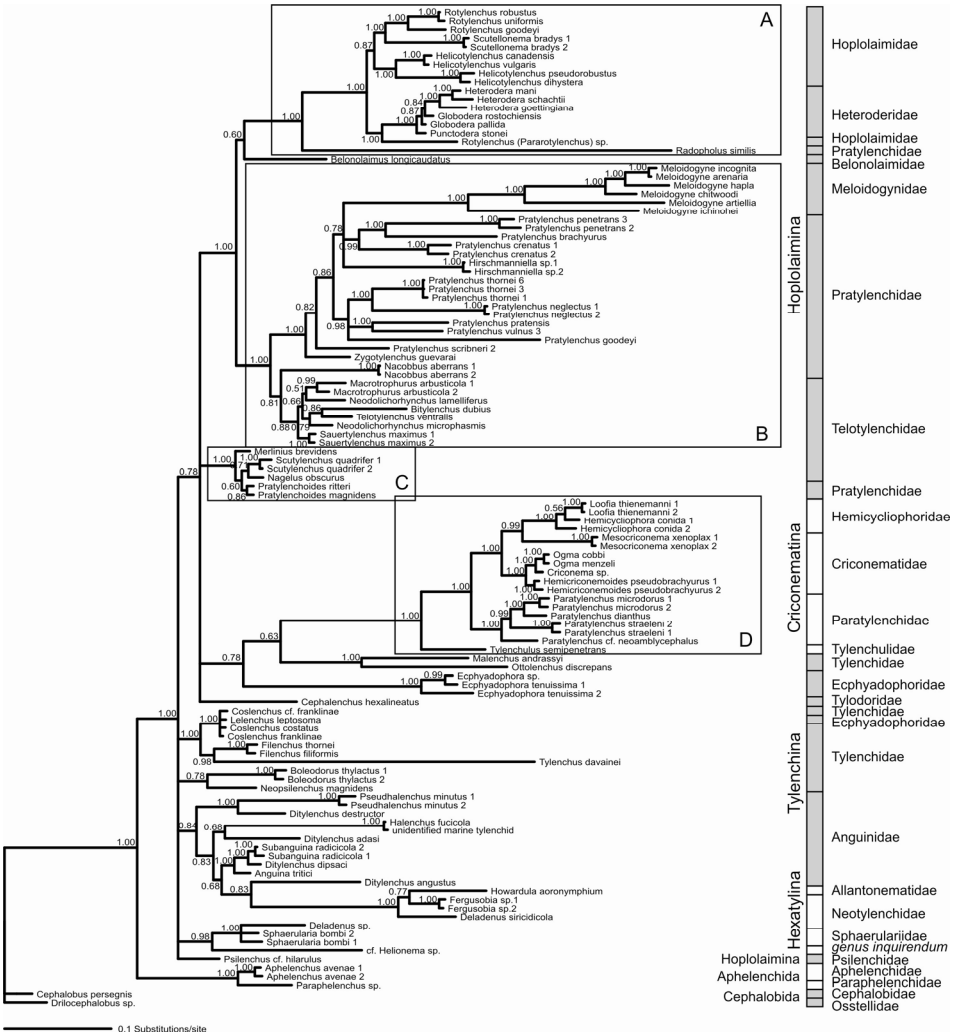

Figure 5.1. Bayesian SSU rDNA tree of the Tylenchida. Numbers near nodes indicate posterior probabilities. Vertical bar indicates families and suborders.

dae - are divided according to their subfamilies sensu Siddiqi (Siddiqi 2000). This also true for the Pratylenchidae with one exception, the Radopholinae, whose representatives, *Radopholus similis*, *Pratylenchoides ritteri* and *P. magnidens*, are positioned in subclades A and C.

Here, we will focus further on the Hoplolaimina, since this is the region of the tree that is best supported and it contains most of the economically important plant parasites.

A. Hoplolaimidae and Heteroderidae

In our analysis, the Hoplolaimidae appear to be paraphyletic as the family Heteroderidae is nested within it. This is caused by the placement of *Rotylenchus (Pararotylenchus) sp*., a member of the subgenus *Pararotylenchus*, as the sister taxon to the Heteroderidae. This placement is remarkable, as one would expect *Pararotylenchus* as the sistertaxon to *Rotylenchus*. From a morphological perspective, the only real difference between *Rotylenchus* and *Pararotylenchus* are the pharyngeal glands (Fortuner 1987); in *Rotylenchus* these glands overlap the intestine mainly dorsally, while in *Pararotylenchus* they form a terminal bulb and do not overlap the intestine. Although the placement of *Pararotylenchus* is well supported (p.p. = 1.00), these abutting pharyngeal glands are not shared by the Heteroderidae. Subbotin *et al*. (2006) also found the Hoplolaimidae to be paraphyletic using LSU rDNA sequences from the expansion segments D2 and D3 (spanning ≈ 560 bp), but this was not supported by significant posterior probabilities. Morphological similarities between these two families already led De Ley and Blaxter (2002) to consider the Heteroderidae – by the authors referred to as the Heteroderinae - as a subfamily of the Hoplolaimidae. Generating additional sequences from *Pararotylenchus* and other Hoplolaimidae and Heteroderidae genera could possibly shed more light on the systematics of these two important plant parasitic families.

B. Meloidogynidae, Pratylenchidae and Telotylenchidae

The current phylogenetic analysis places *Meloidogyne* within the Pratylenchidae. This is consistent with earlier analyses (Holterman *et al*. 2006; Subbotin *et al*. 2006). *Nacobbus* on the other hand, clusters with the Telotylenchidae, albeit with no significant support (posterior probability = 0.81). To the best of our knowledge there are no morphological characters supporting the relationship between *Nacobbus* and the Telotylenchidae to the exclusion of the other Pratylenchidae and *Meloidogyne*. There is, however, a character uniting *Meloidogyne*, *Pratylenchus*, *Zygotylenchus* and *Hirschmanniella* to the exclusion of the Telotylenchidae, Nacobbinae and Radopholinae. The pharyngeal glands in *Meloidogyne*, *Pratylenchus*, *Zygotylenchus* and *Hirschmanniella* are overlapping the intestine ventrally or ventrolaterally, whereas in other Pratylenchidae and

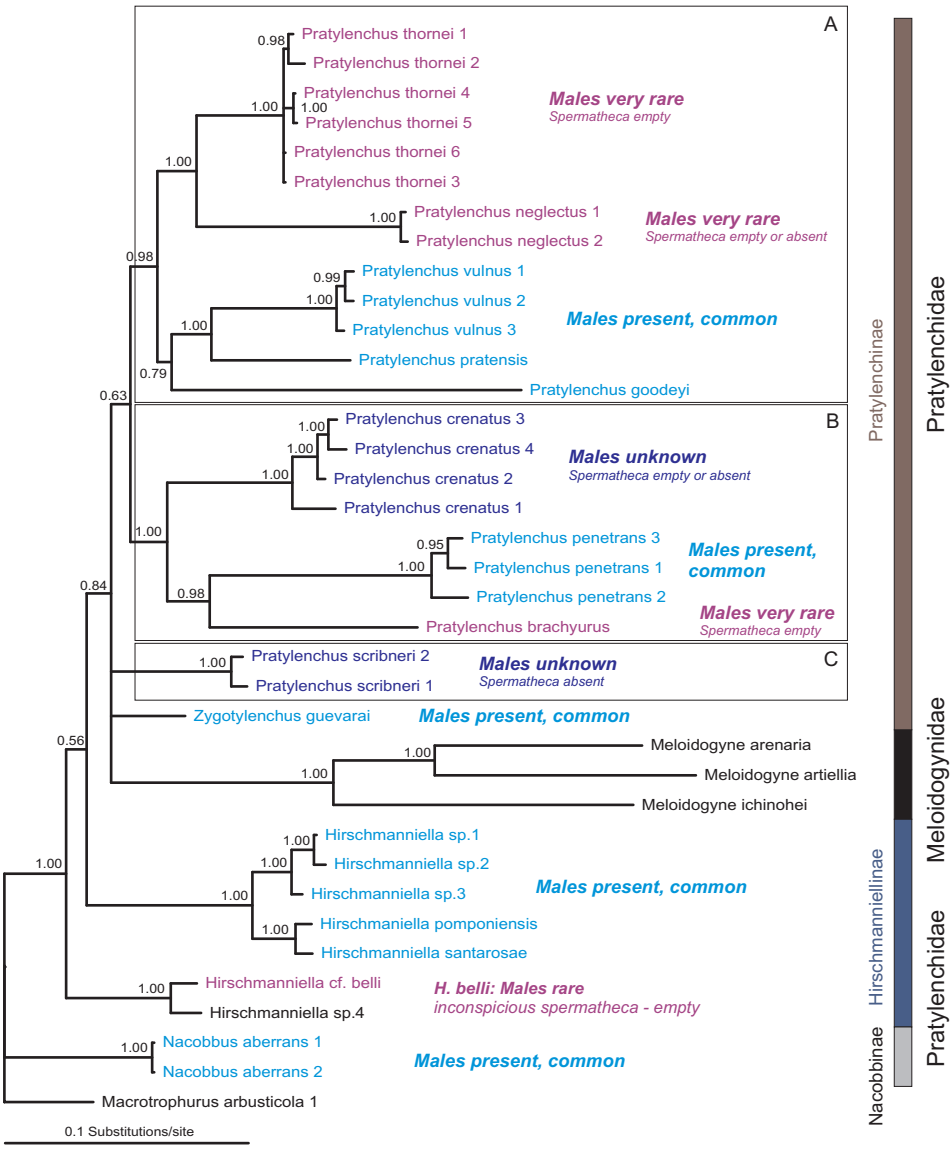

Figure 5.2. Bayesian SSU rDNA tree of the Pratylenchidae. Numbers near nodes indicate posterior probabilities. Vertical bar indicates families and subsubfamilies.

the Telotylenchidae this overlap is dorsally, dorsolaterally or non-overlapping (Siddiqi 2000).

A more extensive Pratylenchidae tree (Fig. 5.2) was constructed to test if the Meloidogynidae really originate from within the Pratylenchidae, and to

verify whether there is indeed an evolutionary trend among *Pratylenchus* species from amphimixis towards multiplication through parthenogenesis as suggested by Ryss (2002). The SSU rDNA Pratylenchidae tree is well able to separate different Pratylenchus species, but the support for the Pratylenchidae backbone remained poor (Fig. 5.2). The inclusion of additional *Hirschmanniella* representatives resulted in a separation between *Hirschmanniella cf. belli* and the other *Hirschmanniella* species (see also Tandingan De Ley *et al*. 2007). A well-supported sister relationship was observed between *Hirschmanniella cf. belli* on the one hand and the other Hirschmanniellinae, Pratylenchinae and Meloidogynidae on the other. The relationship between the Hirschmanniellinae and the Pratylenchinae, and the relatedness between members of the two Pratylenchinae genera - *Zygotylenchus* and *Pratylenchus* - remain to be resolved. Nevertheless, our analysis provided ample support for three *Pratylenchus* clusters: cluster A including *P. thornei, P. neglectus*, *P. vulnus*, *P. pratensis* and *P. goodeyi*, cluster B comprising *P. crenatus*, *P. penetrans* and *P. brachyurus*, and cluster C so far only consisting of *P. scribneri.* Some *Pratylenchus* species multiply through amphixis, whereas other seems to multiply through parthenogenesis (data taken from Handoo and Golden 1989). Ryss (2002) suggested a trend within the genus *Pratylenchus* from sexual to asexual reproduction. On the basis of molecular data we did not observe a clear pattern, but it seems likely that parthenogenesis has arisen multiple times among *Pratylenchus* species.

Radopholus similis

Radopholus similis is another member of the Pratylenchidae which is not placed in the Pratylenchidae in the current phylogenetic analysis. It is placed as the sistergroup of the Hoplolaimidae (Fig. 5.1), this is consistent with an earlier analysis by Subbotin *et al*. (2006) based on the large subunit (LSU) rDNA. The Pratylenchidae are a heterogeneous family (Luc 1987) and *Radopholus* stands out for its strong sexual dimorphism and its distinct lip pattern as seen in SEM (Luc 1987). There are a number of morphological characters that support its placement with the Hoplolaimidae. First of all its strong sexual dimorphism in the cephalic region is shared by the Hoplolaimidae and Heteroderidae, but is absent in the Pratylenchinae, Hirschmanniellinae and Telotylenchidae (Siddiqi

2000). *Radopholus* (and *Radopholoides*, not included) is also the only member of the Pratylenchidae to have a protrusible gubernaculum, a trait shared by part of the Hoplolaimidae (Hoplolaiminae, *Rotylenchus*, *Aphasmatylenchus*; (Siddiqi 2000)). Finally the dorsally overlapping pharyngeal glands, although also present in other Radopholinae and *Nacobbus*, distinguishes *Radopholus* from the Pratylenchinae and *Hirschmanniella*. Again this character is shared by the Hoplolaimidae (with the exception of *Helicotylenchus*, *Rotylenchoides* and *Aphasmatylenchus*; Siddiqi 2000). Given the results of the current and previous (Subbotin *et al.* 2006) molecular phylogenetic analyses and the morphological heterogeneity of the Pratylenchidae, a revision of the family and especially the subfamily Radopholinae seems necessary.

C. Pratylenchoides and Merliniinae

Pratylenchoides is placed in the Merliniinae (Telotylenchidae) in the phylogeny (Fig. 5.1). The Merliniinae are distinguished from the other Telotylenchidae by (amongst other characters) the presence of deirids (sensory organs located in the lateral field) and having six incisures in the lateral field. These characters are shared by *Pratylenchoides* (though some species only have four incisures). Siddiqi (2000) also remarked on the presence of the deirids (unique in the Pratylenchidae) and drew a comparison with *Amplimerlinius* (Merliniinae) with which it shares several other characters (shape of head and tail region, terminally joined lateral field and the pharyngeal glands sometimes forming a pseudo-bulb). This similarity in the tail region was reason for Ryss (1993) to transfer *Pratylenchoides* to the Merliniinae and recognize them as a separate family. He was not followed in this by other authors however. This phylogeny confirms the close relationship between *Pratylenchoides* and the Merliniinae.

Evolution of plant parasitism

The development of plant parasitism in the Tylenchida has traditionally been seen as a gradual evolution from fungal feeding to facultative parasitism of root hairs and epidermal cells into more complex forms of plant parasitism, culminating in the development of sedentary endoparasitism (Luc *et al*. 1987). To investigate this hypothesis, ancestral feeding types were reconstructed among Tylenchida using three different methods: unordered parsimony (Fig

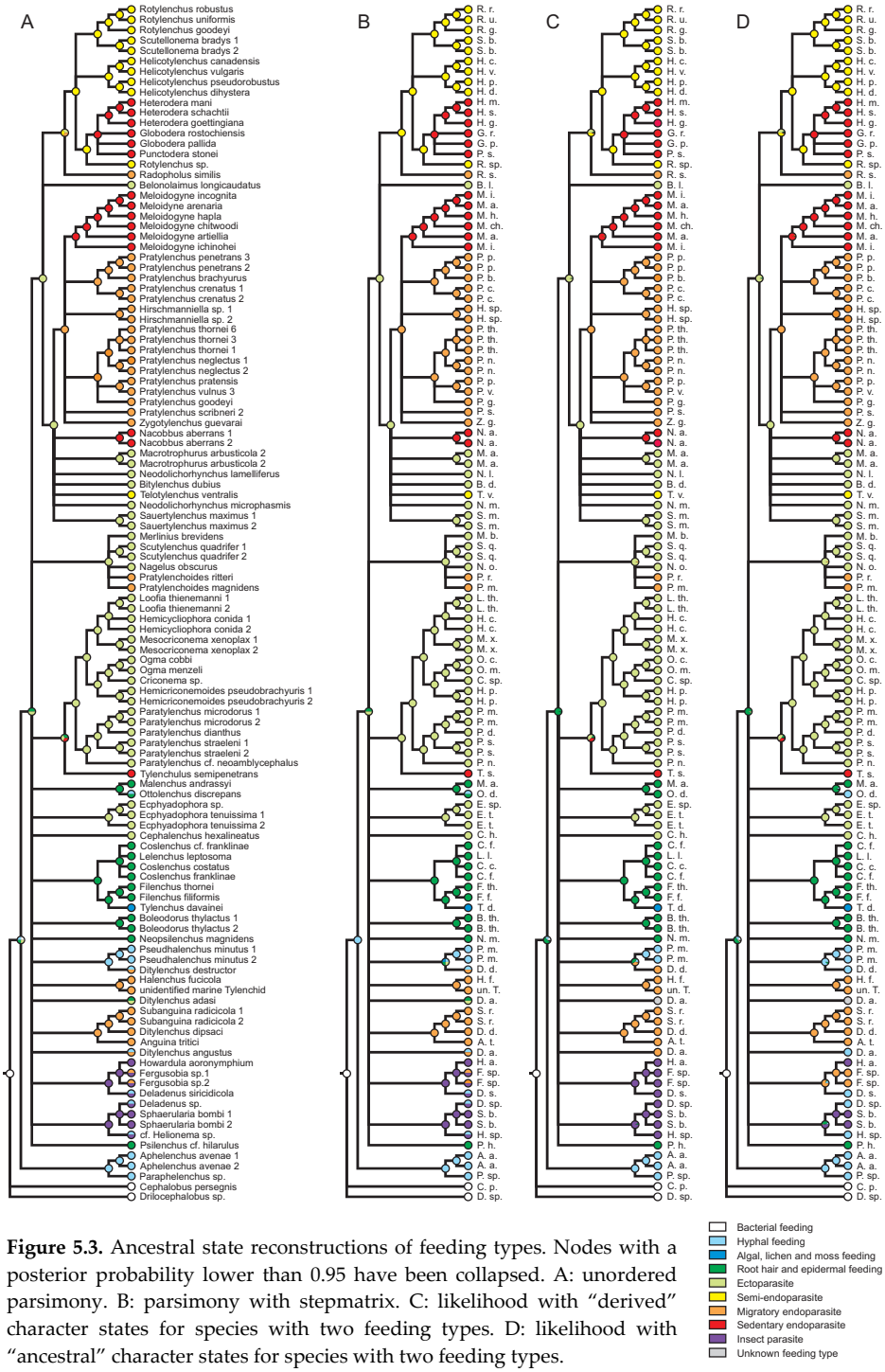

Figure 5.3. Ancestral state reconstructions of feeding types. Nodes with a posterior probability lower than 0.95 have been collapsed. A: unordered parsimony. B: parsimony with stepmatrix. C: likelihood with "derived" character states for species with two feeding types. D: likelihood with "ancestral" character states for species with two feeding types.

Table 5.2. Total number of forward and reverse changes in feeding type and average magnitude of state changes in plant parasitism.

	unordered parsimony	parsimony stepmatrix	likelihood derived characters	likelihood ancestral characters
Forward changes	19.7	20.5	22	21
Reverse changes	12.3	12.6	3	8
Av. magnitude forward changes plant parasitism	1.9	1.87	1.89	1.83
Av. magnitude reverse changes plant parasitism	1.08	1.03	1	1

5.3A), parsimony using a step-matrix (Fig. 5.3B) and likelihood (Fig. 5.3C-D). It seems reasonable to assume that, *e.g.*, the transition from root hair feeding to migratory endoparasitism requires more changes than the evolution from semi-endoparasitism to migratory endoparasitism. Therefore, next to the unordered parsimony analysis where every change is considered equally likely, we designed a stepmatrix for the second parsimony-based analysis (Table 5.1). Likelihood methods don't accept multiple states, and - as some Tylenchids feed on multiple kinds of food sources (*e.g.* on fungi and on plant roots; Yeates *et al*. 1993) - two input matrices were used. Fig. 5.3C is based on the (supposed) most derived trophic states, whereas (supposed) ancestral trophic states were used in Fig. 5.3D. Analysis of basal part of the Tylenchida tree is complicated by the fact that the feeding type(s) of several Tylenchidae and Anguinidae are not known with certainty. They are often considered as obligate plant parasites, while more close investigation could reveal that they can feed on fungi or oomycetes as well. This point is illustrated by the genus *Filenchus,* known as epidermal cell and root hair feeders (Yeates *et al*. 1993), while some *Filenchus* species were recently shown to feed on fungi as well (Okada, Harada, and Kadota 2005).

Our analysis confirmed Luc's hypothesis on the gradual evolution of simple forms of plant parasitism, such as root hair and epidermal feeding and ectoparasitism towards more complex forms of endoparasitism (Fig. 5.3, Table 5.2). First of all the number of forward changes far exceeds the number of reverse changes, especially in the likelihood reconstructions, confirming the trend of development towards more complex feeding types. Secondly, the average magnitude of state changes in plant parasitism is about 1.8-1.9 in the

forward changes and about 1 in the reverse changes, confirming this development is gradual.

Remarkably, endoparasites evolved at least ten times within the order Tylenchida; migratory endoparasitism developed no less than six times and sedentary endoparasitism four times. Only in one instance (*Meloidogyne*) sedentary endoparasitism evolved directly from migratory endoparasitism. The number of independent developments is higher than expected mainly due to the paraphyly of the Pratylenchidae. Athough the development of plant parasitim is usually gradual, endoparasitism seems to have developed directly from several simple forms of plant parasitism such as ectoparasites (giving rise to *Meloidogyne*, Pratylenchidae, *Pratylenchoides*, *Tylenchulus*), epidermal and root hair feeders (from which the Anguinidae evolved) and possibly even insect parasitism (*Fergusobia*, although it is very well possible that the ancestor - just like *Deladenus* - fed on insects and fungi). On the basis of these finding we hypothesize that the potential to develop into endoparasites is present in most if not all the members of the Tylenchida.

Only the parsimony stepmatrix analysis reconstructed the common ancestor of the Aphelenchida and Tylenchida as a fungal feeder. In the other three analyses, we could neither confirm nor discard the hypothesis saying that plant parasitic Tylenchida arose from fungivorous ancestors. Firm conclusions on this hypothesis require a better resolution in the basal part of the Tylenchida tree, and additional information on the feeding behavior of the basal Tylenchida such as theTylenchidae and Anguinidae.

The origin of cell wall-degrading enzymes

Genes encoding cell wall-degrading have been found in five nematode genera (*Globodera*, *Heterodera* (Smant *et al*. 1998), *Meloidogyne* (Rosso *et al*. 1999), *Pratylenchus* (Uehara, Kushida, and Momota 2001) and recently *Bursaphelenchus* (Kikuchi, Shibuya, and Jones 2005)). It has been proposed that these genes may have been acquired through horizontal gene transfer (Keen and Roberts 1998; Yan *et al*. 1998). Cellulases (EC 3.2.1.4) are found in fourteen glycoside hydrolase families (GHF 5, 6, 7, 8, 9, 10, 12, 26, 44, 45, 48, 51, 61, 74), and - remarkably - all Tylenchid cellulases isolated so far belong to a single GH family, namely GHF 5. Hence, the question arises whether these cellulases have

a common (cellulases were present /acquired before separation of subclades A and B (see Fig. 5.1), or a separate origin (cellulases were present / acquired after the separation of subclades A and B). Remarkably, nematode GH5 sequences analysis revealed a clustering of *Pratylenchus penetrans* and *Meloidogyne incognita* cellulases (PpENG1, 2 and MiENG1, 3, 4), and another cellulase subclade comprised most the cyst nematode cellulases (Rehman, 2008). The current SSU rDNA based phylogeny positions *Radopholus similis* at the base of subclade A, whereas the genera *Hirschmanniella*, *Zygotylenchus* and *Nacobbus* are positioned at the basis of the Pratylenchidae and Meloidogynidae branch. *Radopholus similis* harbors GHF5 cellulases (M. Dautova pers. comm.), and the current analysis identifies the genera *Hirschmanniella*, *Zygotylenchus* and *Nacobbus*, and *R. similis* as being highly relevant in addressing question about the origin of Tylenchida cellulases. Apart from this, it would be highly interesting to investigate the two Telotylenchidae branches (basal part of subclade B, and subclade C) for the presence / absence of cellulases and their nature (members of the genus *Pratylenchoides* are endoparasites).

Cellulases are not only found in plant parasitic nematodes but also in fungus or oomycete feeding species as illustrated by the GHF 45 (and not GHF 5) cellulases present in both plant parasitic (*B. xylophilus*) and mycetophagous (*B. mucronatus) Bursaphelenchus* species (Kikuchi *et al*. 2007; *Bursaphelenchus* belongs to the order Aphelenchida).

Although for the reasons mentioned above, no firm statement could be made about the closest living representative of the common ancestor of all Tylenchida, our analysis suggest to that screening of two specific genera namely *Aphelenchus* and *Paraphelenchus* for the presence (and nature) of cellulases could shed more light on the evolution of plant parasitism within the order Tylenchida.

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Appendix A

GenBank accessions of sequences used in this study. Sequences newly generated for this study will be submitted at a later date.

Species	NCBI Accession	Species	NCBI Accession
Anguina tritici	AY593913	Macrotrophurus arbusticola 1	AY284595
Aphelenchus avenae 1	AF036586	Macrotrophurus arbusticola 2	AY284596
Aphelenchus avenae 2	AY284640	Malenchus andrassyi	AY284587
Belonolaimus longicaudatus	AY633449	Meloidogyne arenaria	U42342
Bitylenchus dubius	AY284601	Meloidogyne artiellia	AF248477
Boleodorus thylactus 1	AY593915	Meloidogyne chitwoodi	AY593883
Boleodorus thylactus 2	AY993976	Meloidogyne hapla	AY593892
Cephalenchus hexalineatus	AY284594	Meloidogyne ichinohei	AF442191
Cephalobus persegnis	AY284662	Meloidogyne incognita	AY284621
Coslenchus cf. franklinae	AY284582	Merlinius brevidens	AY284597
Coslenchus costatus	AY284581	Mesocriconema xenoplax 1	AY284625
Coslenchus franklinae	AY284583	Mesocriconema xenoplax 2	AY284626
Criconema sp.	AJ966480	Nacobbus aberrans 1	AF442190
Deladenus siricidicola	AY633447	Nacobbus aberrans 2	AJ966494
Deladenus sp.	AJ966481	Nagelus obscurus	AY593904
Ditylenchus adasi	not subm.	Neodolichorhynchus	AY284598
Ditylenchus angustus	AJ966483	lamelliferus	
Ditylenchus destructor	AY593912	Neodolichorhynchus	not subm.
Ditylenchus dipsaci		microphasmis	
Drilocephalobus sp.	AY284680	Neopsilenchus magnidens	AY284585
Ecphyadophora sp.	AY593917	Ogma cobbi	not subm.
Ecphyadophora tenuissima 1	not subm.	Ogma menzeli	not subm.
Ecphyadophora tenuissima 2	not subm.	Ottolenchus discrepans	AY284590
Fergusobia sp. 1	AY589293	Paraphelenchus sp.	AY284642
Fergusobia sp. 2	AY589295	Paratylenchus cf.	AY284634
Filenchus filiformis	AY284592	neoamblycephalus	
Filenchus thornei	AY284591	Paratylenchus dianthus	AJ966496
Globodera pallida	AY284618	Paratylenchus microdorus 1	AY284632
Globodera rostochiensis	AY593880	Paratylenchus microdorus 2	AY284633
Halenchus fucicola	not subm.	Paratylenchus straeleni 1	AY284631
Helicotylenchus canadensis	AY284605	Paratylenchus straeleni 2	AY284630
Helicotylenchus dihystera	AJ966486	Pratylenchoides magnicauda	AF202157
Helicotylenchus pseudorobustus	AY284606	Pratylenchoides ritteri	AJ966497
Helicotylenchus vulgaris	AY284607	Pratylenchus brachyurus	AY279545

cf. Helionema sp.	not subm.	Pratylenchus crenatus 1	AY284610
Hemicriconemoides	AY284622	Pratylenchus crenatus 2	not subm.
pseudobrachyurus 1		Pratylenchus crenatus 3	not subm.
Hemicriconemoides	AY284624	Pratylenchus crenatus 4	not subm.
pseudobrachyurus 2		Pratylenchus goodeyi	AJ966498
Hemicycliophora conida 1	not subm.	Pratylenchus neglectus 1	not subm.
Hemicycliophora conida 2	AJ966471	Pratylenchus neglectus 2	not subm.
Heterodera goettingiana	not subm.	Pratylenchus penetrans 1	not subm.
Heterodera mani	not subm.	Pratylenchus penetrans 2	not subm.
Heterodera schachtii	AY284617	Pratylenchus penetrans 3	AY279546
Hirschmanniella cf. belli	EF029856	Pratylenchus pratensis	AY284611
Hirschmanniella pomponiensis	EF029854	Pratylenchus scribneri 1	not subm.
Hirschmanniella santarosae	EF029855	Pratylenchus scribneri 2	AY286309
Hirschmanniella sp. 1	AY284614	Pratylenchus thornei 1	AY284612
Hirschmanniella sp. 2	AY284615	Pratylenchus thornei 2	AY284613
Hirschmanniella sp. 3	AY284616	Pratylenchus thornei 3	not subm.
Hirschmanniella sp. 4	EF029857	Pratylenchus thornei 4	not subm.
Howardula aoronymphium	AY589304	Pratylenchus thornei 5	not subm.
Lelenchus leptosoma	AY284584	Macrotrophurus arbusticola 1	AY284595
Loofia thienemanni 1	AY284628	Macrotrophurus arbusticola 2	AY284596
Loofia thienemanni 2	AY284629		

Appendix B

Feeding types and literature sources.

Species	Feeding type [a]	Source[b]	Species	Feeding type [a]	Source[b]
Anguina tritici	ME	Bongers	Mesocriconema	EP	Yeates
Aphelenchus avenae	HF	Bongers	xenoplax		
Aphelenchus avenae	HF	Bongers	Mesocriconema	EP	Yeates
Belonolaimus	EP	Yeates	xenoplax		
longicaudatus			Nacobbus aberrans	SE	Yeates
Bitylenchus dubius	EP	Yeates	Nacobbus aberrans	SE	Yeates
Boleodorus thylactus	ERH	Yeates	Nagelus obscurus	EP	Yeates
Boleodorus thylactus	ERH	Yeates	Neodolichorhynchus	EP	Yeates
Cephalenchus	EP	Yeates	lamelliferus		
hexalineatus			Neodolichorhynchus	EP	Yeates
Cephalobus persegnis	BF	Yeates	microphasmis		
cf. Helionema sp.	IP + HF[c]	Siddiqi	Neopsilenchus	ERH	Yeates
Coslenchus cf.	ERH	Yeates	magnidens		
franklinae			Ogma cobbi	EP	Yeates
Coslenchus costatus	ERH	Yeates	Ogma menzeli	EP	Yeates
Coslenchus franklinae	ERH	Yeates	Ottolenchus discrepans	ERH +	Okada
Criconema sp.	EP	Yeates		HF	
Deladenus siricidicola	HF + IP	Siddiqi	Paraphelenchus sp.	HF	Yeates
Deladenus sp.	HF + IP	Siddiqi	Paratylenchus cf.	EP	Yeates
Ditylenchus adasi	- [d]	Yeates	neoamblycephalus		
Ditylenchus angustus	HF + ME	Ali	Paratylenchus dianthus	EP	Yeates
Ditylenchus destructor	HF + ME	Bongers	Paratylenchus	EP	Yeates
Ditylenchus dipsaci	ME	Bongers	microdorus		
Drilocephalobus sp.	BF	Yeates	Paratylenchus	EP	Yeates
Ecphyadophora sp,	EP	Yeates	microdorus		
Ecphyadophora	EP	Yeates	Paratylenchus straeleni	EP	Yeates
tenuissima			Paratylenchus straeleni	EP	Yeates
Ecphyadophora	EP	Yeates	Pratylenchoides	ME	Yeates
tenuissima			magnicauda		
Fergusobia sp.	ME + IP	Siddiqi	Pratylenchoides ritteri	ME	Yeates
Fergusobia sp.	ME + IP	Siddiqi	Pratylenchus	ME	Yeates
Filenchus filiformis	ERH	Yeates	brachyurus		
Filenchus thornei	ERH	Yeates	Pratylenchus crenatus	ME	Yeates
Globodera pallida	SE	Yeates	Pratylenchus crenatus	ME	Yeates
			Pratylenchus goodeyi	ME	Yeates

Species	Feeding type [a]	Source[b]	Species	Feeding type [a]	Source[b]
Globodera rostochiensis	SE	Yeates	Pratylenchus neglectus	ME	Yeates
			Pratylenchus neglectus	ME	Yeates
Halenchus fucicola	ME	Siddiqi	Pratylenchus penetrans	ME	Yeates
Helicotylenchus canadensis	SME	Yeates	Pratylenchus penetrans	ME	Yeates
			Pratylenchus pratensis	ME	Yeates
Helicotylenchus dihystera	SME	Yeates	Pratylenchus scribneri	ME	Yeates
			Pratylenchus thornei	ME	Yeates
Helicotylenchus pseudorobustus	SME	Yeates	Pratylenchus thornei	ME	Yeates
			Pratylenchus thornei	ME	Yeates
Helicotylenchus vulgaris	SME	Yeates	Pratylenchus vulnus	ME	Yeates
			Pseudhalenchus minutus	HF	Yeates
Hemicriconemoides pseudobrachyurus	EP	Yeates			
			Pseudhalenchus minutus	HF	Yeates
Hemicriconemoides pseudobrachyurus	EP	Yeates			
			Psilenchus cf. hilarulus	ERH	Yeates
Hemicycliophora conida	EP	Yeates	Punctodera stonei	SE	Yeates
			Radopholus similis	ME	Yeates
Hemicycliophora conida	EP	Yeates	Rotylenchus goodeyi	SME	Yeates
			Rotylenchus robustus	SME	Yeates
Heterodera goettingiana	SE	Yeates	Rotylenchus sp.	SME	Yeates
			Rotylenchus uniformis	SME	Yeates
Heterodera mani	SE	Yeates	Sauertylenchus maximus	EP	Yeates
Heterodera schachtii	SE	Yeates			
Hirschmanniella sp.	ME	Yeates	Sauertylenchus maximus	EP	Yeates
Hirschmanniella sp.	ME	Yeates			
Howardula aoronymphium	IP	Siddiqi	Scutellonema bradys	SME	Yeates
			Scutellonema bradys	SME	Yeates
Lelenchus leptosoma	ERH	Yeates	Scutylenchus quadrifer	EP	Yeates
Loofia thienemanni	EP	Yeates	Scutylenchus quadrifer	EP	Yeates
Loofia thienemanni	EP	Yeates	Sphaerularia bombi	IP	Siddiqi
Macrotrophurus arbusticola	EP	Yeates	Sphaerularia bombi	IP	Siddiqi
			Subanguina radicicola	ME	Bongers
Macrotrophurus arbusticola	EP	Yeates	Subanguina radicicola	ME	Bongers
			Telotylenchus ventralis	SME	Yeates
Malenchus andrassyi	ERH	Yeates	Tylenchulus semipenetrans	SE	Yeates
Meloidogyne arenaria	SE	Yeates			
Meloidogyne artiellia	SE	Yeates	Tylenchulus semipenetrans	SE	Yeates
Meloidogyne chitwoodi	SE	Yeates			
			Tylenchus davainei	ALM	Yeates
Meloidogyne hapla	SE	Yeates			

Species	Feeding type [a]	Source[b]	Species	Feeding type [a]	Source[b]
Meloidogyne ichinohei	SE	Yeates	unidentified marine	ME	Siddiqi
Meloidogyne incognita	SE	Yeates	Tylenchid		
Merlinius brevidens	EP	Yeates	Zygotylenchus guevarai	ME	Yeates

[a]: SE: sedentary endoparasite, ME: migratory endoparasite, SME: semi-endoparasite, EP: ectoparasite, ERH: epidermal and root hair feeder, ALM: algal, lichen and moss feeder, HF: hyphal feeder, BF: bacterial feeder, IP: insect parasite

[b]: Ali, R. and Ishibashi, N. 1997 Growth and propagation of the rice stem nematode, *Ditylenchus angustus*, on rice seedlings and fungal mat of *Botrytis cinerea*. Japanese Journal of Nematology 26 (1/2): 12-22

Bongers, T. 1994 De Nematoden van Nederland. Koninklijke Nederlandse Natuurhistorische Vereniging, Utrecht, The Netherlands

Okada, H. *et al*. 2005 Fungal-feeding habits of six nematode isolates in the genus Filenchus. Soil Biology and Biochemistry 37 (6): 1113-1120

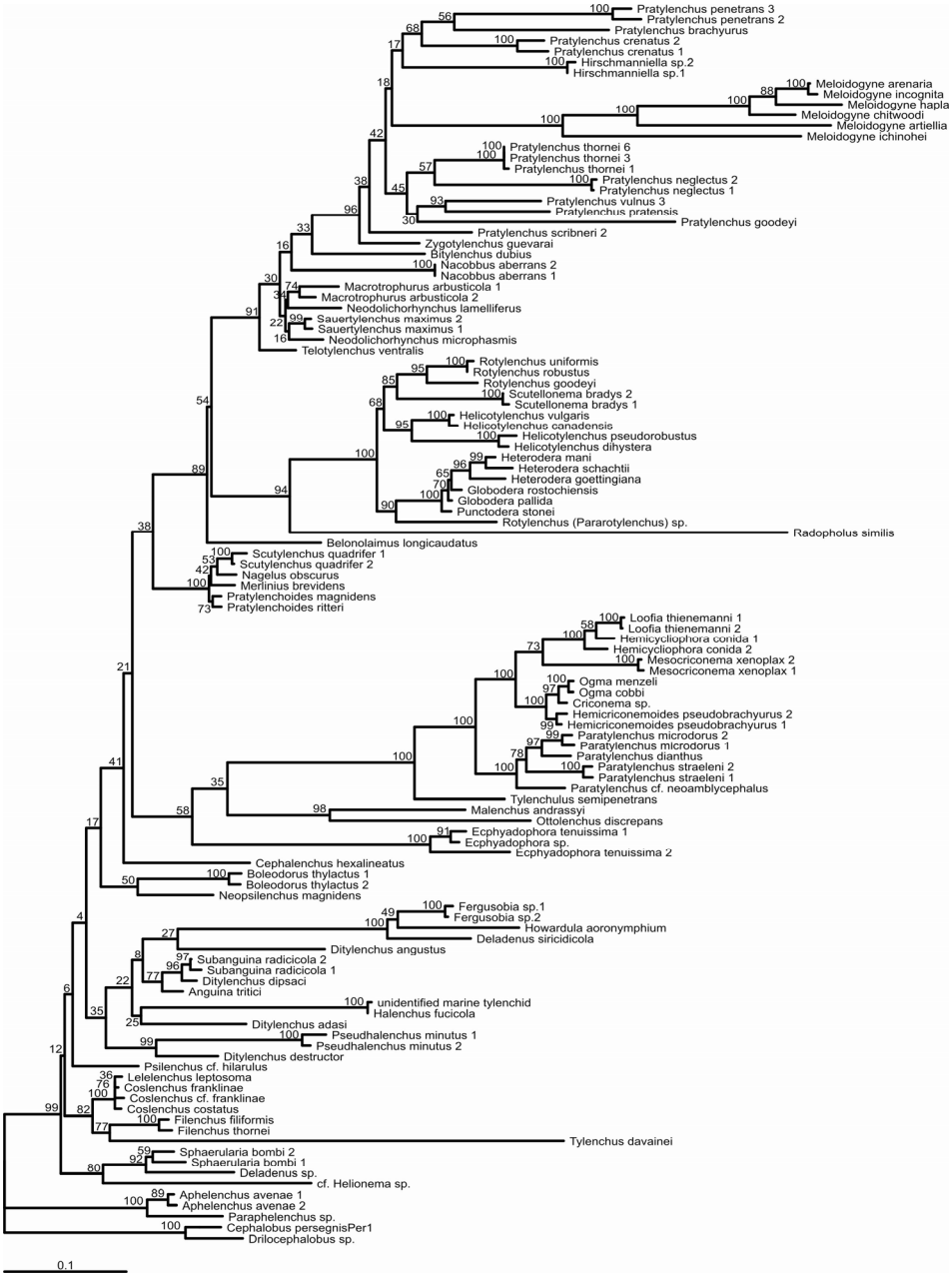
Siddiqi, M.R. 2000 Tylenchida: parasites of plants and insects. CABI Publishing, Wallingford, UK

Yeates G.W. *et al*. 1993 Feeding habits in soil nematode families and genera – an outline for soil ecologists. Journal of Nematology 25 (3): 315-331

[c]: Found once in Poland in peat moss from a forest soil in 1959, described in 1962 by Brzeski (Acta Zoologica Cracoviensia 1962; 7(4): 53-62); found in large numbers in several samples in the Ronde Venen, The Netherlands, in peaty soil; identified by D.J. Hunt, similar to Helionema, but this is tentative due to the destroyed pharynx and brief original description. Stylet shape, presence in several samples and position in tree next to *Deladenus* and *Sphaerularia* suggests this is a fungal feeding stage and it stands to reason (also suggested by Siddiqi, 2000) that an insect parasitic form exists as well. A large number of individuals have been preserved in slides in our collection, all with destroyed pharynx.

[d]: No literature description of feeding type of this particular species available.

Appendix C



Tree with the best likelihood acquired by the maximum likelihood method. Bootstrap values are indicated at nodes (values > 65 are considered robust).

Chapter 6

Towards a transparent, ecological grouping of free living nematodes by searching for stress tolerance-correlated characteristics while accounting for phylogenetic interdependence

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Towards a transparent, ecological grouping of free living nematodes by searching for stress tolerance-correlated characteristics while accounting for phylogenetic interdependence

Biological indicators are highly relevant for assessing the condition of a soil as they are integrative: they reflect the overall impact of physical, chemical and biological changes. Indigenous soil organisms are preferable to other test organisms because the diversity and condition of indigenous soil organisms reflect both acute and chronic effects of soil disturbances. Nematodes are ubiquitous, speciose, easily extractable and present in extremely high numbers. DNA barcode-based community analysis will soon be technically possible - see Chapter 2-3 of this thesis - and a next step would be to define objective criteria for the ecological grouping of free living (= non-parasitic) nematodes. Here we present a framework to study which traits are correlated with a tolerance to stress. For this, a field study on the effects of pH and copper on nematode communities was re-analyzed. Changes in abundances of individual genera were correlated with a number of potentially stress tolerance-related characteristics. The generalized least squares (GLS) method was used to account for the phylogenetic dependence of the data. Mainly due to a high Bonferroni correction, only the relation between the ability to enter a survival stage and tolerance to copper at pH 6.1 was significant. This study did, however, demonstrate the importance of accounting for the effects of phylogenetic dependence in the data. When the phylogeny was taken into account, we observed an average change in P-value of 0.196 for the correlations of stress-related characteristics and Cu or pH tolerance. This research constitutes proof-of-principle for a transparent method to relate stress tolerance to (ecological) characteristics. The usefulness of this powerful method should become even clearer when substantially higher numbers of individuals are analyzed (as facilitated by using DNA barcodes) and when missing data are filled in.

Introduction

Because of their abundance and their (trophic) diversity nematodes occupy important positions in the soil foodweb (De Ruiter, Neutel, and Moore 1998). They also display a great variability in sensitivity to environmental stress (Bongers 1990). As such the composition of nematode communities is considered to be an informative indicator for soil health. However, little is known about biological characteristics that underlie the very wide variation in stress (in)tolerance. Here, we investigate what traits are correlated with stress tolerance in nematodes.

Monitoring of nematode communities as indicator for soil health conditions is widely applied. However, sample size and taxonomic resolution are currently dictated by practical limitations (and are respectively smaller and lower than desired). The morphology of nematodes is relatively conserved, and - therefore - community analysis is time consuming and requires ample expert knowledge. Recent advances in the use of molecular characteristics for the analysis of nematode communities may lift (in part) these practical obstacles (Holterman *et al*. 2008).

Several indexes can be used to describe community diversity, such as the Shannon-Weaver index (Shannon 1948), the Simpson's diversity index (Simpson 1949) or the species richness index (*S*; the number of species present in an ecosystem). An important characteristic of the Maturity Index (MI) as proposed by (Bongers 1990) is the inclusion of ecological characteristics, and over the last decade this MI has been widely used for nematode community analyses. The Maturity Index assigns each nematode family a so called cp-value. This "colonizer-persister" scale ranges from 1 to 5 and corresponds roughly to r-K strategies, with values of 1 and 2 being assigned to the most tolerant r-strategists and values of 4 and 5 to the most sensitive K-strategists. Families assigned to cp-class 1 are enrichment opportunists and while they can outlast periods of adverse conditions they are mainly found in food-rich conditions. For this reason they are often excluded from the MI (Bongers 1999). The MI was demonstrated to be an effective means for the assessing and quantifying the impact of soil pollution on nematode communities (Bongers and Ferris 1999). However, families were assigned to cp-classes mainly on the

basis of expert knowledge and some general observations (Bongers 1999) and there are few clear criteria for each cp-class. In addition, it is known that genera in a single family do not always react similar to stress (Ettema and Bongers 1993) and for some nematode families a refinement of the MI down to genus or even species level would be appropriate. Identification of traits correlated with specific forms of stress tolerance would be helpful for the refinement of the Maturity Index or related indices.

When looking for correlations between traits using data from different species it is important to realize that one of the basic assumptions of the comparative method – *i.e.* the data are independent – is often violated (Felsenstein 1985). If a strong phylogenetic correlation exists in the data, it is important to take this into account. Felsenstein designed a method, named phylogenetically independent contrasts, which takes these confounding effects into account (Felsenstein 1985). However, this method may overestimate the effects of phylogeny (Harvey and Rambaut 2000). Another (intermediate) approach was proposed by Pagel (1999); the generalized least squares (GLS) method.

To study traits correlated with stress tolerance in nematodes, we focused on the effects of copper and pH on nematode communities. The traits involved in this study are body size, reproductive potential, (a)sexual reproduction, feeding type, the ability to form survival stages and cuticle permeability. Body size is an important life history trait which correlates with many processes (Peters 1983). Reproduction can be expected to play a role in stress tolerance, since a high reproduction rate could partially compensate for increased mortality and enhance population recovery from the stress. It is noted that stress tolerant nematodes are often small, have short generation times and produce large numbers of offspring (Bongers 1999). Unfortunately generation times and number of offspring can not be determined for most nematodes, since most nematodes can not (or barely) be cultured. Therefore we looked to the gonad size relative to body size as an alternative measure for fecundity. To the best of our knowledge this has never been done for nematodes, but it has been done in various other animals, such as tardigrades (Guidetti *et al*. 2007), flat worms (Schärer, Sandner, and Michiels 2005) and tree frogs (Rodrigues, Uetanabaro, and Lopes 2005; Rodrigues, Uetanabaro, and Lopes 2007). Asexual

reproduction is often considered a trait for opportunists but this does not necessarily seem to be the case for nematodes (Bongers 1999). Feeding type could also be related to stress tolerance. In the MI most bacterial feeders belong to low cp-classes, while members of cp-class 4 and 5 are often predators or omnivores (Bongers 1999). Entering a survival stage can help to outlast long periods of stress and, finally, changing the cuticle permeability could be an expedient to prevent pollutants from entering the body.

Until now nematode families have been assigned to cp-classes for the Maturity Index on the basis of expert knowledge. Identifying traits which are correlated with a tolerance to stress is a first step towards defining clear objective criteria for the assigning of nematode taxa to cp-classes. Furthermore, having clear criteria will allow for the refinement of the MI from a family to a genus level index. In this study we set out a framework to identify traits important to stress tolerance. We will demonstrate the importance of taking the effects of phylogeny on the data into account. Finally the effectiveness of our framework to identify relevant traits will be discussed.

Materials and Methods

Nematode community data for copper and pH stress

To determine the tolerance of several nematode genera to copper and pH stress, data were used from a field study on the long term effects of copper and pH on nematode communities (Korthals *et al*. 1996). The study site, known as the Bovenbuurt pastures, is located approximately 3 km NNE of Wageningen, The Netherlands. The field soil is slightly loamy sand. After having been used as a permanent pasture for at least thirty years, in 1978 a crop rotation scheme of silage maize, starch potatoes and oats was applied. In 1982 the field was divided into 128 plots of 6 x 11 m. Four copper levels were created by applying 0, 250, 500 and 750 kg Cu/ha. pH levels were adjusted to 4.0, 4.7, 5.4 or 6.1. This resulted in 16 different treatments with 8 replicates per treatment. In March 1992 samples were taken from each plot. A part of the sample was used to determine the pH (pH-KCl) and to determine the total quantity of copper present (Cu-HNO_3) and the available copper concentration (Cu-$CaCl_2$). Nematodes were extracted from another part of the soil sample. Nematodes

were counted under a dissecting microscope and after fixation in formalin at least 150 nematodes were identified using a light microscope. For full details of field history, soil composition, sampling and pH determination and copper extraction we refer to Korthals *et al.* (1996).

Redundancy Analysis

In order to investigate the change in abundance of genera related to pH or copper treatment, a Detrended correspondence analyses (DCA) was performed (Ter Braak 1995). Detrended correspondence analyses (DCA) by segments revealed a gradient of 1.75 standard deviation units for pH and gradients of 1.53, 1.52, 1.33 and 1.65 for Cu at pH levels of 4.0, 4.7, 5.4 and 6.1 respectively, indicating a strong linear response of taxa. Therefore, a redundancy analysis (RDA) was performed (Ter Braak 1995). RDA is the constrained version of a principal component analysis (PCA), meaning that it focuses on the part of the variance explained by the environmental variables only.

RDA analysis was performed on a matrix of 128 samples (8 replicates per treatment) and 71 species using CANOCO for Windows 4.5 (Ter Braak and Smilauer 2002). Nematode abundance data were ln(2x+1) transformed to down-weight high values and approximate a normal distribution. For the pH and Cu values the true pH (pH-KCl) and the available Cu-concentration (Cu-$CaCl_2$) were used. Five RDA analyses were performed. One RDA to determine the tolerance to pH, for this all the plots with a Cu-treatment were excluded from the analysis. To determine the tolerance to Cu a separate RDA was performed for each pH-level (4.0, 4.7, 5.3 and 6.1), excluding the samples for the other pH-levels. The values of the first canonical axis were used as a measure for the tolerance of a genus to the stressor (pH or Cu). A strongly positive or negative value indicated that the variation in abundance was strongly explained by the pH or Cu treatment, *i.e.* the genus under consideration was strongly affected. A value close to zero indicated that abundance was not well explained by the treatment, *i.e.* the genus is tolerant.

The redundancy analyses (RDA) were followed by unrestricted Monte Carlo permutation (MC) tests with 499 permutations was performed to assess the significance of the relation between each environmental variable and community composition (Verdonschot and Braak 1994).

Nematode traits

Six nematode traits were studied for their possible correlation with tolerance to copper and pH stress. These were body size, relative gonad size, (a)sexual reproduction, cuticle permeability, feeding type and the ability to form a survival stage. Wherever possible, only data from species occurring in the Netherlands was used. The cp-values for the maturity index were taken from Bongers (1999).

Body size was determined using Andrassý's formula (Andrássy 1956), using body length and width taken from Bongers (1994). A correction for tail shape was applied as described in Van der Wurff *et al*. (2007). Body size data was log10 transformed to approximate a normal distribution of the data. Feeding types were according to Yeates *et al.* (1993). Survival stages are according to Bongers (1999). For reproduction type it was scored whether a genus can reproduce asexually or not, no distinction was made between genera which can only reproduce asexually and genera which can reproduce both sexually and asexually. Most data came from Bongers (1994) and Lorenzen (1994) with additional data from literature where required. In case the mode of reproduction was not directly known, the presence of males was used as a guideline. If males were unknown or rare, a species was supposed to be able to reproduce asexually. If both sexually and asexually reproducing species were present in a genus, the genus was scored as being able to reproduce asexually.

To assess the permeability of the cuticle, live nematodes were colored with different staining agents. In a preliminary experiment (results not shown) six staining agents (Coomassie R (Merck), Coomassie G (Merck), erytrosin B (Kodak), Trypan blue (Merck), ponceau sodium (Merck), and acid fuchsin (Merck)), were tested with a taxonomically diverse number of species. Three staining agents were selected, one which stained many nematodes (Erytrosin B), one which only stained the nematodes with the most permeable cuticles (Coomassie R) and one which was intermediate between these two (Trypan blue). Living nematodes were transferred to a small well with one drop of staining agent diluted with de-mineralized water. If only natural openings were stained, such as the mouth cavity or the amphids, the nematode was scored as non stained. Testing was done on nematodes isolated directly from

environmental samples, and whenever possible at least five individuals were used for each nematode-stain combination. It is noted that due to low abundance of some genera, not every combination could be tested (fully).

To determine the relative gonad size, pictures were taken from slides in the collection at our laboratory using a light microscope (Zeiss Axioscope) equipped with differential interference contrast optics and a CCD camera (CoolSnap, RS Photometrics, Tucson, AZ). When available, pictures from multiple species per genus were taken. For each species six individuals were used. The area of the total nematode and the gonad was measured using the program Image-Pro Express 4.0 (Media Cybernetics, Bethesda, MD). Relative gonad size was expressed as the proportion of the total area of the nematode that was occupied by the gonads. Relative gonad size data was normally distributed.

Phylogenetic tree

Based on previous analyses (Holterman *et al*. 2006; Holterman *et al*. 2008) a user-tree was defined (Fig. 6.1). Each genus was represented by one full length small subunit ribosomal DNA (SSU rDNA) sequence (GenBank accessions in appendix A). The Rhabditidae were represented by ten species to represent the large variation present in the family. The Diplogasteridae were represented by four sequences. The alignment was created by ClustalW as applied in BioEdit v7.0.1 (Hall 1999) and improved manually using arthropod secondary structure information (http:// bioinformatics.psb.ugent.be / webtools / rRNA / secmodel / index.html in accordance with Wuyts *et al*., 2000). MrBayes 3.1.2 was used to calculate branch lengths. The alignment was divided into a stem and loop partition and the GTR model with invariable sites and gamma correction was used. Parameter values were unlinked between partitions and the rate prior was set to variable. The topology of the user tree was fixed. The analysis was run for three million generations using four independent runs and four chains per run. The burnin was 1,200,000 generations. For the Rhabditidae and Diplogasteridae the average branch length was calculated.

Comparative analysis

For the comparative analysis the program Continuous v.1.0d13 (Pagel 1997;

Pagel 1999) was used. Dummy variables were used for the feeding types. To maximize the amount included data (species with missing data in a trait under analysis are excluded), RDA values and traits were analyzed pair-wise. A standard constant variance random walk model of evolution was used. The parameter λ reveals if the data are predicted by the phylogeny and can be estimated by Continuous. When $\lambda = 0$ Continuous assumes the trait is evolving independent of phylogeny, if $\lambda = 1$ Continuous assumes there is a full correlation between the phylogeny and data (in effect it is using independent contrasts; Felsenstein 1985). Likelihood ratio tests were performed to test if the value of λ estimated by likelihood was significantly different from 1 or 0 for each pair of RDA values and traits. Next a likelihood ratio test was performed to test if a RDA-value and a trait were significantly correlated by constraining the covariances to zero for the H_0. This was done using a λ estimated by Continuous and a λ set to 0 to study the effect of using a phylogenetic correction.

Results and discussion

We have set out a framework to investigate if certain nematode traits – body size, reproductive potential, feeding type, survival stage, asexual reproduction and cuticle permeability – are correlated with tolerance for Cu or pH stress. Furthermore the importance of allowing for the phylogenetic non-independence of the data in the analysis was studied. The results will be discussed in the light of improving the Maturity Index.

Nematode responses

The nematode sensitivity analyses were performed on natural communities, instead of laboratory assays, to mimic a natural response towards stressors. The field site was suitable for this, because the soil was homogenous with respect to other important soil parameters besides pH and copper, such as organic matter and lutum (Korthals *et al*. 1996). Although a multivariate test is not required for testing an, in principle, univariate experimental setup, we choose to use RDA since its use is extremely powerful in natural field conditions, *i.e.*, a soil dominated by multiple (co-)factors. In addition, abundances are patchy by

nature and especially disturbance-sensitive groups, such as predators and omnivores typically have low abundances. Furthermore only a relatively low number of individuals were analyzed per sample. Therefore a univariate statistic, such as logistic regression would simply prove unfruitful as a result of a lack of statistical power and abundance data containing zeroes.

The values of the first canonical axis of the RDA analyses are presented in Table 6.1 together with the data for the nematode traits. If the RDA values are positive, it means there is a positive correlation between abundance and the stress factor (pH or Cu) whereas a negative value means there is a negative correlation. RDA values close to zero (between -0.5 and 0.5) are considered not to be correlated with the treatment, *i.e.* the species are tolerant to the stress. RDA values generally display a positive relationship with cp-values in the case of pH (a higher more neutral pH being the less stressed condition) and a negative relationship with copper. This supports the general use of cp-values at the taxonomic level of family. However, species within, the Cephalobidae and Pratylenchidae show that a family based cp-value is not justified (Table 6.1, Fig. 6.3) in all cases. This is in accordance with earlier findings that some genera in a family may not always behave as would be expected from their cp-values (Ettema and Bongers 1993). One of the genera Ettema and Bongers mentioned as being more abundant than expected was *Aporcelaimellus*, which in our analyses displays RDA values close to zero and therefore is more tolerant than its cp-value of 5 would suggest.

Phylogenetic dependence

As can be seen from the results (Table 6.2) many traits are not phylogenetically independent (λ significantly larger than zero). At the same time, most traits do not evolve entirely according to the tree topology; λ is significantly smaller than 1.0 in most cases. This means that the generalized least squares (GLS) method as applied by Continuous is ideally suited to investigate the data for correlations between traits. A method such as independent contrasts (Felsenstein 1985) would overestimate the effect of phylogeny (in effect it assumes that λ is 1), while the GLS method can take an intermediate approach between independent contrasts and not accounting for phylogenetic dependence at all.

Table 6.1. Data for the different nematode traits and the results of the RDA analysis; * = missing data

[1] : BF: bacterial feeder, HF: hyphal feeder, PP: plant parasite, O: omnivore, P: predator

[2] : asexual means asexual reproduction is possible but not necessarily the main mode of reproduction

Genus	Family	cp-value	log body size (mm³)	relative gonad size	feeding type[1]	survival stage	reproduction[2]	Coomassie R	Erytrosin B	Trypan blue	RDA pH 4.0-6.1	RDA Cu pH 4.0	RDA Cu pH 4.7	RDA Cu pH 5.4	RDA Cu pH 6.1
Cephalenchus	Tylodoridae	*	5.04	0.15	PP	*	asexual	*	*	*	*	*	*	*	*
Psilenchus	Psilenchidae	*	5.40	0.22	PP	*	sexual	-	-	-	*	*	*	*	0.091
Panagrolaimus	Panagrolaimidae	1	5.80	0.28	BF	yes	asexual	*	*	-	0.363	-0.116	-0.327	-0.123	0.254
Rhabditidae	Rhabditidae	1	5.87	0.31	BF	yes	asexual	*	-	-	-0.070	-0.124	0.149	0.278	0.163
Diplogasteridae	Diplogasteridae	1	5.74	0.36	O	yes	sexual	*	-	+	-0.242	-0.207	*	*	0.194
Pristionchus	Neodiplogasteridae	1	6.66	0.29	P	*	sexual	*	-	-	-0.010	-0.224	-0.158	0.348	0.407
Acrobeloides	Cephalobidae	2	5.17	0.36	BF	no	asexual	*	-	-	0.256	-0.747	-0.598	-0.603	0.279
Cephalobus	Cephalobidae	2	5.57	0.28	BF	no	sexual	*	*	-	0.524	-0.158	0.104	-0.133	0.345
Heterocephalobus	Cephalobidae	2	5.43	0.26	BF	no	asexual	*	+	-	-0.196	-0.155	*	0.278	0.203
Eucephalobus	Cephalobidae	2	5.33	0.31	BF	no	sexual	*	+	-	0.381	0.038	-0.134	0.157	0.205
Chiloplacus	Cephalobidae	2	5.86	0.26	BF	no	asexual	*	-	-	-0.330	0.158	0.093	0.513	0.595
Acrobeles	Cephalobidae	2	5.76	0.30	BF	no	sexual	*	+	-	0.743	-0.282	-0.452	-0.796	-0.742
Cervidellus	Cephalobidae	2	5.03	0.29	BF	no	asexual	*	-	+	0.342	-0.614	-0.409	-0.641	-0.554
Drilocephalobus	Osstellidae	2	4.97	0.35	BF	*	sexual	*	-	*	-0.236	-0.559	-0.343	-0.025	0.032
Plectus	Plectidae	2	5.82	0.20	BF	no	asexual	*	-	+	-0.207	-0.637	-0.774	0.183	-0.239
Anaplectus	Plectidae	2	6.19	0.26	BF	no	sexual	*	-	+	0.248	-0.280	-0.086	0.016	-0.343
Eumonhystera	Monhysteridae	2	5.20	0.21	BF	no	asexual	+	+	+	*	*	*	*	-0.124
Monhystera	Monhysteridae	2	5.86	0.35	BF	no	sexual	*	*	*	0.367	*	-0.071	-0.060	-0.305
Ditylenchus	Anguinidae	2	5.78	0.31	HF, PP	yes	sexual	-	-	-	0.163	-0.319	-0.195	0.228	0.313
Pseudhalenchus	Anguinidae	2	4.59	0.26	HF	yes	sexual	*	*	*	-0.491	-0.254	0.077	0.412	0.231

Genus	Family	cp-value	log body size (mm³)	relative gonad size	feeding type[1]	survival stage	reproduction[2]	Coomassie R	Erytrosin B	Trypan blue	RDA pH 4.0-6.1	RDA Cu pH 4.0	RDA Cu pH 4.7	RDA Cu pH 5.4	RDA Cu pH 6.1
Nothotylenchus	Anguinidae	2	5.59	0.30	HF	yes	sexual	*	*	*	0.130	-0.015	0.040	0.391	0.319
Deladenus	Neotylenchidae	2	5.57	0.42	HF	yes	asexual	*	*	*	*	0.355	*	-0.093	*
Aphelenchus	Aphelenchidae	2	5.37	0.25	HF	no	asexual	*	-	-	0.771	-0.037	-0.068	-0.244	0.117
Filenchus	Tylenchidae	2	4.54	0.17	HF, PP	no	asexual	-	-	-	0.089	-0.500	-0.270	0.187	-0.086
Bursaphelenchus	Parasitaphelenchidae	2	5.51	*	HF, PP	yes	sexual	*	*	*	*	*	*	*	*
Aphelenchoides	Aphelenchoididiae	2	5.20	0.21	HF, PP	yes	sexual	-	*	*	-0.624	-0.279	-0.026	0.557	0.645
Seinura	Seinuridae	2	5.12	0.21	P	yes	sexual	*	*	*	*	0.279	0.456	*	*
Paratylenchus	Paratylenchidae	2	4.71	0.23	PP	yes	asexual	-	-	-	-0.061	*	0.058	-0.184	0.155
Ecphyadophora	Ecphyadophoridae	2	4.15	0.14	PP	*	sexual	*	*	*	-0.088	*	-0.139	0.235	*
Malenchus	Tylenchidae	2	4.75	0.21	PP	no	asexual	-	+	-	*	*	*	*	*
Boleodorus	Tylenchidae	2	4.91	0.17	PP	no	sexual	*	*	*	0.309	*	*	-0.064	-0.349
Lelenchus	Tylenchidae	2	4.45	00.18	PP	no	sexual	*	*	*	-0.189	-0.251	0.163	-0.134	-0.045
Coslenchus	Tylenchidae	2	4.81	0.23	PP	no	asexual	*	-	*	0.155	*	*	*	-0.235
Tylenchus	Tylenchidae	2	5.53	0.16	PP	no	sexual	-	-	-	0.324	-0.034	-0.228	-0.376	-0.007
Achromadora	Achromadoridae	3	5.18	0.21	*	no	asexual	-	+	+	0.008	-0.188	-0.060	*	-0.260
Teratocephalus	Teratocephalidae	3	4.89	0.09	BF	no	asexual	*	-	-	0.077	*	*	-0.145	0.091
Euteratocephalus	Metateratocephalidae	3	5.39	0.11	BF	no	asexual	+	*	*	*	*	*	*	*
Metateratocephalus	Metateratocephalidae	3	4.82	0.19	BF	no	asexual	-	*	*	-0.067	*	-0.158	*	*
Cylindrolaimus	Cylindrolaimidae	3	5.52	0.24	BF	*	asexual	-	+	*	0.270	*	-0.096	*	-0.087
Prismatolaimus	Prismatolaimidae	3	5.04	0.12	BF	no	asexual	+	+	+	0.339	-0.123	-0.244	0.077	0.068
Bastiania	Bastianiidae	3	4.98	0.15	BF	no	asexual	-	-	-	0.215	*	-0.158	-0.070	-0.232

Genus	Family	cp-value	log body size (mm³)	relative gonad size	feeding type[1]	survival stage	reproduction[2]	Coomassie R	Erytrosin B	Trypan blue	RDA pH 4.0-6.1	RDA Cu pH 4.0	RDA Cu pH 4.7	RDA Cu pH 5.4	RDA Cu pH 6.1
Diphtherophora	Diphtherophoridae	3	5.73	0.27	HF	no	sexual	+	+	+	0.533	0.168	-0.518	-0.447	-0.335
Tripyla	Tripylidae	3	6.14	0.31	P	no	sexual	*	*	*	*	*	-0.008	*	*
Meloidogyne	Meloidogynidae	3	5.56	*	PP	yes	asexual	-	-	-	*	*	0.152	0.240	*
Pratylenchoides	Pratylenchidae	3	5.61	*	PP	no	sexual	*	*	*	*	0.244	*	*	*
Pratylenchus	Pratylenchidae	3	5.07	0.14	PP	no	asexual	-	-	-	0.254	-0.805	-0.804	-0.304	-0.158
Zygotylenchus	Pratylenchidae	3	5.12	*	PP	no	sexual	*	*	*	-0.130	*	-0.147	-0.311	*
Helicotylenchus	Hoplolaimidae	3	5.44	0.19	PP	no	asexual	-	-	-	0.059	-0.187	-0.255	-0.109	-0.272
Rotylenchus	Hoplolaimidae	3	5.68	0.13	PP	no	sexual	-	-	-	-0.128	-0.106	-0.135	-0.068	-0.166
Bitylenchus	Telotylenchidae	3	5.60	0.23	PP	no	asexual	-	-	-	-0.048	-0.323	-0.528	-0.089	0.035
Neodolichorhynchus	Telotylenchidae	3	5.88	0.19	PP	no	sexual	*	*	*	0.203	*	*	*	-0.192
Merlinius	Telotylenchidae	3	5.21	0.22	PP	no	sexual	-	-	-	0.542	-0.421	-0.318	-0.573	-0.001
Alaimus	Alaimidae	4	5.56	0.06	BF	no	asexual	+	+	+	0.389	*	-0.176	-0.293	-0.438
Paramphidelus	Alaimidae	4	5.27	*	BF	no	asexual	*	+	*	-0.061	*	*	-0.043	-0.079
Tylencholaimus	Tylencholaimidae	4	5.51	0.12	HF	no	asexual	-	*	+	0.017	-0.302	-0.222	-0.427	-0.157
Mesodorylaimus	Dorylaimidae	4	6.33	0.29	O	no	sexual	*	*	+	0.390	*	*	-0.237	-0.366
Prodorylaimus	Dorylaimidae	4	6.33	0.16	O	no	asexual	*	*	+	0.390	*	*	-0.237	-0.366
Eudorylaimus	Qudsianematidae	4	6.42	0.16	O	no	sexual	+	+	+	0.064	-0.188	0.124	-0.193	-0.111
Thonus	Qudsianematidae	4	6.23	0.19	O	no	asexual	+	+	-	0.155	-0.208	-0.171	-0.037	-0.224
Enchodelus	Nordiidae	4	6.63	0.16	O	no	asexual	*	*	*	*	*	*	*	*
Dorylaimoides	Mydonomidae	4	5.83	0.20	O	no	asexual	*	*	*	0.203	*	*	*	-0.192
Thornia	Thorniidae	4	5.92	*	O	no	sexual	*	*	*	*	*	*	*	*
Clarkus	Mononchidae	4	6.13	0.20	P	no	asexual	+	+	+	0.330	*	*	*	-0.359
Prionchulus	Mononchidae	4	6.81	0.23	P	no	asexual	-	+	+	*	*	-0.083	*	*
Coomansus	Mononchidae	4	6.35	0.38	P	no	asexual	-	+	*	-0.027	-0.199	-0.403	-0.310	-0.073

Genus	Family	cp-value	log body size (mm³)	relative gonad size	feeding type[1]	survival stage	reproduction[2]	Coomassie R	Erytrosin B	Trypan blue	RDA pH 4.0-6.1	RDA Cu pH 4.0	RDA Cu pH 4.7	RDA Cu pH 5.4	RDA Cu pH 6.1
Trichodorus	Trichodoridae	4	5.74	0.16	PP	no	sexual	*	+	-	0.355	-0.106	-0.426	-0.620	-0.345
Paratrichodorus	Trichodoridae	4	5.90	0.27	PP	no	asexual	+	+	-	*	*	*	*	*
Aporcelaimellus	Aporcelaimidae	5	6.90	0.18	O	no	asexual	-	+	-	0.182	-0.117	-0.368	-0.238	-0.099
Nygolaimus	Nygolaimidae	5	5.84	0.18	P	no	asexual	+	+	+	0.155	*	-0.096	*	-0.279

Table 6.2. Results of the comparative analysis with Continuous.
[1] : P-values, significant P-values after a Bonferroni correction (0.05/60=8.33e-4) are marked with a * and in bold.

RDA	Trait	λ	λ < 1[1]	λ > 0[1]	Correlation[1] λ estimated	Correlation[1] λ = 0	absolute difference correlation P-values
pH	log10 bodysize	0.38	**0***	**7.43e-5***	0.0960	0.0996	0.004
pH	relative gonad size	0.29	**2.65e-14***	0.0482	0.817	0.700	0.117
pH	bacterial feeder	0.97	0.0419	**7.88e-15***	0.700	0.968	0.268
pH	hyphal feeder	0	**2.22e-16***	1	0.295	0.295	0.000
pH	plant parasite	0.76	**4.21e-5***	**2.22e-7***	0.888	0.457	0.431
pH	omnivore	0.56	**7.33e-9***	**2.85e-6***	0.271	0.175	0.096
pH	predator	0.95	0.0173	**5.06e-7***	0.886	0.731	0.155
pH	survival stage	0.08	**9.44e-7***	0.33	0.0425	0.0283	0.014
pH	asexual reproduction	0.08	**0***	0.186	0.271	0.395	0.124
pH	coomassie R	0.27	**3.88e-9***	0.00351	0.0674	0.0417	0.026
pH	erytrosin B	0.48	**3.11e-12***	**8.70e-17***	0.194	0.250	0.056
pH	trypan blue	0.16	**0***	0.0462	0.699	0.650	0.049
Cu pH 4.0	log10 body size	0.38	**6.40e-13***	0.0124	0.0787	0.176	0.097
Cu pH 4.0	relative gonad size	0.46	**6.08e-9***	0.0203	0.263	0.708	0.445
Cu pH 4.0	bacterial feeder	0.93	0.00443	**3.98e-6***	0.423	0.150	0.273

RDA	Trait	λ	λ < 1[1]	λ > 0[1]	Correlation[1] λ estimated	Correlation[1] λ = 0	absolute difference correlation P-values
Cu pH 4.0	hyphal feeder	0	**1.08e-13***	1	0.982	0.982	0
Cu pH 4.0	plant parasite	0.58	**9.19e-7***	0.0206	0.279	0.538	0.259
Cu pH 4.0	omnivore	0.32	**4.05e-11***	0.00659	0.201	0.165	0.036
Cu pH 4.0	predator	0.89	**1.91e-3***	0.0186	0.678	0.651	0.027
Cu pH 4.0	survival stage	0.17	**6.66e-3***	0.241	0.177	0.267	0.090
Cu pH 4.0	asexual reproduction	0.01	**0***	0.943	0.172	0.173	0.001
Cu pH 4.0	coomassie R	0.39	**1.44e-6***	0.0135	0.344	0.0412	0.303
Cu pH 4.0	erytrosin B	0.45	**4.00e-13***	**1.01e-4***	0.0402	0.00324	0.037
Cu pH 4.0	trypan blue	0.25	**1.41e-14***	0.0453	0.421	0.947	0.526
Cu pH 4.7	log10 bodysize	0.32	**2.78e-15***	0.00411	0.764	0.780	0.016
Cu pH 4.7	relative gonad size	0.51	**1.70e-9***	0.0814	0.758	0.887	0.129
Cu pH 4.7	bacterial feeder	0.94	0.0123	**1.64e-8***	0.763	0.572	0.191
Cu pH 4.7	hyphal feeder	0	**1.11e-16***	1	0.449	0.449	0
Cu pH 4.7	plant parasite	0.62	**7.85e-7***	**2.38e-4***	0.408	0.536	0.128
Cu pH 4.7	omnivore	0.33	**3.86e-12***	0.0128	0.578	0.624	0.046
Cu pH 4.7	predator	0.88	0.00218	**6.97e-4***	0.294	0.216	0.078
Cu pH 4.7	survival stage	0.11	**1.79e-9***	0.194	0.0253	0.0272	0.002
Cu pH 4.7	asexual reproduction	0.02	**0***	0.894	0.159	0.161	0.002
Cu pH 4.7	coomassie R	0.29	**4.99e-7***	0.0167	0.572	0.566	0.006
Cu pH 4.7	erytrosin B	0.44	**3.63e-12***	**4.48e-5***	0.764	0.494	0.270
Cu pH 4.7	trypan blue	0.16	**7.77e-16***	0.111	0.701	0.603	0.098
Cu pH 5.4	log10 bodysize	0.45	**8.21e-13***	**7.17e-5***	0.942	0.285	0.657
Cu pH 5.4	relative gonad size	0.43	**5.53e-9***	0.0226	0.923	0.734	0.189
Cu pH 5.4	bacterial feeder	0.97	0.155	**1.05e-12***	0.596	0.189	0.407
Cu pH 5.4	hyphal feeder	0.04	**3.53e-13***	0.621	0.299	0.247	0.052
Cu pH 5.4	plant parasite	0.71	**4.44e-5***	**2.78e-6***	0.248	0.774	0.526
Cu pH 5.4	omnivore	0.58	**3.89e-5***	**6.96e-7***	0.748	0.199	0.549

RDA	Trait	λ	λ < 1[1]	λ > 0[1]	Correlation[1] λ estimated	Correlation[1] λ = 0	absolute difference correlation P-values
Cu pH 5.4	predator	0.97	0.141	**1.68e-6***	0.900	0.938	0.038
Cu pH 5.4	survival stage	0.13	**5.63e-7***	0.183	0.00942	0.00381	0.006
Cu pH 5.4	asexual reproduction	0.07	**0***	0.328	0.533	0.675	0.172
Cu pH 5.4	coomassie R	0.38	**6.72e-6***	0.00362	0.614	0.816	0.202
Cu pH 5.4	erytrosin B	0.45	**2.30e-13***	**9.92e-5***	0.627	0.220	0.407
Cu pH 5.4	trypan blue	0.14	**0***	0.0796	0.678	0.462	0.216
Cu pH 6.1	log10 bodysize	0.39	**1.11e-16***	**5.93e-6***	0.947	0.144	0.803
Cu pH 6.1	relative gonad size	0.34	**2.11e-13***	0.00223	0.476	0.808	0.332
Cu pH 6.1	bacterial feeder	0.99	0.316	**5.55e-16***	0.994	0.428	0.566
Cu pH 6.1	hyphal feeder	0.10	**1.78e-15***	0.0867	0.0633	0.040	0.023
Cu pH 6.1	plant parasite	0.73	**1.46e-4***	**1.18e-8***	0.272	0.644	0.372
Cu pH 6.1	omnivore	0.56	**7.87e-8***	**3.50e-7***	0.718	0.0776	0.640
Cu pH 6.1	predator	0.97	0.167	**1.89e-8***	0.992	0.613	0.379
Cu pH 6.1	survival stage	0.12	**1.06e-7***	0.0765	0.00178	**2.44e-4***	0.002
Cu pH 6.1	asexual reproduction	0.16	**0***	0.00952	0.677	0.703	0.026
Cu pH 6.1	coomassie R	0.29	**2.47e-8***	0.00182	0.429	0.0483	0.381
Cu pH 6.1	erytrosin B	0.44	**7.22e-14***	**7.74e-6***	0.463	0.0325	0.431
Cu pH 6.1	trypan blue	0.17	**0***	0.0166	0.0260	0.00195	0.024
average							0.196

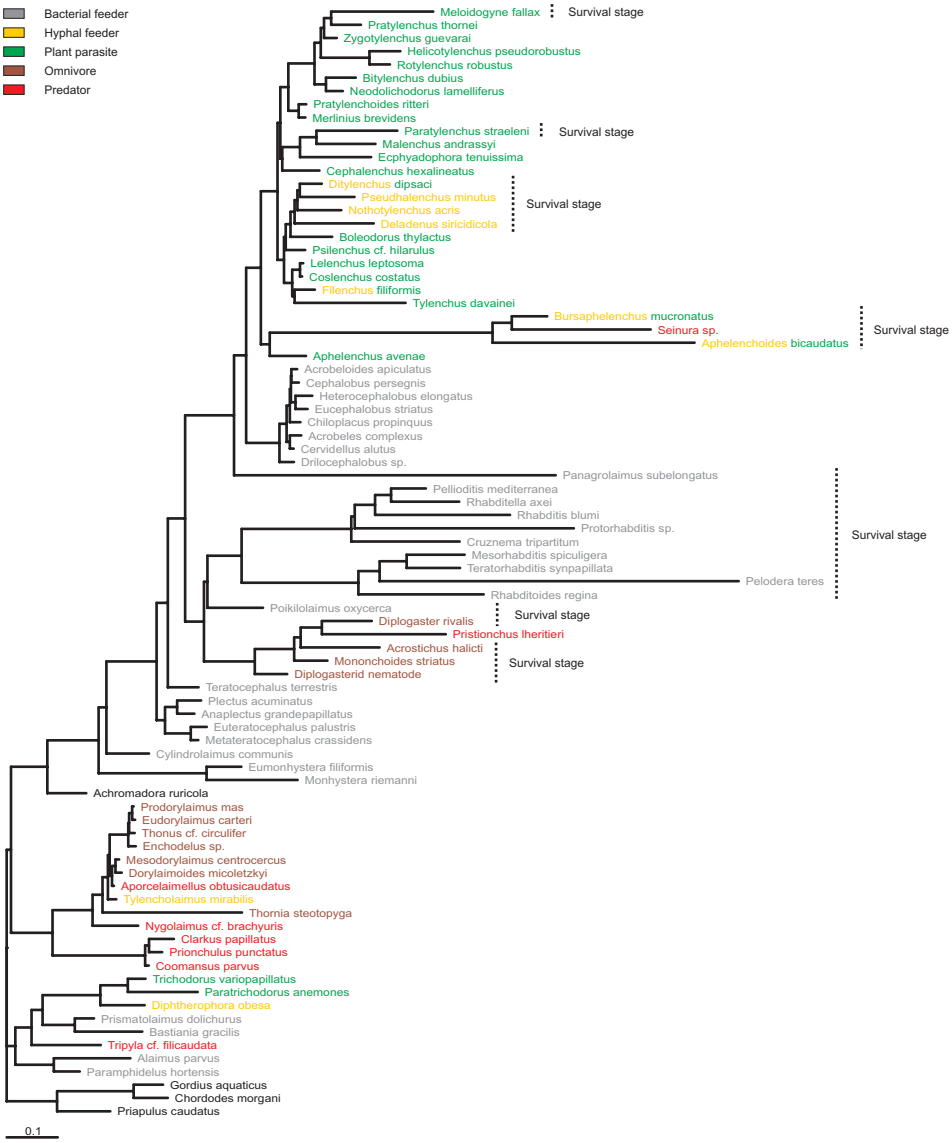

Figure 6.1. Phylogenetic tree of taxa involved in this study. Topology was based on Holterman *et al*. (2006) and Holterman *et al*. (2008), branch lengths were calculated with MrBayes 3.1.2. Feeding types and the ability to enter a survival stage are indicated on the tree.

A phylogenetic dependence of the data can easily be visualized. If one looks for example at bacterial feeding, it has a very high λ in all cases. In the tree, almost all bacterial feeders cluster together. If the ancestor of these bacterial feeders by coincidence also happened to be tolerant to stress, it is very

well possible that its descendents inherited both these traits even though the two traits are not actually correlated. An analysis of these traits without accounting for their shared ancestry would thus probably show the two traits to be correlated even if there is no actual relation between the two. If on the other hand a certain trait is spread over the tree, such as hyphal feeding (λ is 0 or very close to 0 in all cases), shared ancestry is unlikely to play a role in the correlation of this trait with other traits.

Correlations between traits and stress tolerance

Correlations between stress tolerance (as represented by RDA values) and nematode traits were tested in a pairwise fashion. To demonstrate the effect of accounting for phylogeny, the same tests were performed using a maximum likelihood estimate for λ in one case and constraining λ to 0 in the other case. After applying a Bonferroni correction only a single significant correlation was found (Table 6.2). This was between the ability to enter a survival stage and the RDA for copper at pH 6.1 and not using a phylogenetic correction. Given that in this case λ was not significantly different from zero ($P = 2.44 \times 10^{-4}$; λ=0.12), this is a valid result. It seems logical that the ability to enter a survival stage and survive periods of adverse conditions is correlated with a tolerance to stress. Although there are more correlations with a low P-value (survival stage with other RDA's, nematode staining) these are all insignificant because of the large Bonferroni correction.

Accounting for the effects of phylogeny

Although no significant correlations were found when accounting for phylogeny and only one significant correlation when phylogeny was not taken into consideration, the data still demonstrate the importance of accounting for phylogeny. The average absolute difference in P-values between the tests for correlation with and without accounting for phylogeny is 0.196 (Table 6.2) and the changes in the P-values were both positive and negative. As could be expected, this difference becomes even larger with higher λ values (Fig. 6.2) and testing without phylogenetic correction becomes more inappropriate. The importance of allowing for phylogenetic dependence among the data has been demonstrated by other studies (Holden and Mace 1997; Pagel 1999; Espinoza,

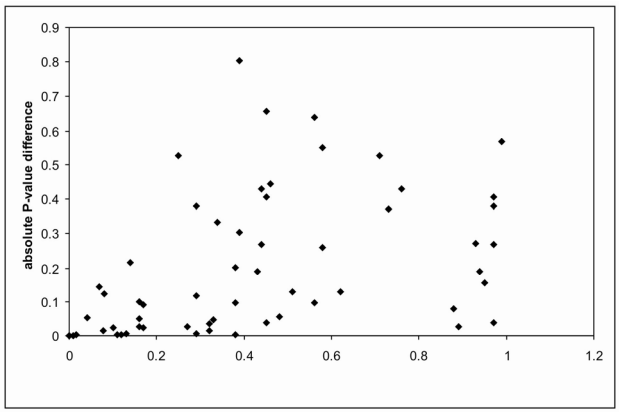

Figure 6.2. The absolute differences of the P-values for the correlations tests with and without phylogenetic correction plotted against λ.

Wiens, and Tracy 2004), in these cases results contradicting established theory were found because the effects of phylogeny were taken into account in the analysis. Yet despite its importance only few authors have allowed for the effect of phylogeny in nematode studies (Morand 1996; Morand and Sorci 1998; Poulin and Morand 2000). Yet the results presented here in Figure 6.2 clearly demonstrate the importance of accounting for the effects of phylogenetic dependence in the data when looking for correlations in traits between nematodes.

Towards experimentally derived cp-classes

What relevance do these results have when it comes to defining more objective criteria for assigning nematodes to cp-classes for the MI? First of all, it demonstrates the importance of allowing for the phylogenetic non-independence of your data when looking for correlations between traits and stress tolerance. For many traits λ is significantly larger than 0 (Table 6.2) and especially if λ becomes larger than about 0.3 the effect on P-values can be substantial (as much as 0.6; Fig. 6.2). Furthermore, the results also demonstrate the suitability of the GLS method for accounting for the effects of phylogeny on the data. The independent contrast method would in most cases overestimate the effect of phylogeny on the data.

Only one significant correlation was found between stress tolerance and

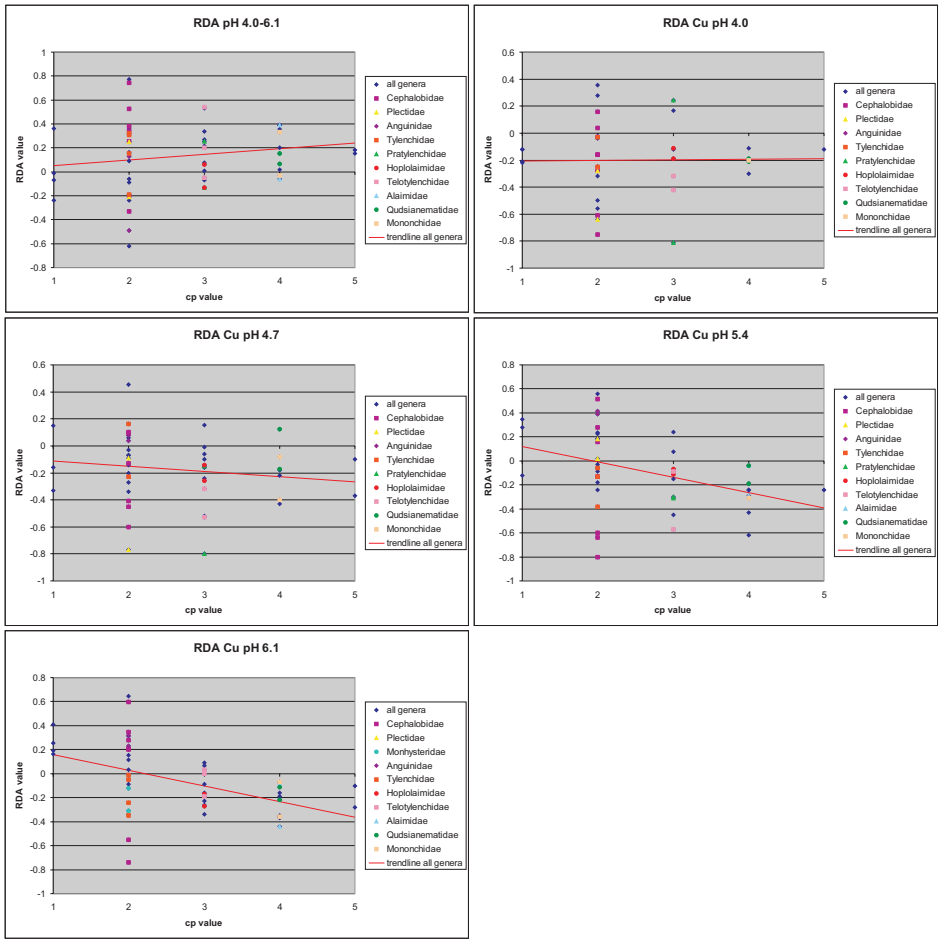

Figure 6.3. RDA values plotted against cp values. Families represented by more than one genus are indicated by separate colors. Trendlines were based on all genera.

nematode traits; the ability to enter a survival stage. This means that for now we cannot design clearer criteria for the cp-classes yet. However, it seems unlikely none of these traits other than the ability to enter a survival stage are correlated with tolerance to stress. It is known that body size correlates with many other life history traits (Peters 1983) and most of the nematodes in a low cp-class are fast reproducing bacterial feeders (Bongers and Ferris 1999). Furthermore, the nematode stains also displayed low P-values in several cases ($0.05 > P > 8.33 \times 10^{-4}$) and it stands to reason that nematodes with a permeable cuticle are more exposed to pollutants in their body than nematodes with a

more or less impermeable cuticle.

What could be done to improve the chances of finding significant correlations? First of all the amount of data from field studies should be increased by analyzing substantially larger numbers of individuals per sample (>> 150 individuals per samples) and by repeatedly sampling over a period of time. This would - at least partially - alleviate the problem of genera not being found in all treatments, resulting in RDA values being calculated for more genera. The development of molecular identification methods would be a great help in increasing the number of nematodes analyzed per sample by a hundredfold or more (Holterman *et al*. 2008). Another possibility would be to identify the nematodes down to species level instead of genus level so that traits such as body size and relative gonad size would no longer have to be averaged over the species in a genus. However this would require a substantial extra effort when identifying nematodes through light microscopy and it is unlikely that molecular identification will be developed to the level of species identification of the entire nematode fauna in the near future. Furthermore it may not be possible to identify all groups of nematodes, such as the Rhabditidae and members of the Dorylaimida, to species level, due to them being notoriously difficult to identify. Finally the missing data for the species traits should be acquired, especially for the data on cuticle permeability. These experiments require living, freshly collected and identified nematodes from field samples (most nematodes are considered to be unculturable), and therefore it is no surprise that the data was incomplete. Filling in the missing data and perhaps combining the results of the different stains into an index may prove to be more informative.

Conclusion

As a first step towards a more objective cp-classification and to refine the Maturity Index from a family level to genus level the traits relevant to tolerance for environmental disturbance have to be identified. We have laid out a framework to identify traits correlated with stress tolerance while taking into account the effects of phylogeny on the data. Accounting for the affects of phylogeny was demonstrated to be very important. Unfortunately our

approach is hampered by incomplete data, especially on the permeability of the cuticle and the tolerance for stress (as represented by the RDA values). Improving on the amount of available data may lead to more significant results and the identification of traits relevant to stress tolerance in the future.

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Appendix A

GenBank accessions of the SSU rDNA sequences used in this study.

Species	NCBI Accession	Species	NCBI Accession
Achromadora ruricola	AY593941	Meloidogyne fallax	AY593895
Acrobeles complexus	AY284671	Merlinius brevidens	AY284597
Acrobeloides apiculatus	AY284673	Mesodorylaimus centrocercus	AY284799
Acrostichus halicti	U61759	Mesorhabditis spiculigera	AF083016
Alaimus parvus	AY284738	Metateratocephalus	AY284686
Anaplectus grandepapillatus	AY284697	crassidens	
Aphelenchoides bicaudatus	AY284643	Monhystera riemanni	AY593938
Aphelenchus avenae	AY284639	Mononchoides striatus	AY593924
Aporcelaimellus	AY284811	Neodolichorhynchus	AY284598
obtusicaudatus		lamelliferus	
Bastiania gracilis	AY284725	Nothotylenchus acris	AY593914
Bitylenchus dubius1	AY284601	Nygolaimus cf. brachyuris	AY284770
Boleodorus thylactus	AY593915	Panagrolaimus subelongatus	AY284681
Bursaphelenchus mucronatus	AY284648	Paramphidelus hortensis	AY284739
Cephalenchus hexalineatus	AY284594	Paratrichodorus anemones	AF036600
Cephalobus persegnis	AY284662	Paratylenchus straeleni	AY284630
Cervidellus alutus	AF202152	Pellioditis mediterranea	AF083020
Chiloplacus propinquus	AY284677	Pelodera teres	AF083002
Chordodes morgani	AF036639	Plectus acuminatus	AF037628
Clarkus papillatus	AY284748	Poikilolaimus oxycerca	AF083023
Coomansus parvus	AY284766	Pratylenchoides ritteri	AJ966497
Coslenchus costatus	AY284581	Pratylenchus thornei	AY284612
Cruznema tripartita	U73449	Priapulus caudatus	Z38009
Cylindrolaimus communis	AY593939	Prionchulus punctatus	AY284746
Deladenus siricidicola	AY633447	Prismatolaimus dolichurus	AY593957
Diphtherophora obesa	AY284838	Pristionchus lheritieri	AF036640
Diplogaster rivalis		Prodorylaimus mas	AY593946
Diplogasterid nematode	AY284689	Protorhabditis sp.	AF083001
Ditylenchus dipsaci	AY593911	Pseudhalenchus minutus	AY284638
Dorylaimoides micoletzkyi	AY284830	Psilenchus cf. hilarulus	AY284593
Drilocephalobus sp.	AY284680	Rhabditella axei	AY284654
Ecphyadophora tenuissima		Rhabditis blumi	U13935
Enchodelus sp.	AY284792	Rhabditoides regina	AF082997
Eucephalobus striatus	AY284666	Rotylenchus robustus	AJ966503
Eudorylaimus carteri	AJ966484	Seinura sp.	AY284651

Eumonhystera filiformis	AY593937	Teratocephalus terrestris	AY284683
Euteratocephalus palustris	AY284684	Teratorhabditis synpapillata	AF083015
Filenchus filiformis	AY284592	Thonus cf. circulifer	AY284795
Gordius aquaticus	X80233	Thornia steatopyga	AY284787
Helicotylenchus pseudorobustus	AY284606	Trichodorus variopapillatus	AY284841
		Tripyla cf. filicaudata	AY284730
Heterocephalobus elongatus	AY284668	Tylencholaimus mirabilis	EF207253
Lelenchus leptosoma	AY284584	Tylenchus davainei	AY284588
Malenchus andrassyi	AY284587	Zygotylenchus guevarai	AF442189

Chapter 7

General discussion

General discussion

The main aim of this thesis was to investigate phylogenetic relationships among nematodes and look at the evolution of traits such as the development of plant parasitism, the colonization of terrestrial habitats and traits relevant to stress tolerance in nematodes. The following sections of the general discussion will summarize the main findings of this thesis and also take a look at possible future avenues of research.

Phylogenetic relationships in the phylum Nematoda

In the first molecular phylogeny spanning the whole phylum Nematoda, Blaxter *et al*. (1998) divided the phylum into five large clades. That study was performed using ≈ 50 SSU rDNA sequences. In our analysis 349 nearly full length SSU sequences were used and twelve major clades were defined on the basis of a series of dichotomies (Chapter 2). The most basal clade (Clade 1) consisted of the Enoplida and Triplonchida. Although the support from the molecular phylogeny was not significant, this position is supported by morphological and developmental features. The embryological development (Schierenberg 2005) and sperm morphology (Justine 2002) of Clade 1 members more resemble that of other animals than other nematodes. The oldest division in the nematodes and one that has dominated nematode taxonomy is the one between the Adenophorea and Secernentea (Chitwood and Chitwood 1933). As was already shown by earlier studies (Aleshin *et al*. 1998; Blaxter *et al*. 1998), the Adenophorea (Clade 1-6) were shown to be paraphyletic with regards to the Secernentea (Clade 7-12). Our analysis showed that the Teratocephalidae (Clade 7) are the most basal members of the Secernentea. The Plectida (Clade 6) were shown to be the closest relatives to the Secernentea.

Although the SSU rDNA gene provided a good resolution for nematode phylogeny in general, poor resolution was present in the order Dorylaimida (Clade 2) where the SSU rDNA was extremely conserved. Therefore a region (±

1000 bp from the 5'-end) of the more variable LSU rDNA was sequenced (Chapter 3). Using LSU rDNA sequences, a molecular phylogeny could be constructed in which 12 subclades could be distinguished. These clades bore very little similarity to classical Dorylaimida taxonomy and most families were proven to be para- or polyphyletic. This was not completely unexpected as Dorylaimida taxonomy has been unstable for decades. The Dorylaimida display a mosaic of characters and it is unclear which are phylogenetically informative and which are homoplaseous. For some of the 12 subclades morphological support could be identified, but not for all.

Another phylogenetic analysis was made of the Chromadoria (not including the Rhabditida) including sequences from taxa that were not available at the time of the original phylum-wide analysis (Chapter 4). These extra sequences included mainly marine nematodes, which were hitherto underrepresented. The original clade division was confirmed with the exception of Clade 5 (Monhysterida and Araeolaimida). Three well-supported subclades could be distinguished. Subclade 5A consisted mainly of members of the Monhysterida, but also included members of the Araeolaimida. Clade 5B included *Isolaimium* (the only genus in the order Isolaimida) and *Aulolaimus* (a member of the Plectida). Clade 5C consisted mainly of members of the Araeolaimida but also included some members of the Monhysterida. *Isolaimium* had traditionally always been placed in the Dorylaimia, but there is clear morphological support for its placement with *Aulolaimus*. The poor support of the backbone in this area of the tree meant that no conclusions could be drawn about the relative positions of these three subclades relative to each other and thus the mono- or paraphyly of Clade 5. The Microlaimoidea and Choanolaimidae could not be placed with any certainty in this analysis and thus were not assigned to any clade.

A more exhaustive analysis of the Tylenchida *sensu* Siddiqi (2000) using the SSU rDNA revealed a large basal polytomy (Chapter 5). However, in the distal part of the tree, consisting of the Hoplolaimina and Criconematina, four well supported subclades could be distinguished. Remarkably, both the cyst (Heteroderidae) and the root knot nematodes (Meloidogynidae) were placed within other families, the Hoplolaimidae and Pratylenchidae respectively, rendering these families paraphyletic. Another surprising finding was the

polyphyly of the Pratylenchidae, with *Radopholus* being closely related to the Hoplolaimidae and *Pratylenchoides* being placed with the Merliinae (Telotylenchidae). Although this is contradictory to classical taxonomy, morphological support for the new positioning could be identified in all these cases.

Properties of the SSU rDNA in nematodes

The SSU rDNA gene has proven itself a very useful gene for exploring the phylogenetic relationships in nematodes. The semi-conserved areas in the gene allowed us to unravel the deep phylogenetic relationships within the phylum, yet at the same time the more variable regions in the gene allowed us to distinguish between families and genera, and - in quite some cases - even species. The Dorylaimida were an exception as the SSU rDNA was exceptionally conserved. Our findings could point at rapid speciation within this order. Although more phylogenetic signal was present in the LSU rDNA (5'-end), we could not completely resolve the relationships between a number of Dorylaimida subclades.

Another distinguishing characteristic of the SSU rDNA in nematodes is the rate of evolution. This rate is not constant and the SSU rDNA in different clades evolve at distinct rates. This was first shown by Aguinaldo *et al*. (1997), who showed that the SSU rDNA in *Caenorhabditis elegans* evolved at a faster rate than the SSU rDNA in the more basal *Trichinella spiralis*. Using a relative rate test, we were able to demonstrate that the rate of evolution in the distal clades (Clades 8-12) was higher than in more basal clades (Clades 1-7; Chapter 2). We hypothesize that there are two main causes for this. First of all, several of the distal clades contain species with a short generation time (Clades 9-11), meaning mutations can accumulate in a shorter time span. Secondly, several of these clades contain plant or animal parasites (Clades 8-10 and 12). A common component of the defense reaction of organism to parasites is the production of free oxygen radicals. These radicals are highly mutagenic and - contrary to free-living nematodes - a parasitic lifestyle would involve regular exposure to these host-produced mutagens.

A further aspect of the use of SSU rDNA is its potential for molecular

identification. Currently identification of nematodes is time consuming, light microscopy-based, and it requires expert knowledge. Because of this the use of nematodes in ecological research and their exploitation for the monitoring of soil disturbance are severely restricted. A molecular system identifying nematodes by their DNA could significantly reduce the time required for the analysis of a sample and could be performed by anybody having a basic knowledge of molecular biology. This would open up the way to the routine analysis of the nematode communities of soil samples. As is demonstrated in Chapter 3 of this thesis, the rDNA cistron is a suitable area for identifying and quantifying nematode families using taxon-specific SNP based primers in combination with real time PCR. Especially in Clades 8-12, primers for identification can be designed that go down to genus or - in many cases – even species level (as demonstrated in Chapter 2 for *Globodera rostochiensis* and *G. pallida*).

Another gene that is widely promoted for the barcoding of animals is Cytochrome *c* oxidase subunit 1 (CO1). The universal primers designed for this gene appear to be robust and CO1 harbors substantial phylogenetic signal (mainly on the third codon position, *e.g*. Hebert, Ratnasingham, and deWaard 2003). Cytochrome *c* oxidase is a relatively complex enzyme that catalyzes the reduction of O_2 into two water molecules in a process called oxidative phosphorylation. In mammals it consists of 13 subunits, and three of them – subunits I, II and III – are encoded by the mitochondrial genome. Usually fragments are amplified ranging from 400 to 650 bp. Among nematodes this fragments was shown to be useful for *e.g.* characterizing the genetic structure of populations of *Geomonhystera disjuncta* (Derycke *et al*. 2007), the structure of *Pellioditis marina* complexes (Derycke *et al*. 2006), or the phylogenetics of a single genus, *Fergosobia* (Ye *et al*. 2007). However, CO1 does not harbor enough characters, and is too variable to be useful for the deduction of deep phylogenetic relationships within the phylum Nematoda.

Development of plant parasitism in nematodes

A long standing hypothesis for the development of plant parasitism in nematodes says that plant parasites evolved from fungal feeding ancestors

(Maggenti 1971) and that plant parasitism gradually evolved from very simple forms of ectoparasitism, such as root hair feeding, to very complex forms of endoparasitism, as can be found in the sedentary endoparasite *Meloidogyne* (Luc *et al*. 1987). Plant parasitism is found in three groups of nematodes, the Trichodoridae (Triplonchida, Clade 1), the Dorylaimida (Clade 2) and the Tylenchomorpha (Clade 12). In all these cases they are closely associated with fungal feeders (Chapters 2 and 3). The plant parasitic Trichodoridae are the sister group to the supposedly fungal feeding Diphtherophoridae (Arpin 1969), but it is unclear what the feeding type of their last common ancestor was (Chapter 2). In the Dorylaimida three plant parasitic groups can be distinguished, the Longidoridae, *Pungentus* and *Longidorella*. The Longidoridae are placed in the basal polytomy in the Dorylaimida tree, thus it is unclear which Dorylaimida are their closest relatives (Chapter 3). However, the fungal feeding Dorylaimida – the Tylencholaimidae and Tylencholaimellidae – are also placed in this polytomy. *Pungentus* and *Longidorella* on the other hand are placed in a clade which consists exclusively of omnivores, so it seems plausible that in these cases plant parasitism did not evolve from fungal feeding but omnivorous ancestors.

The Tylenchomorpha are the only group which contains endoparasites of plants. Ancestral state reconstruction showed that the development of more complex forms of plant parasitism was indeed gradual (Chapter 5). There are however a few instances in which endoparasitism evolved directly from a simple form of plant parasitism such as root hair and epidermal feeding (Anguinidae). Again the development of plant parasitism out of fungal feeding ancestors could not be confirmed. The ancestors of the Tylenchomorpha were reconstructed as plant parasites in most analyses. However, the resolution in the basal portion of the Tylenchomorpha tree was poor and for several of the basal Tylenchids (Tylenchidae, Anguinidae) we do not know for certain whether they are obligate plant parasites or facultative plant parasites which can also feed on fungi. Because of this, no firm conclusion can be drawn on the feeding types of the ancestral Tylenchomorpha.

The colonization of terrestrial habitats by nematodes

Already for decades there is a hypothesis saying that the first nematodes evolved in a marine habitat and that transition to a terrestrial habitat took place only a few times. The Chromadoria (Clades 3-6) contain many marine species as well as terrestrial species and therefore are a good candidate to study this process. Analysis showed that there have been at least 16 major habitat changes (Chapter 4). The change from a thalassic to a limnoterrestial habitat was made at least 11 times in the Chromadoria (excluding the Rhabditida) and on at least one occasion (the Camacolaimoidea) the step from a limnoterrestrial to a thalassic habitat was made. This means that transitions from a thalassic to a limnoterrestrial habitat are common. In fact every clade of the Chromadoria except Clade 4 (Desmorida) contains both thalassic and limnoterrestrial species.

The large number of habitat changes, and the observation that transitions may also take place shallow taxonomic levels also indicate that the transition of a marine to a limnoterrestrial habitat involves a limited number of changes. The main problem for a nematode switching from a thalassic habititat to a terrestrial habitat would be the large fluctuations in osmotic value that can be experienced in the soil whereas the osmotic value of a marine habitat is fairly constant. Since nematodes depend on a pressurized hydroskeleton for movement, fluctuations in the osmotic pressure are a problem. Two mechanisms have been identified which could deal with this problem. The first is the production of osmolytes, such as glycerol, combined with a less permeable cuticle to control the internal water balance. Evidence for this has been found in *C. elegans* (Lamitina *et al*. 2004; Huang *et al*. 2007). The second mechanism is the excretory system of nematodes. The mainly limnoterrestrial Secernentea (Clade 7-12) contain a tubular excretory system which has been proven to play a role in osmoregulation (Wharton and Sommerville 1984). The Adenophorea, which includes most thalassic species, contain a glandular excretory system and it is doubtful if this plays a role in osmoregulation (Wright 1998).

A further difference between limnoterrestrial and thalassic nematodes is that the former tend to have a higher turgor pressure. Possibly as a consequence of this higher turgor limnoterrestrial nematodes have a stronger

cuticle. Radial striae and/or spiral fiber layers in the basal zone of the cuticle are important for delivering radial strength to the cuticle. Notably, the radial striae are always located in the basal cuticle zone in terrestrial nematodes, whereas in all marine nematodes they are located in the cortical cuticle zone. However, these striae are not homologous and the cortical zone plays a role in adding flexibility to the cuticle (Decraemer *et al*. 2003). Yet the strict positioning of radial striae according to the marine or limnoterrestrial habitat is striking and it is tempting to think that this adaptation is functional in the transition from marine to terrestrial habitats.

A framework for assessing the relevance of characters for stress tolerance in nematodes

In Chapter 6 of this thesis a framework was laid out for detecting the correlations between nematode characters and tolerance to different stressors. Nematodes are abundant, occupy several trophical levels and play an important role in the soil food web (De Ruiter, Neutel, and Moore 1998). They also display varying levels of tolerance towards environmental disturbances. This led Bongers (1990) to propose his Maturity Index. This index assigns nematode families to five so-called cp-classes, with low cp-values indicating stress tolerant 'r-strategists' and high cp-values indicating stress sensitive 'K-strategists'. Unfortunately, due to a lack of data, this was mainly done on the basis of expert knowledge and a few general observations (Bongers 1999). Being able to statistically demonstrate correlations between nematode traits and stress tolerance is a first step towards an objective, ecological grouping of nematodes.

For our study existing data from a field study was used which contained pH and Cu gradients (Korthals *et al*. 1996). A redundancy analysis (RDA; Ter Braak 1995) was performed and the values of the first canonical axis were used as a measure for the tolerance to pH or Cu stress. The traits under investigation were body size, relative gonad size, feeding type, survival stage, (a)sexual reproduction and cuticle permeability. One of the basic assumptions of statistical tests is that the data is independent. However, when comparing traits between species this is often not the case (Felsenstein 1985). To account for this phylogenetic dependence a generalized least squares (GLS) method was

used (Pagel 1997; Pagel 1999). Testing showed that many traits were not phylogenetically independent and that phylogeny had to be taken into account. Furthermore it showed that a simpler method as Independent Contrasts (Felsenstein 1985) would overestimate the effects of phylogeny. Therefore the GLS method, which can take an intermediate approach, is well suited correct for the effects of phylogeny. The importance of taken phylogeny into account was demonstrated by comparing the significance of the correlations between traits and stress tolerance with and without accounting for phylogeny. The average absolute difference in P-values was 0.196 and when data was strongly phylogenetically correlated this difference could as much as 0.6.

Only one significant correlation between stress tolerance and nematode traits was found. This was between the ability to enter a survival stage and a tolerance to Cu at pH 6.1. There were two main reasons for this lack of significant correlations. First of all, because of the large number of pairwise tests (60) a large Bonferroni correction had to be applied. The other reason was the amount of missing data, especially for the cuticle permeability and the RDA values. The first was caused by the difficulty in collecting enough live nematodes for staining experiments. The fact that many genera were observed in only a limited number of plots meant that for most genera RDA values could not be calculated for all treatments. Finally it was observed that in general cp-values corresponded with RDA-values. The framework to investigate correlations between nematode traits and stress tolerance while accounting for the effects of phylogenetic dependence, as presented in chapter 6, represents a first step on towards objective, ecological grouping of nematodes.

Possible future avenues of research

Nematode phylogeny

From the viewpoint of nematode systematics, one of the most interesting groups would be Clade 1, seeing as especially the Enoplida are still underrepresented in the trees. Many more sequences are now available on GenBank and a small study was done by Holterman and Holovachov (2007). They found that most suborders of the Enoplida were monophyletic with the exception of the Ironina (unpublished results).

Additional work could also be done on the Chromadoria. A more variable gene such as the LSU rDNA might be able shed more ight on the validity of Clade 5 and the polyphyly of the Monhysterida and Araeolaimida. The position of the Microlaimoidea, Choanolaimidae and the rogue taxa *Prodesmodora*, *Desmoscolex* and *Halichoanolaimus* remains to be resolved as well.

A good number of Dorylaimida species have been sequenced (72 taxa), but given the large diversity of this order (more than 10% of all described nematode species are Dorylaimida; Jairajpuri and Ahmad 1992) this is likely to be just the tip of the iceberg. Including more species, especially from families which so far have been excluded, is almost guaranteed to identify additional subclades to the twelve that have been described in the analysis presented here.

There is also the position of the Aphelenchids in Clade 10. This is likely to be an artifact caused by long branch attraction and their low GC contents. Sequencing of a gene other than ribosomal DNA, is probably required to resolve this issue.

Evolution of plant parasitism

The development of plant parasitsm has been studied in the Tylenchids, Dorylaimida and Triplonchida, but not yet in the Aphelenchids. This group contains both fungal feeders and (facultative) plant parasites and would thus be a suitable group to test the hypothesis that plant parasites evolved from fungal feeders. To better test this hypothesis in the Tylenchids, the resolution in the basal part of the Tylenchida tree needs to be improved. This might be accomplished using the more variable LSU rDNA, as was done for the Dorylaimida. Furthermore one would need to get more clarity on the feeding types of the Tylenchidae and Anguinidae. Experiments such as those performed by Okada *et al*. (Okada, Harada, and Kadota 2005) where *Filenchus* species were cultured on different fungi and media, could prove whether other genera also possess the ability to feed on fungi.

Another aspect of plant parasitism is the presence of genes encoding for cell wall-degrading enzymes in some species. These genes have been found in *Globodera*, *Heterodera*, *Meloidogyne*, *Radopholus* and *Bursaphelenchus*.The main questions are where they originated and whether they were acquired by horizontal gene transfer from bacteria. The most interesting candidates for

screening of the presence of these genes would be the closely related endoparasites *Hirschmanniella*, *Zygotylenchus*, *Nacobbus* and *Pratylenchoides* and the closely related non-endoparasitic families, the Telotylenchidae and Hoplolaimidae (Chapter 5). Considering that genes coding for cell wall-degrading enzymes are also present in *Bursaphelenchus* (albeit from a different gene family), it begs the question if perhaps cell wall-degrading enzymes were already present in the last common ancestor of the Tylenchomorpha. And if this is the case whether they are present and play a role in all fungal feeding and plant parasitic nematodes, including those found in the Dorylaimida and Triplonchida.

Colonization of terrestrial habitats

It has been established that the production and breakdown and or excretion of glycerol plays a role in the osmoregulation in *Caenorhabditis elegans* (Lamitina *et al*. 2004). Supposedly aquaporins, channel foming proteins that allow the transport of water and small solubles, play an important role in this process. It would be interesting to see if the transition to a terrestrial habitat corresponds with the ability to synthesize and break down and/or excrete glycerol.

Stress tolerance in nematodes

The first thing to do improve the chances of finding significant correlations between nematode traits and stress tolerance would of course be filling in the missing data, especially for the cuticle permeability. Perhaps combining the result of the different stainings in an index might help as wel. The other important thing would be the acquisition of RDA values for more genera and more treatments. This could be acquired by more extensive sampling (currently only 150 individuals per sample are identified) and sampling over a period of time. This would help to reduce the amount of 'zeroes' in the data leading to more RDA values being calculated. A molecular identification system (such as demonstrated in chapters 2 and 3) would be of great help in increasing sampling size.

Conclusion

In this thesis we have endeavoured to make a significant contribution to the field of nematode taxonomy. A phylogenetic tree was constructed of 349 taxa spanning the entire phylum Nematoda using the small subunit ribosomal DNA. Twelve major clades could be distinguished with the Enoplida and Triplonchida forming the most basal clade. Further trees were constructed for the Dorylaimia, Chromadoria and Tylenchomorpha. For the Dorylaimida a tree using the more variable large subunit ribosomal DNA was constructed. In most cases nematode relationships could be elucidated with good support, although some areas in the trees remained unresolved. Furthermore, the suitability of ribosomal DNA for a (semi-) quantative molecular identification method was demonstrated. Plant parasitism has arisen several times within the Nematoda (once in the Triplonchida, at least twice in the Dorylaimida and at least twice in the Tylenchomorpha) and in most cases plant parasites were associated with fungal feeding nematodes. The generally accepted hypothesis that plant parasites evolved from fungal feeding ancestors could not be corroborated however. Analysis revealed that transitions from a thalassic to a limnoterrestrial habitat (and *vice versa*) have taken place at least 11 times in the Chromadoria and these transitions are apparently fairly easy to achieve for nematodes. Finally a framework was laid out to study correlations between nematode traits and stress tolerance and the importance of accounting for the effects of phylogeny was demonstrated. This is a first step towards a transparent, ecological grouping of free-living nematodes.

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Summary

Nematodes – "eel worms"; members of the phylum Nematoda – can be considered as a success story within the Metazoa (multicellular, heterotrophic eukaryotes in which cells lack cell walls): they are speciose and – probably - the most numerous group of multicellular animals on our planet. Nematodes are present in virtually all terrestrial, freshwater and marine habitats. Nematodes are trophically diverse; they may feed on bacteria, fungi/oomycetes, algae and protozoa, other nematodes or on a combination of these (omnivores), or live as facultative or obligatory parasites of plants or animals. As they are abundant, ubiquitous and occupy several trophic levels, they play an important role in the soil food web. Nematode parasites of animals affect billions of humans and livestock, while plant parasites such as cyst, root knot and lesion nematodes cause large agricultural losses worldwide.

Despite their undisputed ecological and economical relevance, the systematics of the phylum Nematoda is far from established. One of the aims of this research was to further elucidate nematode phylogeny using molecular data. First a phylogenetic tree was constructed of 349 taxa, spanning the entire phylum Nematoda, on the basis of full length small subunit ribosomal DNA (SSU rDNA) sequences. A series of mostly well-supported bifurcations defined twelve major clades, whereas the most basal clade was defined by representatives of the Enoplida and Triplonchida. Our analysis confirmed the paraphyly of the Adenophorea. Furthermore it was found that the SSU rDNA from representatives of the distal clades evolved at a higher rate than the SSU rDNA from the basal clades. In the meantime, a substantial number of sequences was added to our overall SSU rDNA nematode alignment - both public data (GenBank) and data generated by ourselves (≈ 1,500 sequences in total; February 2008). It is noted that the clade division as proposed in 2006 on the basis of "only" 349 taxa still seems to be valid.

Subsequent research focused on three specific groups; Dorylaimia, Chromadoria and Tylenchomorpha. Within the suborder Dorylaimina, the SSU rDNA provided an exceptionally low phylogenetic signal, and - therefore – a

part (≈ 1,000 bp) of the more variable large subunit ribosomal DNA (LSU rDNA) was analyzed. In most cases nematode relationships could be elucidated with good support, although some areas in the trees remained unresolved. Generally speaking the results of molecular phylogenetics corresponded fairly well with classical nematode taxonomy. The main exception was the order Dorylaimida where twelve subclades could be distinguished which bore little resemblance to classical taxonomy. Furthermore the suitability of ribosomal DNA for a (semi-) quantative molecular identification method was demonstrated using quantitative PCR (q-PCR) and primers designed to specifically amplify members of the order Mononchida and the potato cyst nematodes *Globodera pallida* and *G. rostochiensis*.

Plant parasitism has arisen several times within the phylum Nematoda (once in the Triplonchida, at least three times in the Dorylaimida and at least twice in the Tylenchomorpha). The long-standing and generally accepted hypothesis states that plant parasites evolved from fungal feeding ancestors. However, while in most cases plant parasites were associated with fungal feeding nematodes, this hypothesis could neither be confirmed nor denied with the results of our phylogenetic analyses. In the case of two Dorylaimida (*Pungentus* and *Longidorella*), however, the ancestor was probably an omnivore. The analysis of this problem was substantially hampered by the lack of knowledge on feeding behavior of basal Tylenchomorpha.

Presumably, the common ancestor of the nematodes lived in a marine environment and - if this assumption is correct - the transition to a limnoterrestrial environment must have taken place at least once. Surprisingly, analysis of the Chromadoria (minus the Rhabditida) revealed that transitions from a thalassic to a limnoterrestrial habitat (and *vice versa*) have taken place at least 11 times in the Chromadoria. Given their frequency these transitions are apparently fairly easy to achieve for nematodes and the possible adaptations involved were discussed.

Nematodes vary widely in their responses to environmental disturbance, making them good bio-indicators of soil health. Yet it is not known with certainty which traits are responsible for tolerance to stress in nematodes. A framework was laid out to study correlations between nematode traits and stress tolerance. Furthermore the importance of accounting for the confounding

effects of phylogeny was demonstrated. This is a first step towards a transparent, ecological grouping of free-living nematodes.

It is worthwhile mentioning that - on the basis of the rDNA-based molecular framework described in this PhD thesis - DNA sequences signatures were identified for nearly all North-West European terrestrial and freshwater nematodes families. The relationship between quantitative PCR signal and numbers of individuals has been established for nearly all families and a first testing of DNA barcode-based community analysis is planned for spring 2008.

Samenvatting

Nematoden – "aaltjes" in alledaags Nederlands; leden van het fylum Nematoda – kunnen als een succes verhaal binnen het dierenrijk beschouwd worden: er zijn zeer veel soorten en ze zijn - waarschijnlijk - de meest talrijke meercellige dieren op onze planeet. Nematoden zijn aanwezig in vrijwel alle terrestrische, zoet- en zoutwater milieus. Nematoden zijn trofisch divers; soorten kunnen zich voeden met bacteriën, schimmels/oomyceten, algen en protozoa, andere nematoden of een combinatie hiervan (omnivoren) of ze leven als obligate of facultatieve parasieten van planten of dieren. Omdat ze algemeen zijn, in grote aantallen in de bodem leven en verschillende trofische niveaus innemen, spelen ze een belangrijke rol in het bodem-ecosysteem. Dierparasieten infecteren jaarlijks miljarden mensen en dieren, terwijl plantenparasieten zoals cysten-, wortelknobbel- en wortellesie-aaltjes wereldwijd grote schade in de landbouw veroorzaken.

Ondanks hun ecologische en economische relevantie is de taxonomie van nematoden alles behalve stabiel. Eén van de doelen van dit onderzoek was om de fylogenie (evolutionaire verwantschapsrelaties) van nematoden verder te onderzoeken met behulp van moleculaire data. Er is een fylogenetische boom gemaakt van 349 soorten die het hele fylum Nematoda beslaan. Dit werd gedaan op de basis van de bijna volledige sequenties van het "small subunit" ribosomaal DNA (SSU rDNA) gen. Aan de hand van een serie - over het algemeen goed ondersteunde - T-splitsingen in de boom konden twaalf grote claden onderscheiden worden (een clade bestaat uit alle soorten die afstammen van een gemeenschappelijke voorouder). De meest basale clade in de boom bestond uit vertegenwoordigers van de ordes Enoplida en Triplonchida. De nematoden werden traditioneel altijd in twee klasses verdeeld: de Secernentea en de Adenophorea. Onze analyse bevestigde dat de Adenophorea geen monofyletische groep zijn, maar dat de Secernentea binnen deze groep vallen. Verder werd vastgesteld dat de claden aan het uiteinde van de boom sneller evolueren dan de basale claden. Inmiddels is er een groot aantal sequenties toegevoegd aan de SSU rDNA database (≈ 1500 sequenties, februari 2008),

zowel publiek beschikbare data (GenBank) als door ons gegenereerde data. Vooralsnog lijkt de cladenindeling zoals we die op basis van 349 sequenties hebben voorgesteld stand te houden.

Vervolg onderzoek richtte zich op drie groepen: de Dorylaimia, de Chromadoria en de Tylenchomorpha. Binnen de suborde Dorylaimina bevatte het SSU rDNA bijzonder weinig fylogenetisch signaal en daarom werd voor deze groep een gedeelte (≈ 1000 baseparen) van het meer variabele "large subunit" ribosomaal DNA (LSU rDNA) gen geanalyseerd. In de meeste gevallen konden de relaties tussen soorten opgehelderd worden met een goede ondersteuning door de data, hoewel sommige gedeeltes van de bomen onopgehelderd bleven. Over het algemeen kwamen de resultaten van de moleculaire fylogenie goed overeen met de klassieke nematoden taxonomie. De voornaamste uitzondering was de orde Dorylaimida. Binnen deze orde konden twaalf subcladen onderscheiden worden die weinig tot geen overeenkomst vertoonden met de klassieke taxonomie. Verder werd de geschiktheid van ribosomaal DNA aangetoond voor de (semi-) kwantitatieve identificatie van nematoden. Dit werd gedaan met behulp van kwantitatieve PCR (q-PCR) en primers die ontworpen waren om specifiek leden van de orde Mononchida of aardappelcystenaaltjes (*Globodera pallida* en *G. rostochiensis*) te amplificeren.

Planten parasitisme is in de loop van de evolutie meerdere keren ontstaan binnen het fylum Nematoda: eenmaal binnen de Triplonchida, tenminste driemaal in de Dorylaimida en minimaal twee keer binnen de Tylenchomorpha. De algemeen geaccepteerde hypothese is dat planten parasieten geëvolueerd zijn uit voorouders die zich op schimmels voedden. In de meeste gevallen zijn plant parasitaire soorten inderdaad nauw verwant aan schimmeletende soorten, echter de hypothese dat planten parasieten geëvolueerd zijn uit schimmeleters kon niet worden bevestigd - maar ook niet worden ontkracht – aan de hand van de hier gegenereerde moleculaire fylogenieën. De analyse werd gehinderd door het feit dat voor veel basale Tylenchomorpha weinig bekend is over hun voedingspatroon. In twee gevallen, de Dorylaimieden *Longidorella* en *Pungentus*, kon worden aangetoond dat de voorouder waarschijnlijk geen schimmeleter maar een omnivoor was.

De voorouder van alle nematoden leefde waarschijnlijk in een zoutwater habitat. Als deze veronderstelling correct is dan moet de overstap

naar een terrestrische of zoetwater habitat dus minimaal eens, maar waarschijnlijk vaker gemaakt zijn. Analyse van de subklasse Chromadoria (met uitsluiting van de orde Rhabditida) liet zien dat de overstap van een zout-/brakwater milieu naar een terrestrisch/zoetwater milieu (en *vice versa*) tenminste elf maal heeft plaats gevonden binnen deze groep. Gezien de hoge frequentie waarmee dit onder nematoden plaats heeft gevonden, is deze overstap is dus blijkbaar vrij gemakkelijk te maken voor nematoden. De mogelijk benodigde fysiologische en/of morfologische aanpassingen zijn verder bediscussieerd.

Nematoden zijn zeer talrijk, weinig mobiel, en relatief gemakkelijk uit de grond te extraheren. Als daarbij in beschouwing wordt genomen dat soorten daarnaast sterk variëren in hun respons op verstoringen van hun leefomgeving, lijkt het redelijk te stellen dat ze goede indicatoren zijn voor de bodemgezondheid. Het is echter nog niet met zekerheid bekend welke eigenschappen verantwoordelijk zijn voor stress tolerantie in nematoden. In dit onderzoek is een raamwerk opgesteld om de correlaties tussen nematoden eigenschappen en stress tolerantie te onderzoeken. Bovendien is aangetoond hoe belangrijk het is om hierbij rekening te houden met de invloed van de fylogenetische relaties op de data. Dit is een eerste stap op weg naar een transparante, ecologische groepering van vrij-levende (niet-parasitaire) nematoden.

Op basis van het moleculaire raamwerk beschreven in dit proefschrift, zijn specifieke DNA sequenties geïdentificeerd voor (vrijwel) alle Noordwest-Europese terrestrische en zoetwater nematoden families. De correlatie tussen het kwantitatieve PCR (q-PCR) signaal en het aantal nematoden aanwezig in een monster is vastgesteld voor een groot deel van deze families en de eerste testen voor gemeenschapsanalyses bij nematoden op basis van DNA barcodes staan gepland voor de lente van 2008.

Dankwoord

Eindelijk is het dan zover, de laatste loodjes van mijn proefschrift. In januari 2002 kwam ik als student op de leerstoelgroep Nematologie binnen. Na hier een afstudeervak gedaan te hebben ben ik eind 2002 als AIO verder gegaan en daar heb ik geen moment spijt van gehad. Ik heb hier ruim 6 jaar, eerst als student en later als AIO, met veel plezier gewerkt en ik wil bij deze dan ook graag een aantal mensen bedanken die hier mede verantwoordelijk voor zijn.

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Martijn

Curriculum Vitae

Martijn Hermanus Maria Holterman werd op 12 augustus 1979 geboren in Zwolle. In 1997 haalde hij zijn VWO-diploma aan het Florens Radewijns College te Raalte. In dit zelfde jaar begon hij zijn studie Plantenveredeling en Gewasbescherming aan de Wageningen Universiteit met een specialisatie in ecologische gewasbescherming. In 2000 begon hij aan zijn eerste afstudeervak getiteld "Host specificity and biocontrol of *P. ultimum*, *R. solani* and *H. schachtii* by DAPG-producing *Pseudomonas* spp." bij het Laboratorium voor Fytopathologie onder begeleiding van dr. Jos Raaijmakers en ir. Maria Bergsma-Vlami. Hierna ging hij voor een stage van een half jaar naar Adelaide, Australie, alwaar hij onderzoek deed naar de genotypische en fenotypische diversiteit van Pseudomonas brassicacaearum van tarwe veld gronden bij het CSIRO onder begeleiding van dr. Maarten Ryder en dr. Paul Harvey. In 2002 volgde een tweede afstudeervak bij het Laboratorium voor Nematologie getiteld "Development of a new molecular tool (MLPA) to describe nematode community composition" onder begeleiding van dr. André van der Wurff. Hierna volgde een korte aanstelling als toegevoegd onderzoeker op dezelfde leerstoelgroep op het DREAM EU-project waar gekeken werd naar de naar genen coderend voor celwand afbrekende enzymen en de mogelijke rol van horizontale gen overdracht. In december 2002 ging hij verder als AIO onder begeleiding van dr. ir. Hans Helder en dr. André van der Wurff met als onderwerp de fylogenie van nematoden op basis van ribosomaal DNA en de biologische implicaties hiervan. Dit resulteerde na vijf jaar in het proefschrift dat nu voor u ligt.

List of publications

Refereed journals

Holterman, M., A. van der Wurff, S van den Elsen, H. van Megen, T. Bongers, O. Holovachov, J. Bakker, J. Helder. 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.

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Patent

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Holterman, M., S. van den Elsen, H. van Megen, A. van der Wurff, J. Helder. 2003. Phylogeny of the phylum Nematoda based on small subunit ribosomal DNA sequences. 42nd annual meeting of the Society of Nematologists. Cornell University, Ithaca, New York, July 12-17, 2003.

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Holterman, M., A. Doroszuk, J. Helder, A. van der Wurff. 2006. A comparative analysis of traits involved in stress tolerance in nematodes. 28th international symposium of the European Society of Nematologists. Blagoevgrad, Bulgaria, June 5-9, 2006.

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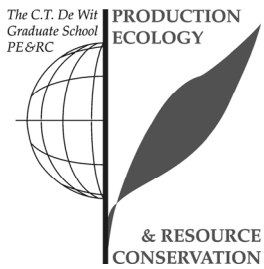
Holterman, M., O. Holovachov. 2007. Phylogeny and biogeography of Triplonchida. APS SON joint meeting, Society of Nematologists 46th annual meeting. San Diego, California, July 28-August 1, 2007.

Mullin, P., M. Holterman. 2007. Molecular phylogeny of Dorylaimia. APS SON joint meeting, Society of Nematologists 46th annual meeting. San Diego, California, July 28-August 1, 2007.

PE&RC PhD Education 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 (5.6 ECTS)
- Phylogenetic relationships in nematodes using the 18S rDNA (2003)

Post-Graduate Courses (6.3 ECTS)
- Soil ecology: linking theory to practice; PE&RC (2003)
- Basic and advanced statistics; PE&RC (2003/2004)
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- Scientific writing; Language Centre (2004)

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- Project management & time planning; Stoas GreenWise (2003)
- Career perspectives; Meijer & Meijaard (2006)

Discussion Groups / Local Seminars and Other Scientific Meetings (5.3 ECTS)
- Farewell symposium of prof. August Coomans; University of Gent (2003)
- Discussion group: in the tracks of evolution (2003-2005)
- In the tracks of evolution symposium (2005)
- Benelux congress of Zoology (2005)
- Current themes in ecology meetings (2005 and 2006)
- Soil & water symposium (2007)

PE&RC Annual Meetings, Seminars and the PE&RC Weekend (2.4 ECTS)
- Introduction weekend (2003)
- PE&RC days (2003, 2005 and 2007)
- Scientific publishing: an introductory workshop for PhD students and young authors (2004)

International Symposia, Workshops and Conferences (16 ECTS)
- 42nd Annual meeting of the Society of Nematodes; Ithaca, New York (2003)
- 27th International symposium European Society of Nematologists; Rome, Italy (2004)
- 10th Congress of the European Society for Evolutionary Biology; Krakow, Poland (2005)
- 28th International symposium European Society of Nematologists; Blagoevgrad, Bulgaria (2006)
- 46th Annual meeting of the Society of Nematologists; San Diego, California (2007)
- Nematode tree of life workshop (2007)

The research presented in this thesis was performed at the Laboratory of Nematology, Wageningen University, Binnenhaven 5, 6709 PD Wageningen.

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