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Molecular Ecology Resources

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https://doi.org/10.1111/j.1471-8286.2007.01963.x

 doi: 10.1111/j.1471-8286.2007.01963.x

BARCODING ARTICLE

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

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Abstract

Indigenous communities of soil-resident nematodes have a high potential for soil health assessment as nematodes are diverse, abundant, trophically heterogeneous and easily extractable from soil. The conserved morphology of nematodes is the main operational reason for their under-exploitation as soil health indicators, and a user-friendly biosensor system should preferably be based on nonmorphological 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 (SSU rDNA) sequences 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 rDNA (≈ 1000 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 polymerase chain reaction). Our results constitute proof-of-principle for the concept of DNA sequence signatures-based monitoring of stress sensitive nematode families in environmental samples.

Keywords: LSU ribosomal DNA, nematode, RT-PCR, soil community analysis, SSU ribosomal DNA

Received 2 July 2007; revision accepted 1 August 2007

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 metre (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 *et al*. 1998; Bongers & Ferris 1999). Furthermore, nematodes

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themselves serve as a food source for a wide range of soil inhabitants (Bongers & 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 & 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 six 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 freshwater habitats at family or even genus level is ecologically relevant (Ettema & Bongers 1993; Bongers 1999; Bongers *et al*. 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 of 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 & 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 h), and because only adults are taken into consideration (juveniles cannot always be identified). It is concluded that a nematode-based biosensor should be based on nonmorphological 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 12 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 (≈ 1000 bp from the 5′-end). In many cases (45), both the SSU (full length) and LSU (fragment) rDNA were sequenced from the same individual nematode. In this study, we present SSU and LSU rDNA-based phylogenetic analyses 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 work on the phylogeny of the Nematoda (Holterman *et al*. 2006), and new sequences were acquired as described in this study. SSU rDNA sequences were deposited at GenBank under Accession nos EF207244

to EF207254. For a full list of sequences used for this study, see Tables S1 and S2, Supplementary material.

DNA extraction, LSU rDNA amplification and sequencing

Single nematodes were transferred to a 0.2 mL polymerase chain reaction (PCR) tube containing 25 μL sterile water. An equal volume of lysis buffer containing 0.2 M NaCl, 0.2 M Tris-HCl (pH 8.0), 1% (v/v) β-mercaptoethanol and 800 μg/mL proteinase K was added. Lysis took place in a Thermomixer (Eppendorf) at 65 °C and 750 r.p.m. for 2 h 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× 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; 5× (94 °C, 30 s; 45 °C, 30 s; 72 °C, 70 s) followed by 35× (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 (Table S1) and aligned using the CLUSTAL W 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, namely*: 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 1000 bp from the 5′-end onwards) and contained 1309 aligned positions (including gaps).

Phylogenetic analyses

The SSU rDNA trees were constructed using Bayesian inference. MODELTEST selected the general time reversible (GTR) model with invariable sites and gamma distribution as the best fitting models for both SSU data sets. In essence the data set was analysed as described in Holterman *et al*. (2006), except for now the gamma parameter was included. The Dorylaimia SSU rDNA data set was run for 2 000 000 generations and the burn-in was 100 000 generations (Fig. 1). The Dorylaimida SSU rDNA data set was run for 1 800 000 generations and the burn-in was 500 000 generations (Fig. 2).

Three different methods were used to construct a phylogenetic tree from the LSU rDNA; neighbour-joining (NJ), maximum parsimony (MP) and Bayesian inference (BI). MODELTEST 3.06 (Posada & Crandall 1998) was used to determine the most appropriate nucleotide substitution model. Both the likelihood ratio test and the Akaike information criterion selected the GTR model with invariable sites (I) and a γ-shaped distribution of substitution rates (Γ) as the best fitting substitution model. The neighbour-joining tree was constructed using PAUP* 4.0b10 (Swofford 1998) using the model parameters calculated by MODELTEST. The resulting tree was bootstrapped 1000 times. The MP tree was also constructed using PAUP*, the default parameters were used. This tree was bootstrapped 1000 times as well. The Bayesian tree was constructed using the program MRBAYES 3.1.2 (Ronquist & 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 analysed 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 million 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 version 1.2.1 (Rambaut & Drummond 2005) was used to check if all parameters had converged. The program MACCLADE version 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. 1 (M1–M5), subclade-specific SSU rDNA-based primers were

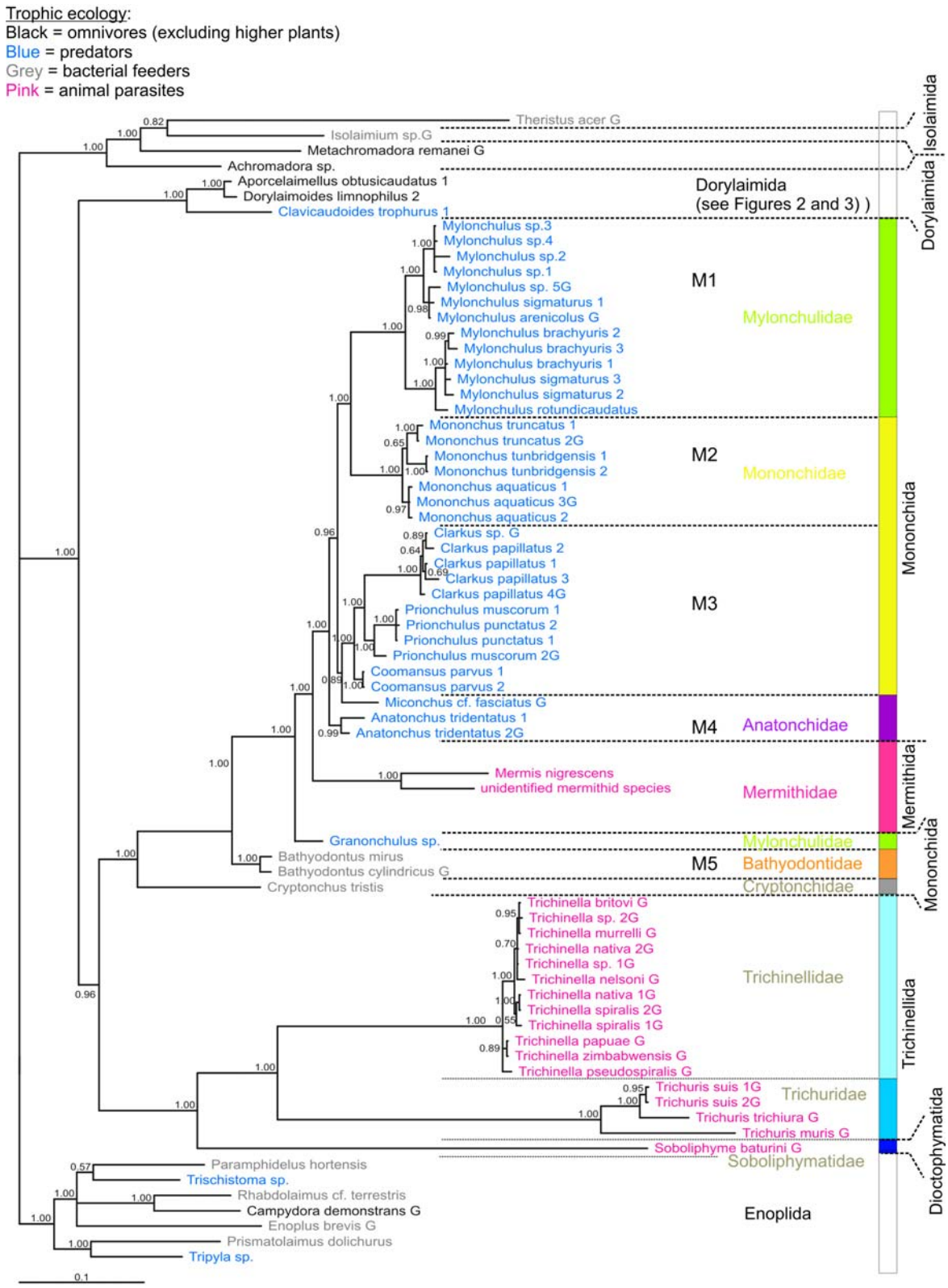

Fig. 1 SSU rDNA-based Bayesian phylogeny of the subclass Dorylaimia (Clade 2 in Holterman *et al*. 2006). Numbers near nodes represent posterior probabilities. The coloured bar indicates to which order, family, and subclade (M1–M5) a species belongs. The colour of the species name indicates the feeding type (according to Yeates *et al*. 1993). A 'G' behind the species name indicates the sequence was acquired from GenBank.

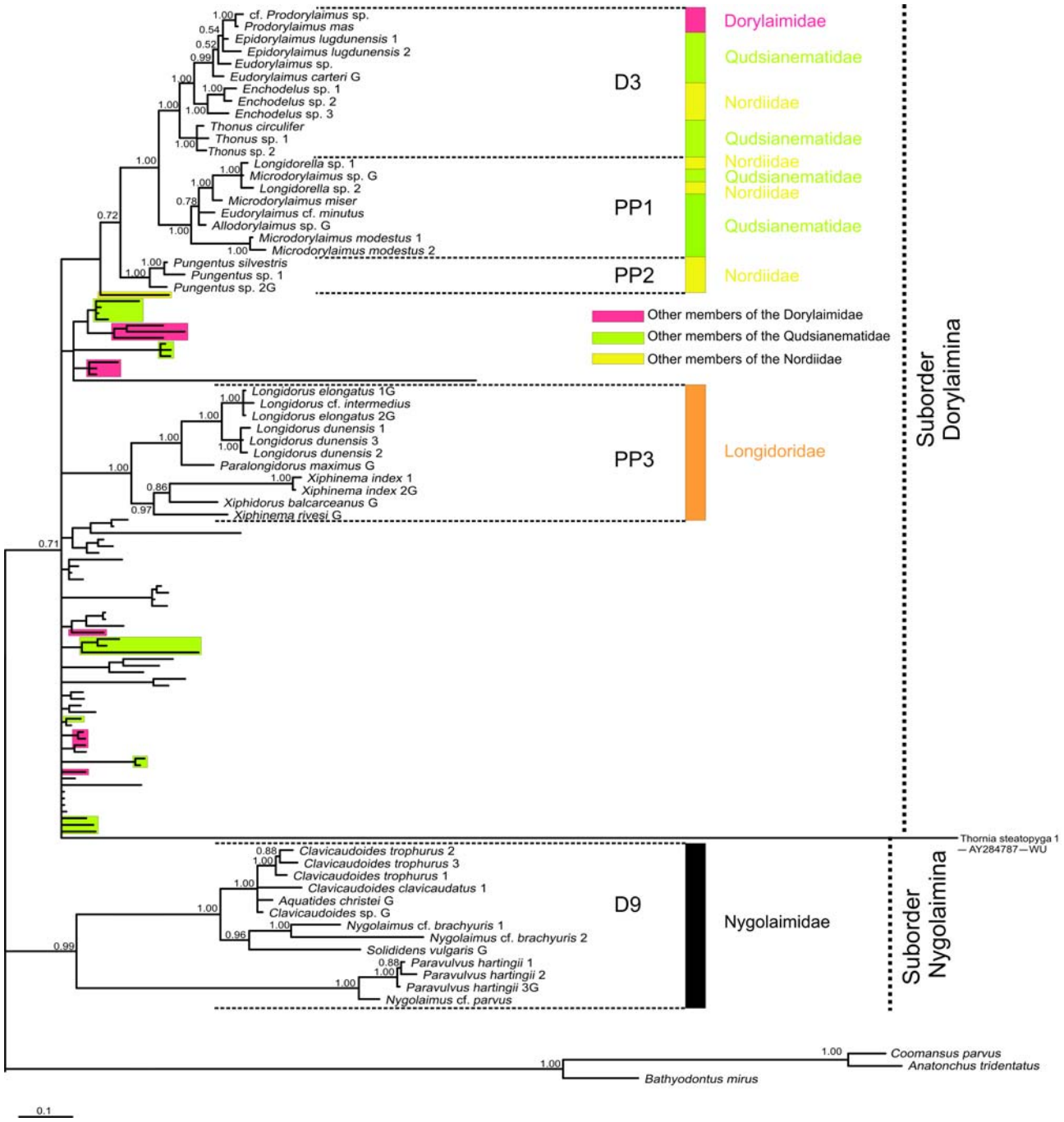

Fig. 2 SSU rDNA-based Bayesian phylogeny of the order Dorylaimida. Numbers near nodes represent posterior probabilities. The coloured bars indicate to which suborder, family and subclade a species belongs. Only species names belonging to well-resolved subclades are given (for detailed phylogenetic tree see Fig. S1). A 'G' behind the species name indicates the sequence was acquired from GenBank.

designed using the software package ARB (Ludwig *et al.* 2004). An alignment of about 1200 full-length SSU rDNA sequences covering a substantial part of the nematode biodiversity in terrestrial and freshwater habitats was used as input file to identify subclade-specific sequence motives. Potential close nontargets were selected by changing the ARB mismatch setting to — at most — four nucleotides. One or two mismatches were always considered as close nontargets 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.

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

Subclade identifier	Primer combination (F, forward; R, reverse)	Close nontargets	Annealing temperature (°C)
M1	F: 5′-CGATCCGTCGGTGTTAAATAT*T R: 5′-CTCG*AGCTGATGACTCGA*A	*Prionchulus punctatus* 1 *Mononchus truncatus* 1 Haliplectus sp. 1 Prismatolaimus dolichurus 2 Pratylenchus pratensis	63
M2	F: 5′-CGCATTTATTAGACCAAAACCAG* R: 5′-TAGAAGACCCAGTTAAACTCCTT*	*Unidentified mermithid species* Mesocriconema xenoplax 1 Malenchus andrassyi 1 Ditylenchus dipsaci 9	64
M3	F: 5′-CGAGCTTCTTAGAGGGACAG* R: 5′-CCAATTCTTACCAGAAAAGGTTTTAA	*Mylonchulus rotundicaudatus* 2** *Opisthodorylaimus sylphoides*** *Granonchulus* sp. 1 Diphtherophora obesa 1 Trischistoma sp. 1** Eumonhystera filiformis 1** Steinernema glaseri1** Prochromadora sp. 1**	65
M4	F: 5′-CGATCCGTCGGTGTTAAG* R: 5′-CCAATTCTTACCAGAAAAGGTTTTAA	*Mylonchulus* sp. 1 *Mylonchulus sigmaturus* 3 *Mylonchulus brachyuris* 2 *Mylonchulus rotundicaudatus* 2 *Mononchus truncatus* 1 *Clarkus papillatus* 1 *Coomansus parvus* 1 *Granonchulus* sp. 1 *Bathyodontus mirus* 1 *Cryptonchus tristis* 1 Prionchulus punctatus 2 Prismatolaimus dolichurus 2 Euteratocephalus sp. 1	62
M5	F: 5′-GACGAAGAATTTTATATGTTTTTTGTG* R: 5′-GGTTGTAAAGCACACTGCTATTC*	*Anatonchus tridentatus* 1 *Granonchulus* sp. 1 *Coomansus parvus* 1	63

*LNA; **starts giving a positive signal in later cycles.

Subclades are defined in Fig. 1, and for each of the subclades, the closest nontargets are shown in Table 1. Primer combinations as presented in Table 1 were tested using cloned SSU rDNA fragments. Bacterial clones harbouring a TOPO TA vector with an SSU rDNA fragment of interest were grown at 37 °C in 2 mL of LB medium supplemented with 100 μg/mL of ampicillin. Plasmid extraction was performed using the Wizard Plus Minipreps DNA Purification System (Promega). DNA concentrations were measured with a NanoDrop spectrophotometer and adjusted to 10 ng/μL. For Q-PCR application 3 μL of 1000× 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 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 s, subclade-specific annealing temperature (Table 1) for 1 min and 72 °C for 30 s.

Results and discussion

Currently, nematode community analysis for ecological soil classification invariably includes time-consuming light microscopy-based identification (mostly till family level) and counting of relatively small samples (typically 75 up

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

Phylogeny of the subclass Dorylaimia

Six orders of the Dorylaimia are represented in our analysis (Figs 1 and 2): Dorylaimida, Mononchida, Mermithida (insect parasites), Trichinellida (animal parasites), Dioctophymatida (animal parasites) and Isolaimida (an order that comprises only a single family with one genus; *Isolaimium*). SSU rDNA sequence data offer a remarkably good resolution within the Mononchida, Mermithida and Trichinellida (Fig. 1), whereas the phylogenetic resolution among representatives of the Dorylaimida was poor (Fig. 2). Addition of sequences from representatives of the Cryptonchidae and the Soboliphymatidae — both positioned basally in two major branches (see Fig. 1) — resulted in intraclade 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 withinorder relationships of Mermithida, Trichinellida, and Dioctophymatida will not be discussed here. The order Isolaimida was placed outside the Dorylaimia, a confirmation of a result that was recently published by Mullin *et al*. (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 harbouring 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. 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 & 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 sister group of *Mylonchulus*, separate from *Coomansus*, *Clarkus* and *Prionchulus* (M2 in Fig. 1). There is morphological support for this separation. Both *Mononchus* and *Mylonchulus* have well-developed tail glands and a (reduced) spinneret (a cuticular cone connected to the caudal glands). On the contrary, *Coomansus*, *Clarkus* and *Prionchulus* (M3 in Fig. 1) have only rudimentary tail glands and no spinneret (Zullini & 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 & 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. 1) are placed at the base of a subclade that includes most Mononchidae and Mylonchulidae. Our analysis suggests that it is probably not possible to define Anatonchidae-specific DNA sequence signatures that would cover all members of the genera *Anatonchus* and *Miconchus*.

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

Dorylaimida — SSU rDNA-based phylogenetic relationships

Within the Dorylaimida, two suborders are distinguished, the Dorylaimina and the Nygolaimina (De Ley *et al*. 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. 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 (≈ 1000 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 three members of the Mononchida. The LSU trees show a better resolution within the Dorylaimida, although a large basal polytomy still remains. The Bayesian tree (Fig. 3), parsimony tree (Fig. S2, Supplementary material) and neighbour-joining tree (Fig. S3, Supplementary material) are nearly identical, and therefore, only the Bayesian tree is depicted here. All parameters of the evolutionary model for the Bayesian tree had converged after a burn-in of 200 000 generations.

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

D1. Being quite heterogeneous from a morphological point of view, D1 shows several general (although not totally common) patterns (Fig. S4, Supplementary material). (i) It groups several long-tailed taxa which currently are dispersed in separate 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. 2). Although these taxa share several characters, none of these characters are unique for this subclade (Fig. S4). These shared characteristics are: (i) guiding ring simple (double in *Enchodelus* and *Prodorylaimus*), (ii) pharynx widening near or slightly behind the middle, (iii) vagina sclerotized, and (iv) tail shape equal in both sexes. Although none of these characteristics is D3-specific by itself, their combination is fairly unique. The only other genus which combines all these four 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. It consists solely of the genus *Pungentus*.

D4. This 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 *et al*. 2005) — and based on these results it seems reasonable to reinstate this family.

D5–D9, PP3. 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

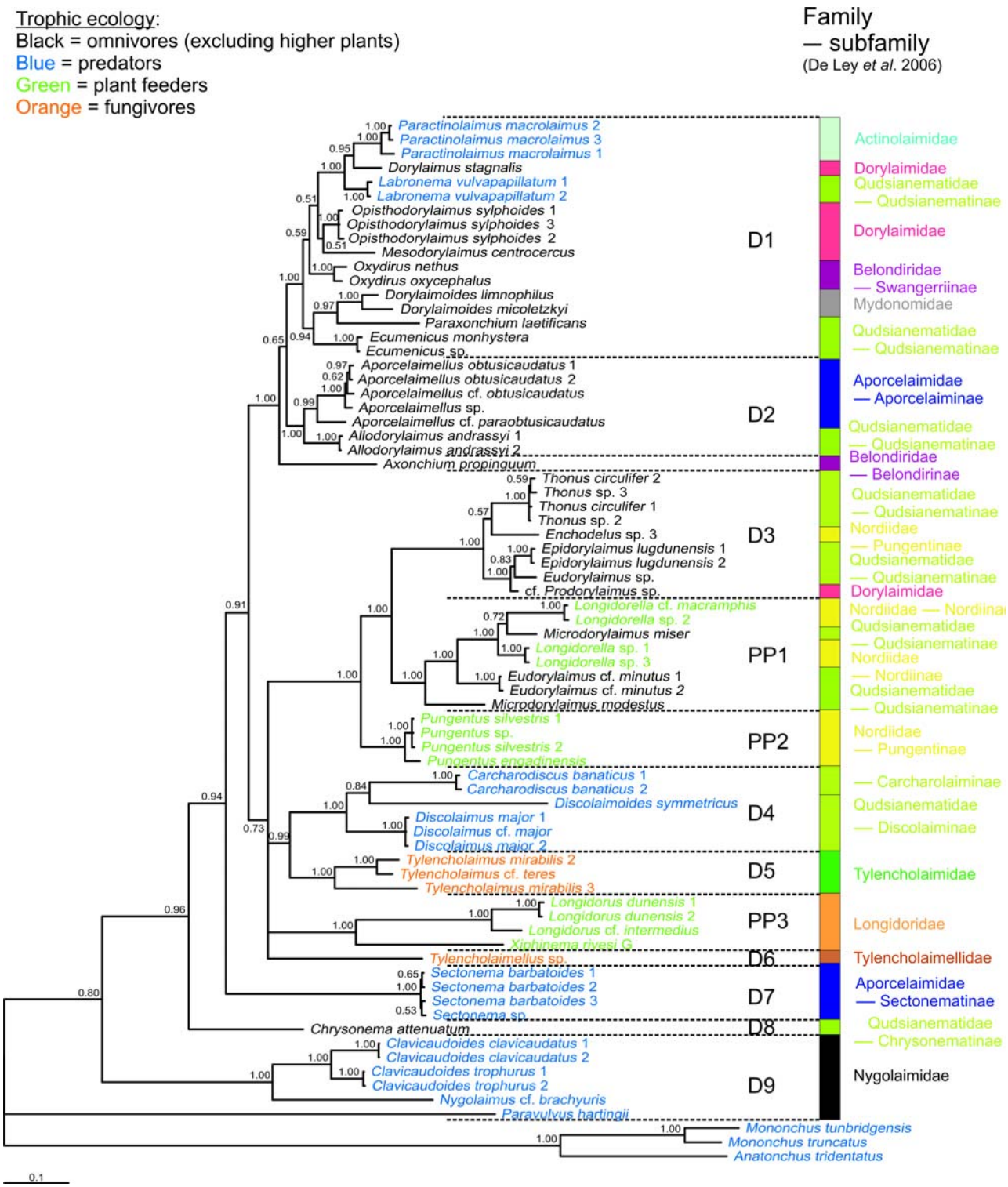

Fig. 3 LSU rDNA-based Bayesian phylogeny of the order Dorylaimida. Numbers near nodes represent posterior probabilities. The coloured bar indicates to which (sub)family a species belongs. The Dorylaimida are divided into 12 subclades (D1–D9, PP1–PP3). The colour of the species name indicates the feeding type (according to Yeates *et al*. 1993).

here by a single LSU rDNA sequence only, corresponds to the Qudsianematidae subfamily Chrysonematinae. D9 covers Nygolaimidae (Nygolaimina) and this subclade could be clearly distinguished on the basis of SSU rDNA sequence data as well (Fig. 2).

The origin of plant parasitism within the order Dorylaimida

Plant parasitism arose at least three times independently during the evolution of the phylum Nematoda, one time within the order Dorylaimida (Longidoridae), and two times in the (infra)orders Triplonchida and Tylenchomorpha (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 1200 full length SSU rDNA sequences from all over the phylum Nematoda) were determined for the five subclades (M1–M5) within the order Mononchida as defined in Fig. 1. On the basis of these signatures, primers were designed that would work under similar annealing temperatures, and these were tested for their specificity (Fig. 4). It is noted that close nontargets as given in Table 1 do not necessarily belong to the Dorylaimia. On the contrary, except for M5, all primer combinations tested were shown to have close nontargets 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. 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 Clades 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 possible uncertainties about specificity by the development of SSU-rDNA-based suborder Dorylaimina-specific primers. In the 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, parallel investigations 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 freshwater nematode communities.

Conclusion

Although the potential of nematodes as indicators for the ecological condition of soil and freshwater sediments is widely recognized (e.g. Bongers & Ferris 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

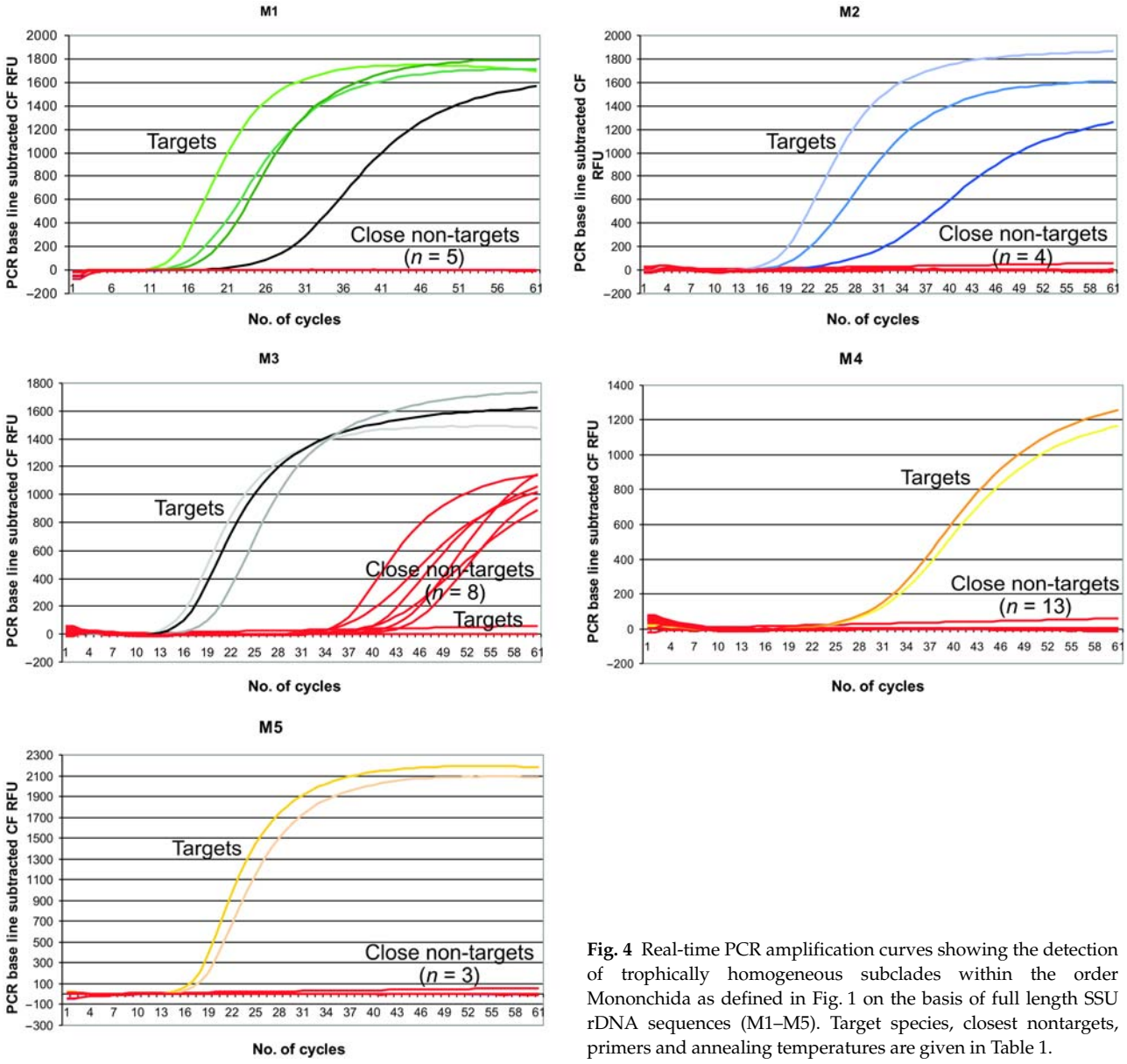

Fig. 4 Real-time PCR amplification curves showing the detection of trophically homogeneous subclades within the order Mononchida as defined in Fig. 1 on the basis of full length SSU rDNA sequences (M1–M5). Target species, closest nontargets, primers and annealing temperatures are given in Table 1.

tool as presented here offers great perspectives for the exploitation of this group as it lifts — at least in part — the need for specialist taxonomic expertise, detects all developmental stages (instead of — mainly — adults), facilitates the analysis of relatively large and numerous soil and/or sediment samples, and greatly reduces the sample-handling time.

Acknowledgements

This work was supported by the Dutch Technology Foundation (STW) grant WBI4725. Further support was received from SenterNovem, an agency of the Dutch Ministry of Economic Affairs (IS043076).

References

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

Anderson DT (2001) *Invertebrate Zoology*, 2nd edn. Oxford University Press, New York.

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

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

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

Bongers T (1994) *De nematoden van Nederland*, 2nd edn. Koninklijke Nederlandse Natuurhistorische Vereniging, Utrect, The Netherlands.

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

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

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

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

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

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

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

Dmowska E, Ilieva-Makulec K (2004) Past and present status of nematode community indicators. In: *Proceedings of the Fourth International Congress of Nematology, 8–13 June 2002, Tenerife, Spain* (eds Cook R, Hunt DJ), pp. 487–501. Brill, Leiden, The Netherlands.

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

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

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

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

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

Jairajpuri MS, Ahmad W (1992). *Dorylaimida*. Brill, Leiden, The Netherlands.

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

Maddison DR, Maddison WP (2000) *MACCLADE 4: Analysis of Phylogeny and Character Evaluation*. Sinauer Associates, Sunderland, Massachusetts.

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

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

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

Premachandran D, Vonmende N, Hussey RS, McClure MA (1988) A method for staining nematode secretions and structures. *Journal of Nematology*, **20**, 70–78.

Rambaut A, Drummond A (2005) TRACER version 1.2.1. Available at http://evolve.zoo.ox.ac.uk/.

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

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

Siddiqi MR (1969) Crateronema n. gen. (Crateronematidae n. fam.) Poronemella n. gen. (Lordellonematinae n. subfam.) and Chrysonemoides n. gen. (Chrysonematidae n. fam.) with a revised classification of Dorylaimoidea (Nematoda). *Nematologica*, **15**, 81–&.

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

Trudgill DL (1976) Observations on feeding of *Pungentus thornei* on roots of *Petunia hybrida*. *Plant Pathology*, **25**, 117–&.

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

Yeates GW (1987) Nematode feeding and activity — the importance of development stages. *Biology and Fertility of Soils*, **3**, 143–146.

Yeates GW, Bongers T, De Goede RGM, Freckman DW, Georgieva SS (1993) Feeding-habits in soil nematode families and genera — an outline for soil ecologists. *Journal of Nematology*, **25**, 315–331.

Zullini A, Peneva V (2006) Order Mononchida. In: *Freshwater Nematodes: Ecology and Taxonomy* (eds Abebe E, Traunspurger W, Andrassy I), pp. 468–496. CABI Publishing, Wallingford, UK.

Supplementary material

The following supplementary material is available for this article:

Table S1 GenBank Accession numbers of SSU sequences.

Table S2 GenBank Accession numbers of LSU sequences.

Fig. S1 Complete Bayesian SSU tree Dorylaimida

Fig. S2 Maximum parsimony LSU tree

Fig. S3 Neighbour-joining LSU tree

Fig. S4 Character tracing Dorylaimida LSU tree

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