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Assessing the diversity and spatial distribution of nematodes in the Store Mosse National Park (Sweden) using metabarcoding

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Abstract

Nematode taxa of the Store Mosse National Park in the south of Sweden were surveyed using DNA metabarcoding. Samples were collected from a range of media across all the five vegetation types the park spans. A total of 50 samples consisting of soil, litter, lichens, sphagnum, roots, wood, moss, fungus and anthill materials were analysed. Nematodes were characterised using a ~350 bp region of their 18S ribosomal RNA gene that include V7 and V8 variable domains. The analysis identified 47 families, 76 genera (21 new to Swedish fauna) and 60 species (31 new to Swedish fauna). Some nematodes showed a strong association with certain medium types, especially at the species level. The results showed a strong justification for our strategy of sampling different medium types. Soil and litter communities, which were the most diverse, showed high levels of stability with good balance of all the various trophic and coloniser-persister groups.

Keywords

litter, molecular marker, national park, Nematoda, soil, vegetation

Introduction

Nematodes represent a highly species-rich group that occur across a wide range of habitats with astonishing abundance (Holterman et al., 2006; van den Hoogen et al. 2019). There exists a great amount of morphological, genomic and functional diversity among nematodes, allowing them to play diverse roles in the ecosystem. The variety of ways in which they respond to conditions of their environment is well documented and constitutes an important area of nematological research (Bongers 1990). In fact, local nematode communities often reflect the prevailing physical, climatic, biogeographical and chemical conditions of their environment (Cobb 1915; Yeates 1984; Neher 2001). Because of this, nematodes have been used as effective biological entities for assessing the conditions of their environment. This is typically accomplished through morphological identification of individual nematodes within the community to the levels of family or genus which are subsequently assigned to functional groups. However, morphological identification of bulk nematode samples is time-consuming and requires expertise, the availability of which has been on a decline for some years now (Coomans 2002). On a small scale of a few samples, this can easily be carried out by a skilled taxonomist or with the help of a good identification key, by a keen observer of morphological characters to at least the family level. For survey studies involving tens to hundreds of samples, however, analysing and characterising nematodes in each sample quickly becomes an almost insurmountable undertaking. Molecular identification methods provide faster and more accurate alternatives that require very little to no taxonomic expertise and can be easily automated (Blok 2005; Ahmed et al. 2016). Most popular among these was DNA barcoding, which involves the use of a targeted DNA region for discriminating species (Floyd et al. 2002; Blaxter 2003; Hebert et al. 2003; Blaxter et al. 2005). Early studies on DNA barcoding using the Sanger sequencing approach were constrained in their throughput – the number of species they can identify within a given time or sequence run is limited (Creer et al. 2010). For this reason, they have very limited practical application for assessing nematode communities. This limitation was finally overcome with the advent of next-generation sequencing (NGS) technology (Creer et al. 2010; Taberlet et al. 2012).

One exciting concept that emerged because of NGS technology is DNA metabarcoding. Metabarcoding involves the use of a standardised, typically short genomic region to characterise organisms from bulk samples. This approach has revolutionised the way we tackle questions related to biodiversity assessment (Guardiola et al. 2015). With its promise of providing a speedy method of assessing community structure, metabarcoding quickly saw wide adoption across many fields of meiofaunal studies (Porazinska et al. 2010a; Bik et al. 2012). Within nematology, most of the earlier studies on metabarcoding sought to evaluate the performance of different genomic regions and to establish robust pipelines for analysing bulk nematode samples (Porazinska et al. 2009; Creer et al. 2010). Porazinska et al. (2009) assessed the suitability of metabarcoding for nematode community analysis and established a benchmark for future nematode metabarcoding studies. The authors used “mock” nematode communities to test the ability of the markers (NF1/18Sr2b of the SSU rDNA, D3Af/D3Br of the LSU rDNA) to recover the sampled taxa in

these mock communities. The SSU rDNA-based primer they described was well regarded for its wide taxonomic coverage (Ahmed et al. 2019). While very useful in samples containing mostly the targeted group, for samples with mixed taxa including non-targets, the broad taxonomic coverage of these primers proved to be an issue. Because of this, there have recently been several attempts to develop alternative primers within the nuclear rDNA (Sapkota & Nicolaisen 2015; Waeyenberge et al. 2019; Kenmotsu et al. 2020; Sidker et al. 2020; Kawanobe et al. 2021). Similar benchmarking studies have been carried out on nematodes in freshwater and marine habitats (Holovachov et al. 2017; Macheriotou et al. 2019; Schenk et al. 2020).

An aspect of metabarcoding that has been the subject of several mock community studies concerns the utility of sequence abundance information for inferring species abundance (Amend et al. 2010; Porazinska et al. 2010a; Elbrecht & Leese 2015; Thomas et al. 2016; Lamb et al. 2019). Most of these studies have reported varying degrees of divergence between taxa abundance and read frequencies. In other words, the most abundant taxa do not often give the most reads and vice versa. Comparing biomass, instead of abundance, to sequence read frequency, however, has been shown to result in a better correlation for multiple marker regions (Schenk et al. 2020). Several factors have been implicated as leading causes of this bias. These include primer mismatch, biomass or size difference between individuals, DNA extraction bias where cuticles of some taxa hamper efficient tissue lysing, PCR bias, and for repetitive regions, copy number differences (Amend et al. 2010; Bik et al. 2013; Deagle et al. 2013). Quality filtering of reads during the bioinformatic analysis step can also contribute to the discordance between sequence read frequencies and taxa abundance. Attempts at mitigating this issue have taken different forms. In diatoms, for example, a correction factor based on cell biovolume has been applied to minimise the magnitude of the deviation between sequence reads abundance and taxa abundance (Vasselon et al. 2018). Methods that involve gene enrichment and no PCR amplification have also been suggested to eliminate all PCR-associated bias (Zhou et al. 2013). Using this approach in a study on freshwater macroinvertebrates, Dowle et al. (2016) were able to obtain a strong correlation between biomass and read abundance. In addition to being more accurate at quantifying taxa, this approach may be the way to recover taxa which cannot be amplified by the current primer set. The constraint here, however, is the increased cost and workload that may come with this method (Krehenwinkel et al. 2017). The use of presence-absence data as a safe and reliable substitute for abundance data is common. Here, since taxa occurrence and not read counts of taxa are used, read proliferation due to PCR bias does not have any effect. Of course, the biggest limitation to this approach is it ends up inflating the influence of very rare taxa and ignoring the fact that some of the differences in sequence read numbers have real biological bases (Deagle et al. 2019). Moreover, most nematode community indices can only be applied with data on abundance and hence have limited use for presence-absence data. Despite the substantial efforts in resolving the issue of abundance, there is currently no practical approach to predicting abundance from the read abundance. But does this suggest that read abundance data have no use at all? According to Deagle

et al. (2019), in spite of their sensitivity to recovery bias, relative read frequencies provided a more accurate representation of the population level diversity, compared to presence/absence data.

As in other fields, metabarcoding has seen various applications in studying nematode biodiversity. Porazinska et al. (2010b) were among the first to apply metabarcoding to study the diversity of nematodes. Using metabarcoding, they examined the diversity of nematodes within different habitats, soil, litter and canopy of Costa Rica. They concluded that nematode diversity was higher in tropical regions than in temperate regions, contrasting a previously held notion that suggested the contrary. As a follow-up to this study, the main authors published another paper where they demonstrated that nematode species richness in the tropical rainforest was three times more than it is in temperate rainforest, thus further supporting their earlier finding (Porazinska et al. 2012). In their contribution to understanding the latitudinal differences in the diversity of nematodes, Kerfahi et al. (2016) also used a metagenetic approach to examine nematodes from the tropical rainforest of Malaysia and the arctic tundra of Svalbard. They observed no difference between the two ecosystems. The fact that only soil samples were used in their analysis may explain this observed difference with Porazinska et al. (2010b; 2012). It is often rare in applied studies to have both morphological and metabarcoding used complementarily, but Treonis et al. (2018) combined the two approaches in a study on nematode communities under different cropping systems. Their results indicated that metabarcoding provided better resolution beyond the family level, recovering families that were not detected with morphological analysis. Metabarcoding also resulted in under- or overestimating the prevalence of some nematode families.

A strong consensus exists across most studies regarding the ability of metabarcoding to recover more taxa and reveal deeper taxonomic resolution when compared to the traditional morphological approach. We aim to leverage this by using metabarcoding to reveal the extent of nematode diversity within the Store Mosse National Park in the south of Sweden. To our knowledge, this is the first study into the nematode diversity of this park. The park spans five different vegetation types and at least eight different soil types. We sampled across all five vegetation types, with some more heavily sampled than others because they were easily accessible. To better capture the diversity of nematodes, we collected not just mineral soil, but other media such as litter, lichens, sphagnum, roots, decomposed wood, moss, fungus and samples from below anthills. Sampling across vegetation types or medium types was not carried out evenly. Therefore, the number of samples varied across these two variables. Our goal with this study was also to use the nematode community structure to infer the status of the different areas of the park, in terms of how pristine or perturbed they are. Given its protected nature, we hypothesise that conditions will generally incline toward the former.

Material and methods

Study site

All samples were collected within the Store Mosse National Park, located in the traditional province of Småland or present-day county of Jönköping situated in the southern part of Sweden (Fig. 1). Store Mosse is one of the largest bog complexes in southern Sweden (Martínez Cortizas et al. 2021), covering approximately 7,682 hectares, most of which is wetlands. The bog consists mostly of high swamps with a few areas of open swamps (Naturvårdsverket 2015). Standing between 160–170 m above sea level, the area records an annual average of +6ºC of temperature and 766 mm of precipitation (Kylander et al. 2013). Store Mosse became a national park in 1982 following a long campaign initiated by Prof. Edvard Wibeck for the area to be protected. The peat layers covering its high swamps are believed to have accumulated over a period of nearly 10,000 years (Ryberg et al. 2022).

Sample collection and processing

Sampling was carried out in 2021 over two days, the 13th and 14th of October. Samples were collected from 50 spots across all vegetation types in the Store Mosse National Park (Figs 1, 2, Table 1). Samples consisted of nine different types of media (Table 2). These diverse types of media were chosen to capture even those taxa found only in certain specific environments, and not in the commonly sampled mineral soil. At the same time, all samples were collected not too far from and along the roads and trails, in order to minimise our impact on undisturbed habitats. Whenever possible, samples were collected using a corer with inner diameter of 16 mm, collecting 100 ml for each sample. Samples were then stored at 6ºC until extraction. Dense samples were manually disintegrated prior to extraction in order to facilitate nematode isolation. Nematodes were extracted from 100 ml of media using the Whitehead tray method (Whitehead and Hemming 1965). The set-up was taken down after 48 hours and the extracts were collected in water suspensions. Through a series of centrifugation, the suspensions were reduced to volumes of about 50 µl inside microcentrifuge tubes. Each sample at this stage contained, in about 50 µl volume of suspension, total assemblage of individuals of different taxa obtained from the extraction. The reduced volume of suspension was used to limit the chances of non-metazoan eukaryotes becoming dominant in the samples.

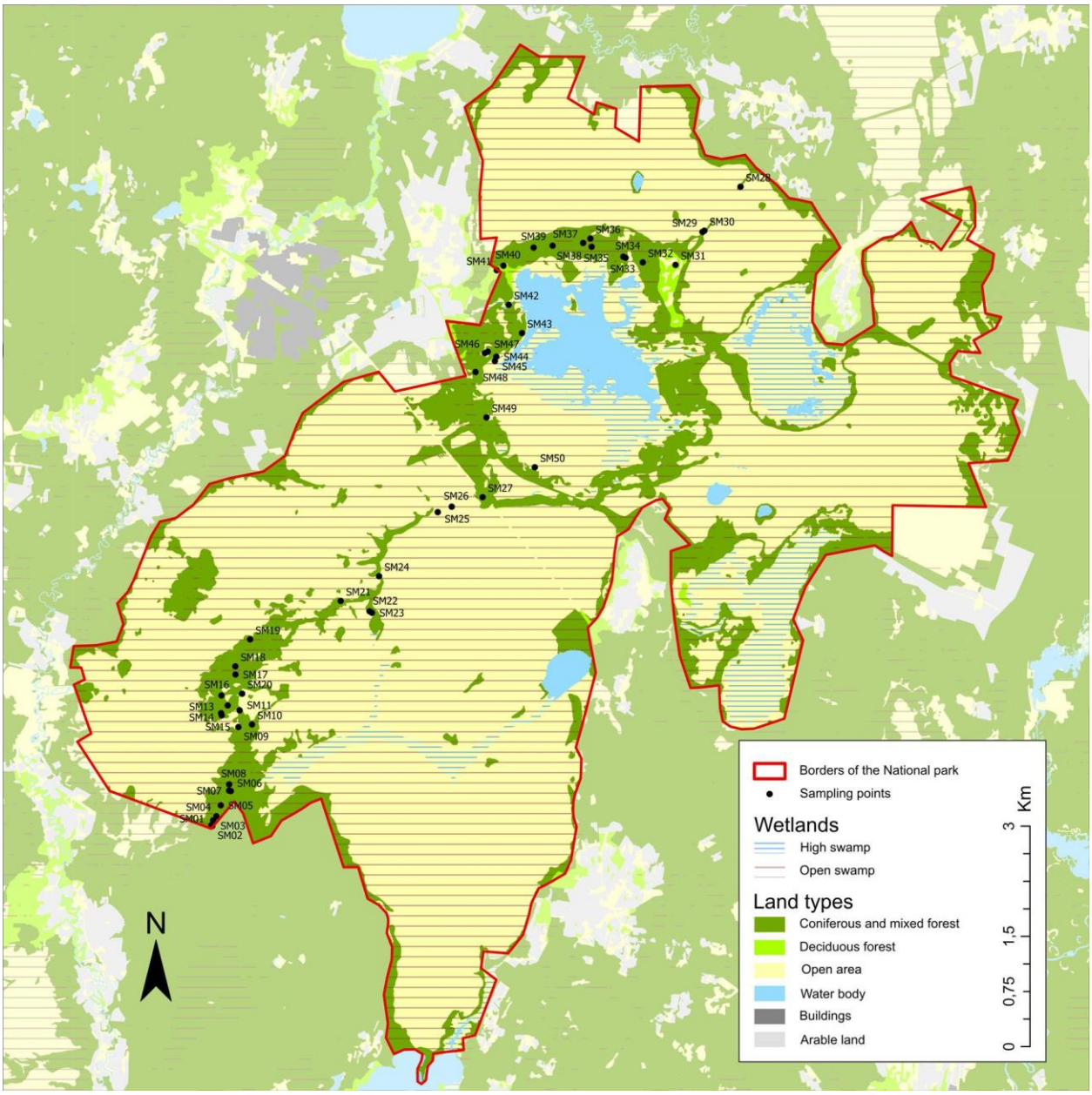

Figure 1. Map of Store Mosse National Park (Sweden) showing all sampling points and the different vegetation covers.

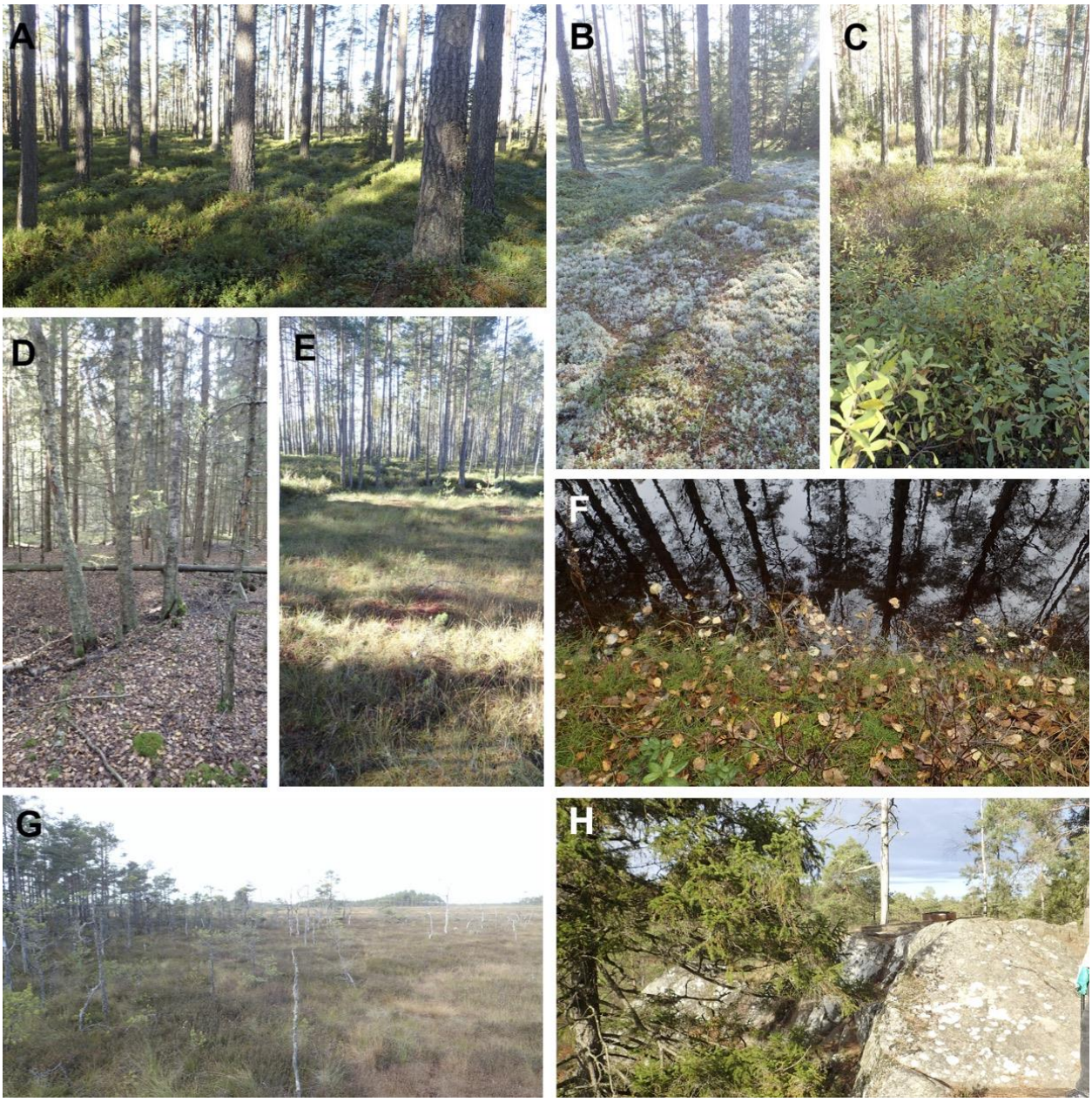

Figure 2. Images showing the different habitats where samples were collected. A. Lingonberries vegetation under a Pine forest. B. Pine forest with lichen and moss ground cover. C. Pine forest with lingonberries, sphagnum and other bushes as ground cover. D. Fir and birch forest with litter covering the ground surface. E. Open area within a pine forest covered with sedge ground cover. F. The bank of an artificial channel within a coniferous forest. G. Grassland with lower sphagnum cover. H. Granite outcrops.

Table 1. Sampling data.

Sample	GPSLatitude	GPSLongitude	Vegetation	Lower vegetation	Medium
SM01	57°14'50.66"N	13°52'15.58"E	Coniferous forest on dry land	Blueberries	litter
SM02	57°14'50.30"N	13°52'16.46"E	Coniferous forest on dry land	Blueberries	litter
SM03	57°14'52.84"N	13°52'17.03"E	Coniferous forest on dry land	Lingonberries	litter
SM04	57°14'54.68"N	13°52'19.58"E	Coniferous forest on dry land	Lingonberries	litter
SM05	57°14'59.48"N	13°52'23.02"E	Coniferous forest on dry land	Lingonberries	soil
SM06	57°15'5.83"N	13°52'30.97"E	Coniferous forest on dry land	Lingonberries	soil
SM07	57°15'6.01"N	13°52'29.57"E	Coniferous forest on dry land	N/A	lichens
SM08	57°15'8.77"N	13°52'29.54"E	Coniferous forest on high swamp	Blueberries	soil
SM09	57°15'34.06"N	13°52'36.29"E	High swamp	Grass	sphagnum
SM10	57°15'35.27"N	13°52'47.30"E	Coniferous forest on dry land	None	litter
SM11	57°15'41.39"N	13°52'37.03"E	Coniferous forest on high swamp	Blackberries	litter
SM12	57°15'43.47"N	13°52'27.28"E	Coniferous forest on dry land	Lingonberries	litter
SM13	57°15'40.00"N	13°52'22.00"E	Coniferous forest on dry land	Juncus/Carex	roots
SM14	57°15'40.57"N	13°52'23.06"E	Coniferous forest on dry land	N/A	lichens
SM15	57°15'39.04"N	13°52'22.61"E	Coniferous forest on high swamp	N/A	wood
SM16	57°15'47.83"N	13°52'22.07"E	Coniferous forest on dry land	none	litter
SM17	57°15'57.15"N	13°52'33.17"E	Coniferous forest on dry land	Blackberries	fungus
SM18	57°16'0.75"N	13°52'32.96"E	Coniferous forest on high swamp	N/A	wood
SM19	57°16'12.72"N	13°52'44.62"E	Coniferous forest on high swamp	Lingonberries	litter
SM20	57°15'48.75"N	13°52'38.81"E	Coniferous forest on dry land	Grass	soil
SM21	57°16'30.28"N	13°53'57.63"E	Coniferous forest on dry land	Grass	sphagnum
SM22	57°16'25.93"N	13°54'21.23"E	Coniferous forest on high swamp	Calluna	litter
SM23	57°16'25.30"N	13°54'23.15"E	Coniferous forest on dry land	N/A	moss
SM24	57°16'41.43"N	13°54'28.43"E	Coniferous forest on dry land	N/A	litter
SM25	57°17'10.01"N	13°55'15.29"E	High swamp	Crow berry	litter
SM26	57°17'12.49"N	13°55'26.57"E	High swamp	Crow berry	litter
SM27	57°17'16.91"N	13°55'51.50"E	Coniferous forest on dry land	Lycopodium	soil
SM28	57°19'35.22"N	13°59'17.21"E	Coniferous forest on high swamp	Crow berry	litter
SM29	57°19'15.11"N	13°58'46.81"E	High swamp	Carex	litter
SM30	57°19'15.62"N	13°58'48.33"E	High swamp	N/A	wood
SM31	57°19'0.41"N	13°58'25.33"E	Deciduous forest	Grass	soil
SM32	57°19'1.31"N	13°57'58.73"E	Coniferous forest on high swamp	Cranberries	litter
SM33	57°19'3.24"N	13°57'44.68"E	Coniferous forest on high swamp	Equisetum	soil
SM34	57°19'3.68"N	13°57'42.74"E	Coniferous forest on high swamp	N/A	anthill
SM35	57°19'7.82"N	13°57'16.96"E	Coniferous forest on high swamp	Blackberries	moss
SM36	57°19'11.44"N	13°57'15.84"E	Coniferous forest on high swamp	Blackberries	wood
SM37	57°19'9.45"N	13°57'9.97"E	Coniferous forest on dry land	N/A	anthill
SM38	57°19'8.03"N	13°56'45.18"E	Coniferous forest on dry land	Blackberries	litter
SM39	57°19'7.09"N	13°56'29.78"E	Coniferous forest on dry land	Blackberries	fungus

SM40	57°18'58.91"N	13°56'5.45"E	Deciduous forest	Grass	soil
SM41	57°18'56.86"N	13°56'0.07"E	Deciduous forest	N/A	lichens
SM42	57°18'41.73"N	13°56'10.24"E	Open swamp	Semiaquatic plants	soil
SM43	57°18'29.40"N	13°56'21.44"E	Coniferous forest on dry land	N/A	fungus
SM44	57°18'18.85"N	13°56'0.85"E	High swamp	N/A	moss
SM45	57°18'16.89"N	13°55'59.73"E	High swamp	N/A	wood
SM46	57°18'20.36"N	13°55'51.59"E	Coniferous forest on high swamp	N/A	soil
SM47	57°18'20.98"N	13°55'53.82"E	Coniferous forest on high swamp	N/A	soil
SM48	57°18'11.92"N	13°55'44.20"E	Coniferous forest on dry land	Blackberries	moss
SM49	57°17'52.02"N	13°55'53.66" E	High swamp	N/A	wood
SM50	57°17'30.45"N	13°56'33.55"E	High swamp	Grass	litter

Table 2. Summary of vegetation covers of sampled sites and the types of media collected.

Medium	Vegetation				
	Coniferous forest on dry land	Coniferous forest on high swamp	Deciduous forest	High swamp	Open swamp
Litter	SM1, SM2, SM3, SM4, SM10, SM12, SM16, SM24, SM38	SM11, SM19, SM22, SM28, SM32		SM25, SM26, SM29, SM50	
Soil	SM5, SM6, SM20, SM27	SM8, SM33, SM46, SM47	SM31, SM40		SM42
Lichens	SM7, SM14		SM41		
Sphagnum	SM21			SM9	
Roots	SM13				
Decomposed wood		SM15, SM18, SM36		SM30, SM45, SM49	
Moss	SM23, SM48	SM35		SM44	
Fungus	SM17, SM39, SM43				
Anthill	SM37	SM34			

DNA Extraction, PCR and NGS

Genomic DNA extraction was performed on each sample using the Qiagen QiAmp DNA Micro kit. Briefly, 130 µl of ATL buffer was added to each sample (50 µl), followed by the 20 µl of proteinase K. The mixture was then vortexed and incubated overnight in an incubating microplate shaker at 56 ºC and 300 rpm. Pure DNA was isolated from the lysed samples following the manufacturer's instructions for genomic DNA extraction for blood and tissue samples using the Qiagen QiAmp DNA Micro kit. The PCR primers used were the NF1 (5 'GGTGGTGCATGGCCGTTCTTAGTT 3', matching the 5' end of the 38th helix) and 18Sr2b (5' TACAAAGGGCAGGGACGTAAT 3', matching the 3' end of the 32nd helix) (Porazinska et al.

2009), with Illumina adapter sequences ligated at their 5' ends. Amplification was performed in a 25 µl reaction mixture using Illustra Hot Start Mix RTG0.2 ml reaction kit (GE Healthcare Life Sciences, Sweden). The reaction mixture consisted of 1 µl (0.4 µM) of each primer, 2 µl of template DNA and 21 µl of nuclease-free water. The cycle conditions set were as previously described in Ahmed et al. (2019). Following the initial PCR reaction, the amplicons were all purified using Agencourt AMPure XP (Beckman Coulter, California, United States). The purified products were sent to Macrogen Europe B.V. (Amsterdam, the Netherlands) for subsequent library preparation and NGS. In brief, index PCR was performed where unique indices were attached to amplicons of each sample. Library size distribution was checked by running on Agilent Technologies 2100 Bioanalyzer using a DNA 1000 chip. Quantification of the library was performed using qPCR according to the Illumina qPCR quantification protocol guide. The samples were then multiplexed, before Illumina MiSeq 2x300 bp sequencing. Raw data is available at NCBI Sequence Read Archive under the BioProject ID PRJNA923582.

Bioinformatics

Analysis of the raw NGS data was performed using a 64-bit USEARCH v11.0.667 (Edgar 2010), licensed to the senior author (academic non-profit licence). Raw reads were merged using the *-fastq_mergepairs* command (options: the minimum length of overlap between the forward and reverse reads was set to 150 bp, the maximum number of mismatches within the overlapping region set to 10, the minimum percentage identity at the overlapping region set to 80). Following this, the merged reads were filtered using the usearch command *-fastq_filter* (options: maximum expected error per sequence was set to 1, minimum length of the sequences set to 250). Using the *-fastx_uniques* command, the remaining reads were reduced to unique sequences in order to speed up the clustering step. The output file consisted of unique sequences, each with its size appended to its description line. This was followed by clustering using the UNOISE algorithm, as implemented in the *-unoise3* command, to obtain amplicon sequence variants (ASVs), also referred to as zero-radius operational taxonomic units (ZOTUs) by Edgar (2016). The use of ASVs has been shown to recover a higher number of correct biological sequences than the UPARSE-OTU algorithm. All parameters for the clustering command were set to their default values. This included a *-minsize* value of 8 which ensured that only ZOTUs with an abundance of 8 or higher were retained. Low *-minsize* values tend to introduce more errors in the predicted low-abundance biological sequences (see https://www.drive5.com/usearch/manual/cmd_unoise3.html). The usearch command *-otutab* was then used to create a ZOTU table, a table with ZOTUs shown as records, and their read frequencies in each sample shown in separate fields. Using the *-sintax* command and 18S rDNA sequences from the PR2 database version 4.14.0 (Guillou et al. 2013) as reference, the ZOTUs were assigned taxonomy. A pattern matching script was used to extract ZOTUs matching Nematoda from the sintax taxonomy assignment. These ZOTUs were then searched for and extracted from the ZOTU sequences and ZOTU table. The extracted nematode ZOTUs were then assigned a taxonomy, this time using a custom high-quality nematodes-only curated database with more accurate taxonomies based on the classification of De Ley and Blaxter

(2004). Initially, the high posterior probability score cut-off set for the sintax taxonomy resulted in ZOTUs associated with Dorylaimidae and Qudsianematidae failing to be assigned to the correct families. Blast search results were therefore used to complement the sintax taxonomy. A phylogenetic tree of the nematodes was generated for mafft-aligned (Katoh and Standley 2013) ZOTUs using FastTree (Price et al. 2010). All parameters were left at their default settings for mafft alignment, and for FastTree analysis, the 'gtr' model was used. The ZOTU table, taxonomy file, phylogenetic tree and sample metadata file (data on the vegetation type, medium, soil type etc for each sample) were exported into R for further statistical analyses.

Statistical analyses

Computation of community indices, CP (coloniser-persister) groupings and feeding group designations were performed using NINJA (Sieriebriennikov et al. 2014; https://shiny.wur.nl/ninja/), an online tool for nematode faunal analyses. Taxa not recognized by NINJA were replaced by their closest relative acceptable to NINJA or where possible replaced by a higher-ranking taxon (e.g. *Basilaphelenchus* replaced by Aphelenchoididae). Abundance information was based on sequence read counts for each taxon in a sample. For comparisons, samples were categorised in terms of the different vegetation types and the different media from which the nematodes were collected. All analyses were carried out using R version 4.0.5 (R Core Team 2021) inside RStudio (RStudio Team 2022). Alpha diversity within the vegetation types and the medium types were computed based on Chao1 diversity index using the phyloseq package McMurdie and Holmes (2013).

Results

General statistics

A total of 7,040,489 paired reads were generated. On average 85% of the paired reads were successfully merged per sample. Following filtering, 5,943,062 reads were left. Clustering using UNOISE and setting the *-minsize* parameter to 8 resulted in a total of 2,569 ZOTUs. The sintax algorithm assigned 31.8% (899 in total) of the ZOTUs to Nematoda, 18.7% were unassigned, and the remainder were assigned to other eukaryotic lineages (Fig 3). By design, each taxonomic rank assignment in the sintax output is accompanied by a posterior probability value, which indicates the statistical support for that particular assignment. For this analysis, only assignments with posterior probability scores of >=0.8 were considered. The primer pair used for the DNA amplification has been shown to demonstrate a wide taxonomic coverage–capable of amplifying even Archaean DNA. However, because nematodes were first isolated from the medium most of the non-target DNA were excluded, that otherwise would have dominated the samples had DNA extraction been performed directly on the sample medium. Furthermore, getting rid of most of the suspension by concentrating the samples to about 50 µl ensured that the incidence of fungal DNA was significantly suppressed. The nematodes extraction method used also recovered a noticeable

number of tardigrades, platyhelminths, rotifers and arthropods (Fig. 3), which, however, were not analysed in details.

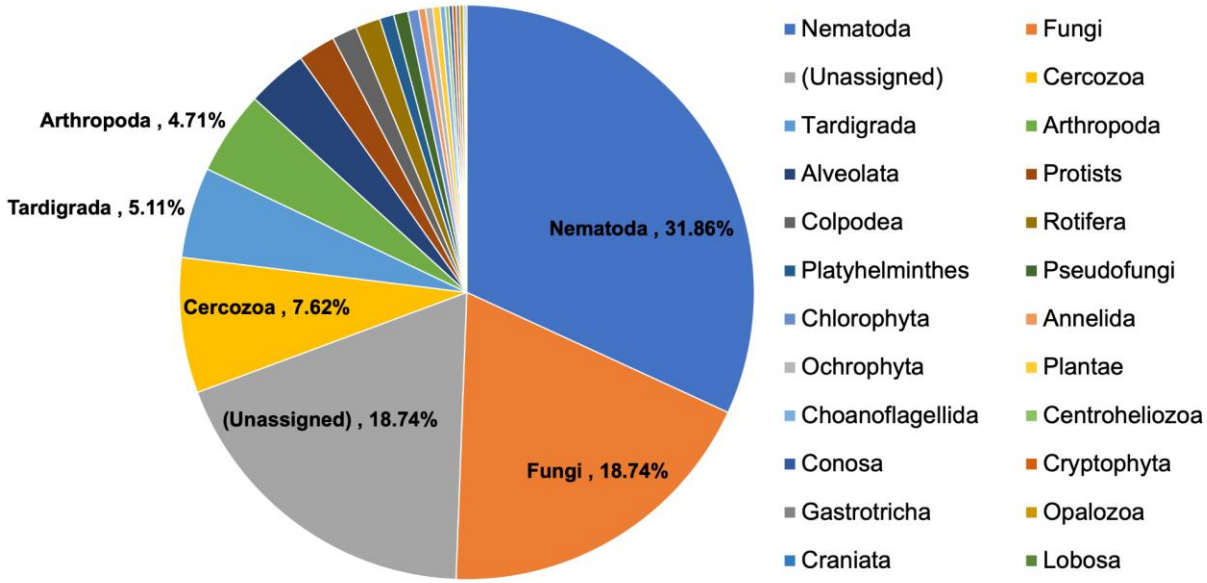

Figure 3. Relative abundance of ZOTUs associated with major groups of eukaryotes in all 50 samples combined, including ZOTUs that were not assigned to any group of eukaryotes.

Nematode diversity

The analysis recovered 46 nematode families in total, with Tylenchidae represented by the greatest number of ZOTUs (Fig. 4). At the genus level, the percentage of nematode ZOTUs that were assigned taxonomy with enough support averaged about 40% and in some samples was as high as 78%. Across the 47 families recovered, there were 76 genera identified in the analysis (Table 3). The most represented families were Aphelenchoididae (7 genera), Rhabditidae (8 genera) and Tylenchidae (11 genera). Most of the families had more than one genus present. Identification to the species level was accomplished for 46 of the genera. Sixty nematode species were identified in total representing all five trophic groups. *Aphelenchoides* and *Malenchus* were the most diverse genera with four species associated with both. Among these 60 identified species, 31 are new to the fauna of Sweden (Dyntaxa): *Acrobeloides varius, Aglenchus agricola, Aphelenchoides blastophthorus, Aphelenchoides heidelbergi, Baldwinema ardabilense, Cephalenchus hexalineatus, Choriorhabditis cristata, Crassolabium circuliferum, Diploscapter coronatus, Ditylenchus adasi, Ecphyadophora tenuissima, Filenchus facultativus, Helicotylenchus pseudorobustus, Hexatylus viviparus, Irantylenchus vicinus, Laimaphelenchus penardi, Malenchus bryanti, Malenchus neosulcus, Malenchus pressulus, Miculenchus muscus, Oscheius*

dolichura, *Paravulvus hartingii*, *Paurodontella gilanica*, *Potensaphelenchus stammeri*, *Tylencholaimus teres*, *Tylencholaimus zhongshanensis*, *Tylenchorhynchus parvulus*, *Tylenchus arcuatus*, *Tylenchus naranensis*, *Tylolaimophorus typicus*, *Veleshkinema iranicum*. Similarly, the following 21 genera have not been reported in Sweden until now (Dyntaxa): *Baldwinema*, *Basilaphelenchus*, *Choriorhabditis*, *Crassolabium*, *Diploscapter*, *Discotylenchus*, *Ektaphelenchoides*, *Heterorhabditis*, *Hexatylus*, *Irantylenchus*, *Laimaphelenchus*, *Miculenchus*, *Neodolichorhynchus*, *Oscheius*, *Paravulvus*, *Paurodontella*, *Poikilolaimus*, *Potensaphelenchus*, *Rhabditophanes*, *Schistonchus*, *Veleshkinema*.

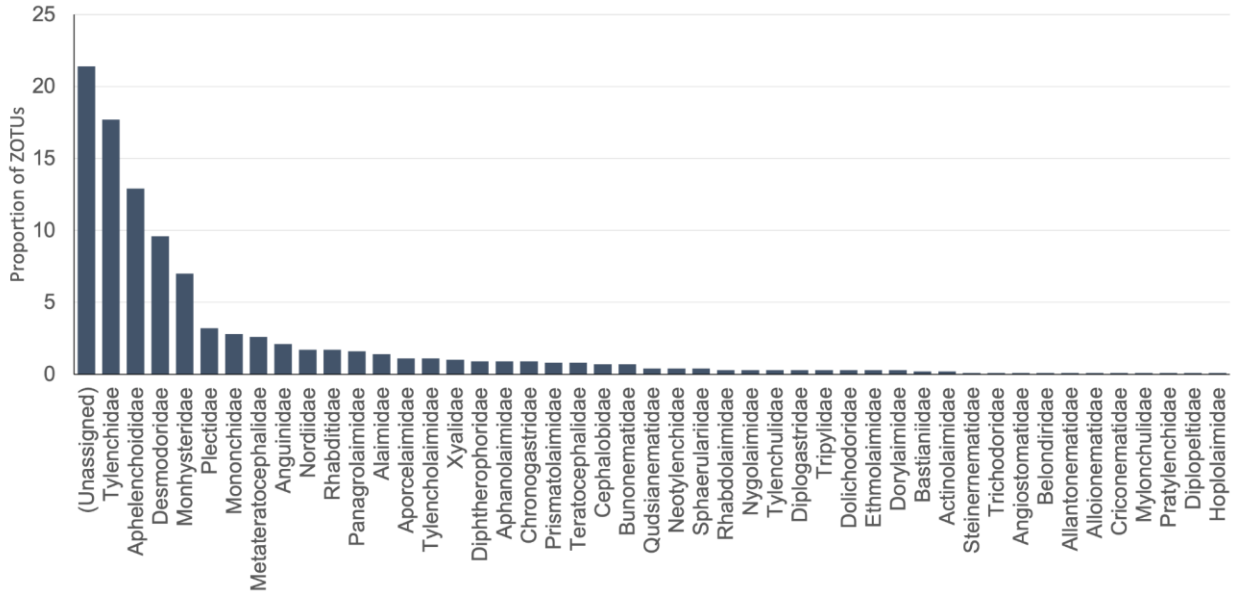

Figure 4. Proportions of total ZOTUs assigned to various nematode families including those unassigned at the family level across all samples.

Table 3. List of genera and species of nematodes identified across all samples. The families for which genus assignment could not be achieved are not represented in this table. Number of ZOTUs identified for each taxon in parenthesis.

Family	Genus	Species
Actinolaimidae (2)	*Paractinolaimus* (1)	
Alaimidae (12)	*Alaimus* (4)	*Alaimus parvus* (1)
Alloionematidae (1)	*Rhabditophanes* (1)	
Angiostomatidae (1)	*Angiostoma* (1)	*Angiostoma margaretae* (1)
Anguinidae (25)	*Anguina* (4)	
	Ditylenchus (14)	*Ditylenchus adasi* (1), *D. destructor* (1)
Aphanolaimidae (9)	*Aphanolaimus* (4)	*Aphanolaimus aquaticus* (2)

Aphelenchoididae (115)	*Aphelenchoides* (68)	*Aphelenchoides blastophthorus* (1), *A. heidelbergi* (5), *A. ritzemabosi* (3), *A. saprophilus* (1)
	Basilaphelenchus (15)	
	Bursaphelenchus (1)	
	Ektaphelenchoides (2)	
	Laimaphelenchus (16)	*Laimaphelenchus penardi* (6)
	Potensaphelenchus (3)	*Potensaphelenchus stammeri* (1)
	Schistonchus (1)	
Aporcelaimidae (11)	*Aporcelaimellus* (9)	*Aporcelaimellus obtusicaudatus* (7)
Bunonematidae (5)	*Bunonema* (5)	*Bunonema reticulatum* (1), *B. richtersi* (1)
Cephalobidae (6)	*Acrobeloides* (4)	*Acrobeloides varius* (1)
Chronogastridae (6)	*Chronogaster* (6)	
Desmodoridae (86)	*Prodesmodora* (86)	
Diphtherophodridae (8)	*Diphtherophora* (1)	
	Tylolaimophorus (5)	*Tylolaimophorus typicus* (5)
Diplogastridae (5)	*Pristionchus* (3)	
Diplopeltidae (1)	*Cylindrolaimus* (1)	
Dorylaimidae (5)	*Crassolabium* (1)	*Crassolabium circuliferum* (1)
	Prodorylaimus (1)	
Ethmolaimidae (1)	*Ethmolaimus* (1)	*Ethmolaimus pratensis* (1)
Hoplolaimidae (2)	*Helicotylenchus* (1)	*Helicotylenchus pseudorobustus* (1)
Metateratocephalidae (21)	*Metateratocephalus* (16)	*Metateratocephalus crassidens* (7)
	Euteratocephalus (2)	*Euteratocephalus palustris* (1)
Monhysteridae (61)	*Eumonhystera* (59)	*Eumonhystera filiformis* (3)
	Geomonhystera (2)	
Mononchidae (25)	*Clarkus* (3)	*Clarkus papillatus* (2)
	Mononchus (10)	*Mononchus truncatus* (7)
	Prionchulus (9)	*Prionchulus muscorum* (9)
Mylonchulidae (1)	*Mylonchulus* (1)	
Neotylenchidae (4)	*Hexatylus* (4)	*Hexatylus viviparus* (3)
Nordiidae (11)	*Enchodelus* (5)	
	Pungentus (1)	
Nygolaimidae (6)	*Paravulvus* (2)	*Paravulvus hartingii* (2)
Panagrolaimidae (13)	*Baldwinema* (1)	*Baldwinema ardabilense* (1)

	Panagrolaimus (7)	
Plectidae (34)	*Plectus* (25)	*Plectus minimus* (1), *P. tenuis* (6)
	Tylocephalus (2)	*Tylocephalus auriculatus* (1)
Pratylenchidae (1)	*Pratylenchus* (1)	*Pratylenchus crenatus* (1)
Prismatolaimidae (8)	*Prismatolaimus* (8)	*Prismatolaimus dolichurus* (3)
Rhabditidae (20)	*Choriorhabditis* (1)	*Choriorhabditis cristata* (1)
	Diploscapter (1)	*Diploscapter coronatus* (1)
	Heterorhabditis (1)	
	Oscheius (1)	*Oscheius dolichura* (1)
	Pellioditis (1)	
	Poikilolaimus (2)	
	Protorhabditis (1)	
	Rhabditis (10)	
Rhabdolaimidae (3)	*Rhabdolaimus* (3)	
Sphaerulariidae (5)	*Paurodontella* (2)	*Paurodontella gilanica* (2)
	Veleshkinema (2)	*Veleshkinema iranicum* (2)
Steinernematidae (1)	*Steinernema* (1)	*Steinernema kraussei* (1)
Telotylenchidae (1)	*Neodolichorhynchus* (1)	
	Tylenchorhynchus (2)	*Tylenchorhynchus parvulus* (2)
Teratocephalidae (7)	*Teratocephalus* (7)	*Teratocephalus deconincki* (2)
Trichodoridae (1)	*Paratrichodorus* (1)	*Paratrichodorus pachydermus* (1)
Tripylidae (3)	*Tripyla* (2)	
Tylenchidae (151)	*Aglenchus* (1)	*Aglenchus agricola* (1)
	Basiria (1)	
	Cephalenchus (3)	*Cephalenchus hexalineatus* (2)
	Coslenchus (1)	*Coslenchus costatus* (1)
	Discotylenchus (1)	
	Ecphyadophora (7)	*Ecphyadophora tenuissima* (2)
	Filenchus (16)	*Filenchus facultativus* (7), *F. misellus* (1)
	Irantylenchus (4)	*Irantylenchus vicinus* (2)
	Malenchus (68)	*Malenchus acarayensis* (4), *M. bryanti* (3), *M. neosulcus* (17), *M. pressulus* (6)
	Miculenchus (24)	*Miculenchus muscus* (5)

	Tylenchus (9)	*Tylenchus arcuatus* (2), *T. naranensis* (1)
Tylencholaimidae (8)	*Tylencholaimus* (6)	*Tylencholaimus mirabilis* (3), *T. teres* (1), *T. zhongshanensis* (1)
Tylenchulidae (2)	*Paratylenchus* (2)	
Xyalidae (10)	*Theristus* (9)	*Theristus agilis* (8)

Nematode communities across medium types and vegetations

Soil and litter samples had high ZOTU richness. However, this is most likely because there were more samples collected for these medium types. The two medium types showed comparable richness (Fig. 5a). Moss samples were the closest to these two in terms of richness. Fungus, lichens, and decomposing wood samples, on the other hand, had low alpha diversity scores. The two most heavily sampled vegetations, coniferous forest on dry land and coniferous forest on high swamp showed a wide range of diversity across sampled spots. Between them, there was a significant difference in their alpha diversity (Fig. 5b).

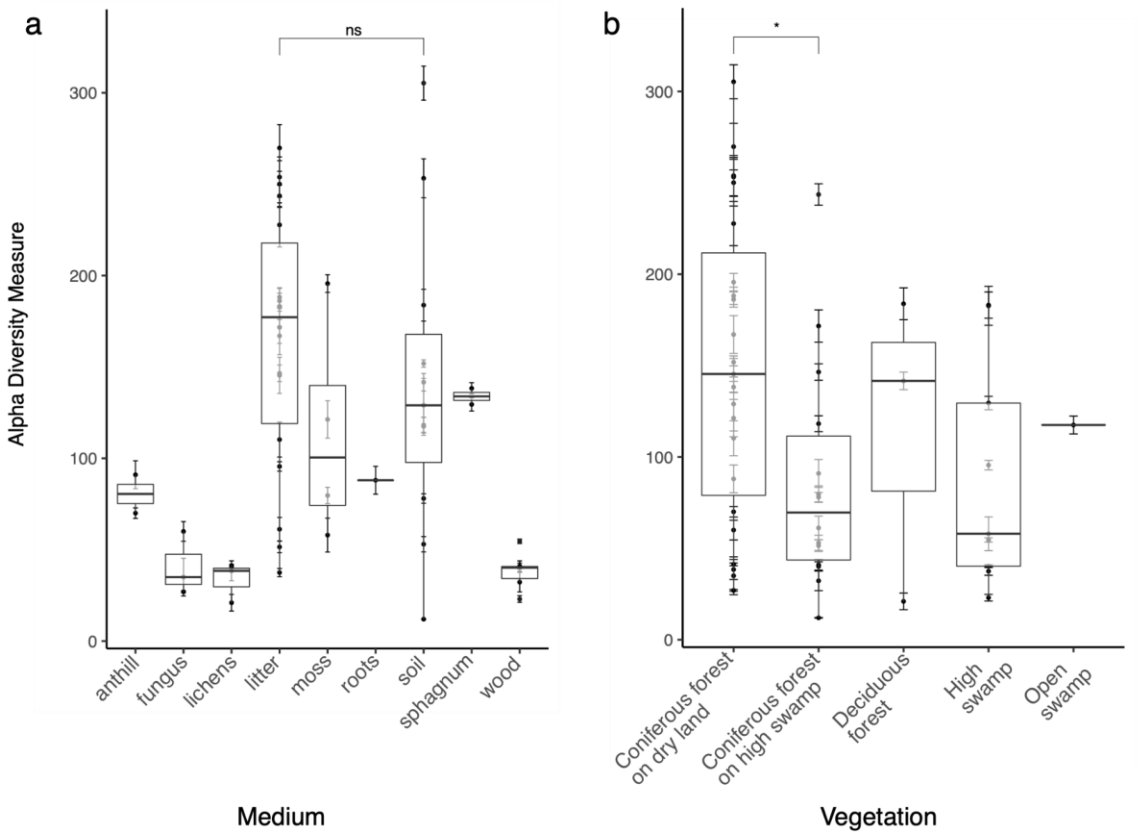

Figure 5. Chao1 measures of α-diversity of nematodes in different samples. a) Medium types. Statistical significance of the difference between alpha diversity for litter and soil samples was tested using the Wilcoxon test. ns = not significant. b) Vegetation types. Statistical significance of

the difference between alpha diversity for Coniferous Forest on dry land and Coniferous Forest on high swamp samples was tested using the Wilcoxon test. * = significant.

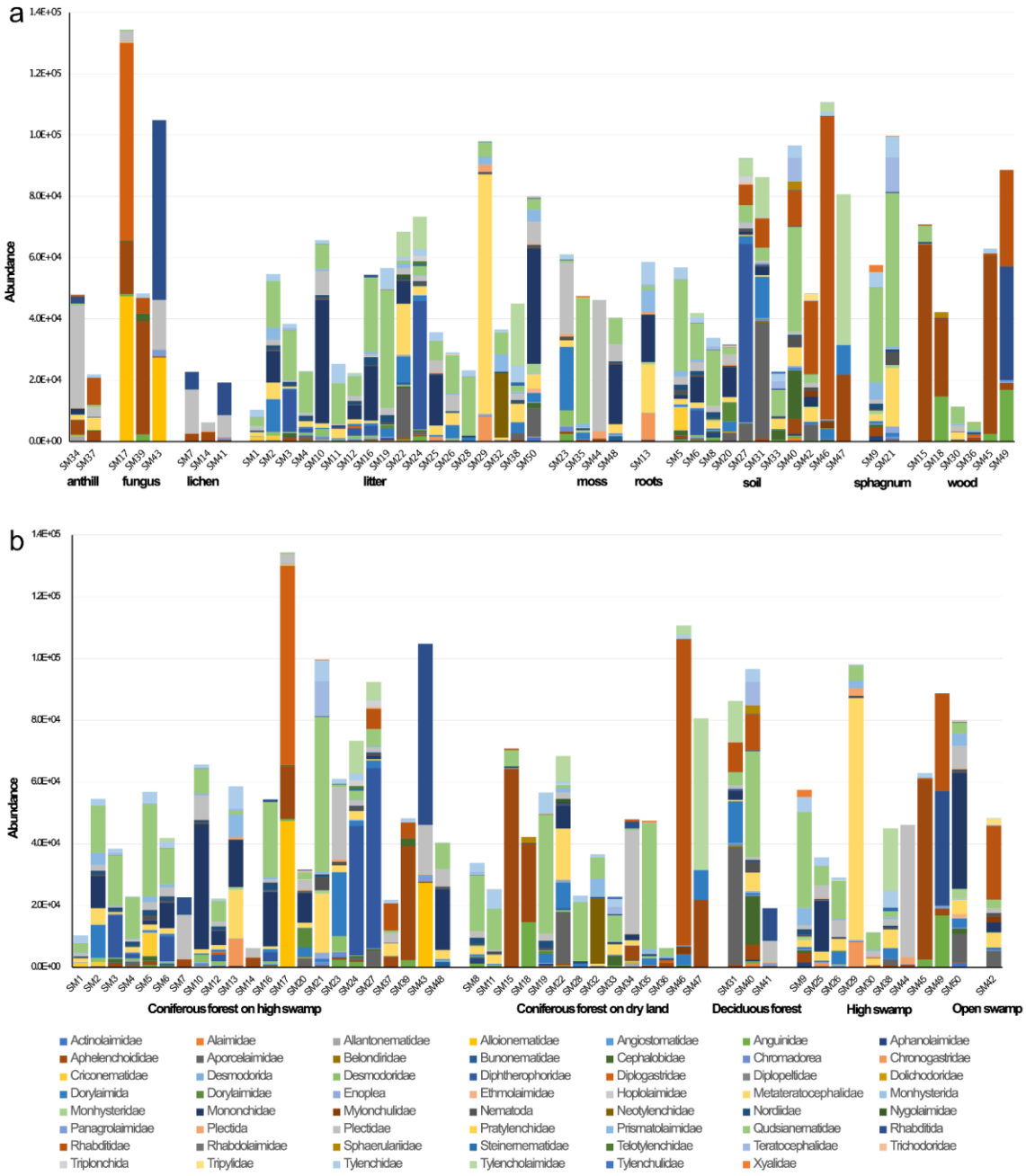

Figure 6. Read distribution among nematode families. Each bar corresponds to a sample. Samples are aggregated into various medium types (a) and vegetation types (b). Not all taxa were resolved to the species level. These are represented at the order or class rank.

ZOTUs associated with Qudsianematidae were dominant in most of the samples, especially the litter and soil samples (Fig. 6a). Rhabditidae and Plectidae dominated the two samples collected

from the anthills. Fungus samples produced very high number of reads associated with Diplogastridae, Alloionematidae, Aphelenchoididae, unidentified Rhabditida and Plectidae. Aside from being the most dominant family in litter samples, Qudsianematidae was also the most prevalent, occurring in each of the eighteen litter samples (Fig. 6a). Sequence reads associated with Plectidae, Mononchidae, Qudsianematidae and an unidentified Dorylaimida dominated the moss samples. There were also a few Anguinidae and Metateratocephalidae reads present. In the single root sample collected, Tylenchidae, Prismatolaimidae, Mononchidae, Metateratocephalidae and Chronogastridae were dominant. Qudsianematidae was the most prevalent family in the soil samples, absent only in two of eleven samples. It was also the dominant family in four of the soil samples. Next in terms of prevalence was Tylenchidae, followed by Rhabditidae, then another dorylaimid family, Tylencholaimidae. The taxonomic compositions of the two sphagnum samples were similar, except for Teratocephalidae and Xyalidae, which were found only once with significant abundance. As expected, the family Aphelenchoididae dominated the wood samples. One of the wood samples had a substantial number of reads belonging to Anguinidae, Rhabditidae along with an unidentified Rhabditida. Tylenchidae was also found in three of the six wood samples. Nematode distribution across samples within the same vegetation types did not show same level of similarity observed in samples from the same medium types, indicating a lack of correlation between community structure and vegetation type (Fig. 6b).

In terms of prevalence, most taxa showed wide distribution across multiple medium types and vegetations (Figs 7, 8). *Rhabditis* (soil), *Poikilolaimus* (wood), some species of *Basilaphelenchus* (wood) and an unidentified Dorylaimida (soil) were confined to only one medium type. *Heterorhabditis* (coniferous forest on dry land), Criconematidae (coniferous forest on dry land), some unidentified species of Dorylaimida (coniferous forest on high swamp) and unidentified Triplonchida (coniferous forest on dry land) were also associated with just one vegetation type.

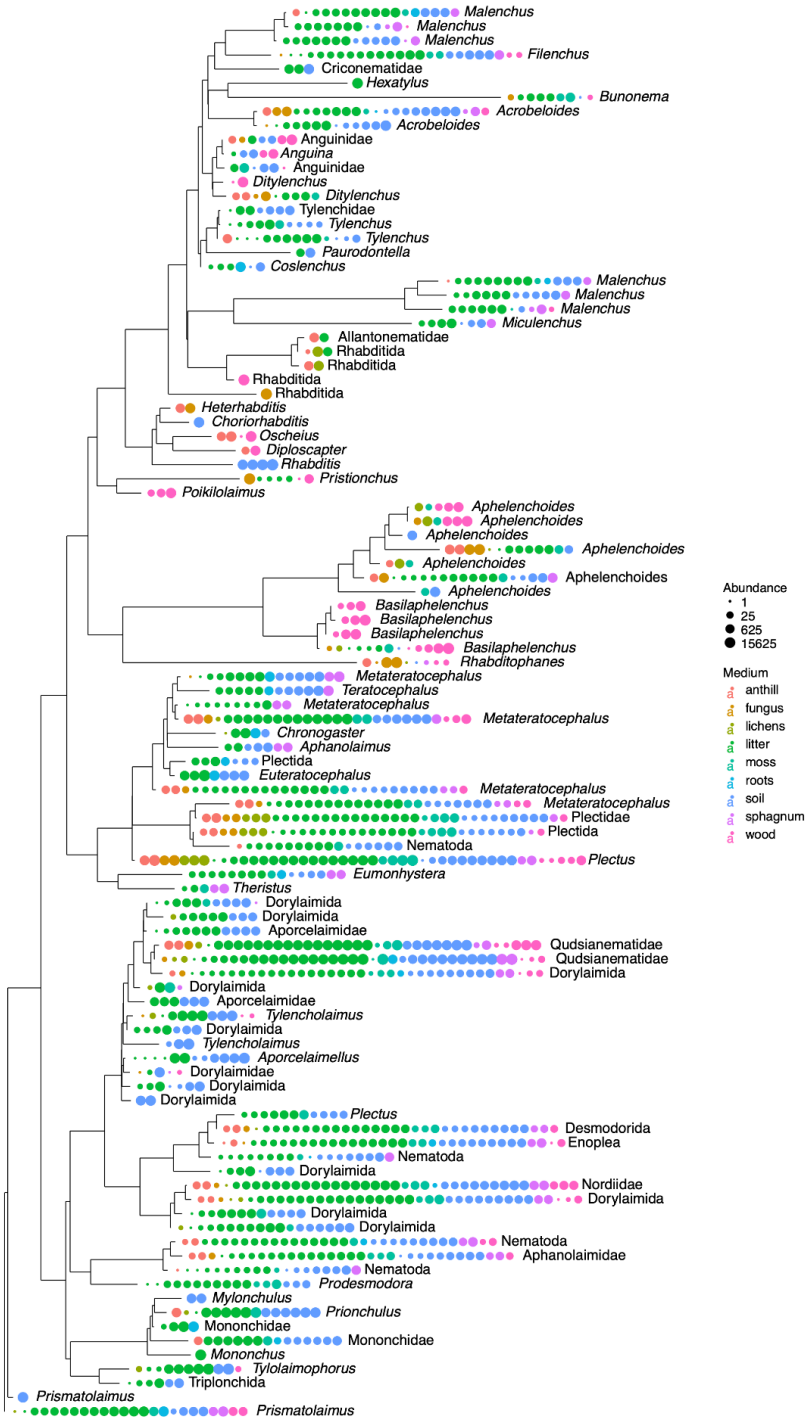

Figure 7. Maximum likelihood tree of the 100 most dominant ZOTUs showing their prevalence and abundance across different samples and medium types. Leaf nodes are labelled with the assigned taxa (genus where possible) of the ZOTUs. Circles represent the samples, and the diameters of the circles indicate the abundance of the taxon in samples. Circles of the same colour indicate samples from the same medium type.

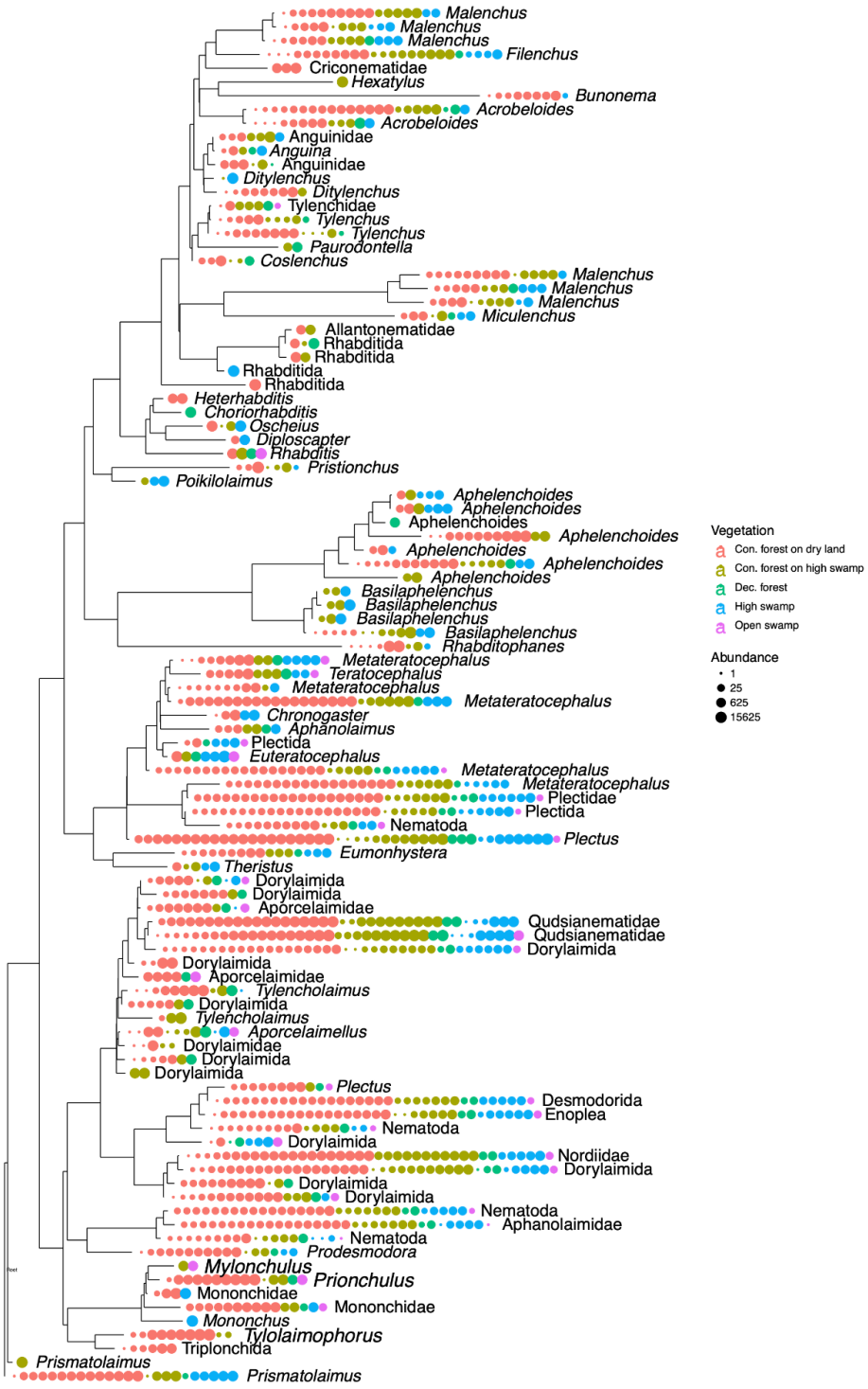

Figure 8. Maximum likelihood tree of the 100 most dominant ZOTUs showing their prevalence and abundance across different samples and vegetation types. Leaf nodes are labelled with the assigned taxa (genus where possible) of the ZOTUs. Circles represent the samples, and the

diameters of the circles indicate the abundance of the taxon in samples. Circles of the same colour indicate samples from the same vegetation type. Con. = Coniferous, Dec. = Deciduous.

Nematode trophic groups and coloniser-persister groups

All five major trophic groups described by Yeates et al. (1993) were recovered in each of the medium types. Their distribution, however, varied across the medium types (Fig. 9a). Fungivores were most dominant in decomposing wood samples, but very low in moss, sphagnum, and root samples. Bacterivores occurred with significant abundance across all sample types, particularly in anthill, lichen, moss, and root samples. The occurrence of predators was very low in lichen and sphagnum samples. Omnivores had high abundances in the samples associated with litter, moss, soil, and sphagnum. They were also well represented in the wood samples. The occurrence of herbivores appeared to be in concert with that of fungivores in the different medium types, except for decomposing wood samples where almost half of all reads came from the latter. Bacterivore-linked c-p groups 1 and 2 dominated the reads in fungus, lichen, and decomposing wood samples (Fig. 9b). Fungus samples were dominated by c-p 1 nematodes, suggesting high level of enrichment. Soil samples showed a uniform distribution of all five c-p classes, a feature of a pristine or relatively undisturbed community. Litter samples also showed a c-p class distribution similar to that of soil samples.

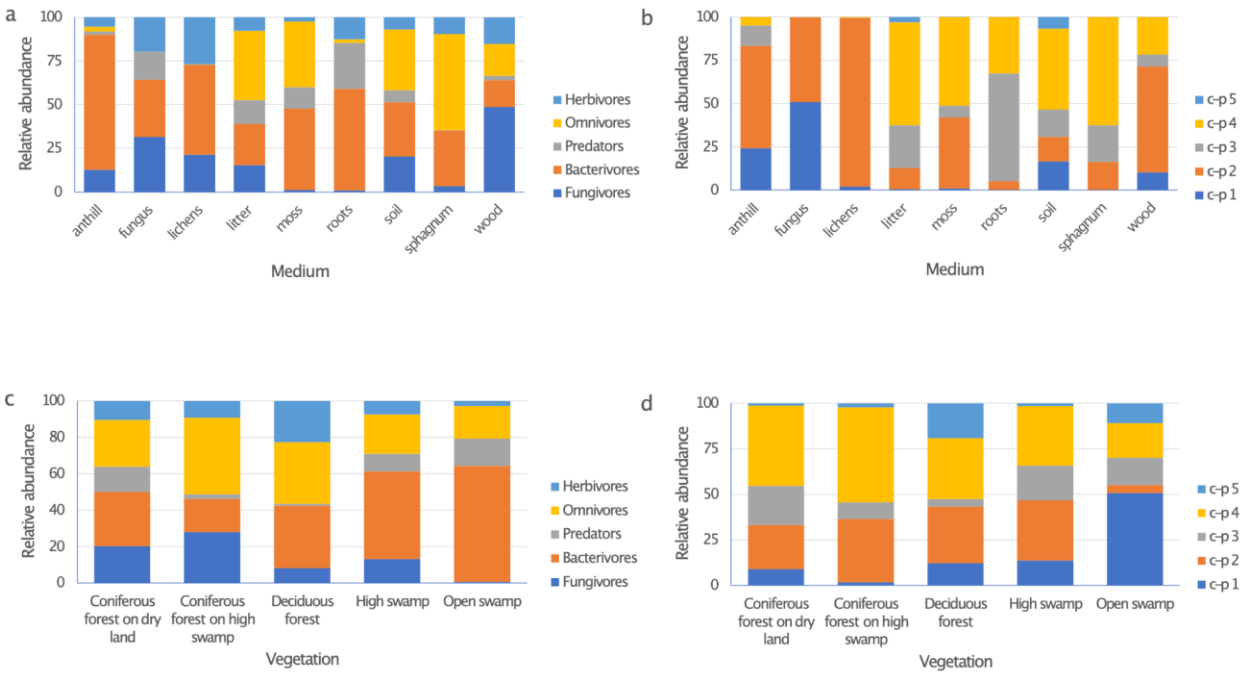

Figure 9. Distribution of nematode trophic and coloniser-persister groups in different medium and vegetation types.

There was also a uniform representation of all trophic groups in coniferous forest on dry land, and to some extent in high swamp vegetation types (Fig. 9c). The single sample representing open swamp was dominated by bacterivorous nematodes, representing over 60% of the nematode assemblage. Predators and omnivores together represented about a third of the assemblage. Fungivores and herbivores were very few. In coniferous forest on high swamp and deciduous forest samples, all the trophic groups were well represented except predators which occurred with very low abundance in both vegetation types. The single open swamp sample was dominated by c-p 1 nematodes (Fig. 9d). All c-p classes, except c-p 5 were well represented in the high swamp samples. In the two coniferous forest types, c-p 4 nematodes were the dominant group. The good structure indicators, c-p 4 and c-p 5 nematodes constituted a high proportion of the assemblage associated with deciduous forest vegetation (Fig. 9d).

Analysis of disturbance levels and food web in the communities

Using the c-p triangle to depict the stability/enrichment/stress conditions of the communities, most samples appeared to be in good stable conditions (Figs 10a, 10b). The soil and litter samples, where most of the diversity occurred, showed the highest stability while at the same time showing very low levels of enrichment. Wood and lichen samples were generally depicted as stressed, except for a few samples of decomposing wood. The two anthill samples were in low stability states. Based on the interpretation by Ferris et al. (2001), most of the samples regardless of the medium type were either in a matured or maturing food web state (Fig. 10b). Lichen was the only medium for which all samples were in a degraded, depleted state, with high C:N ratio. The two sphagnum samples fell within the matured and fertile category, with moderate C:N ratio. Soil and litter samples were mostly concentrated within the high structure quadrants (maturing to matured food web), although with varying degrees of enrichment. Fungus samples showed low maturity and appeared to be highly disturbed in some samples and enriched in others. Their decomposition was generally not fungal but bacterial driven. The only root sample collected depicted a food web that was matured and fertile. The wood samples varied greatly in the states of the food webs they depicted, while some appeared matured and fertile/N-enriched, others were highly disturbed and moderate to heavily enriched. This can be explained by the fact that wood decomposition is a complex multistage process that includes different organisms during different times, with wood at late stages becoming similar to litter and soil. The two anthill samples were in quite opposite conditions, one in a maturing state while the other was in a degraded and nutrient-depleted state.

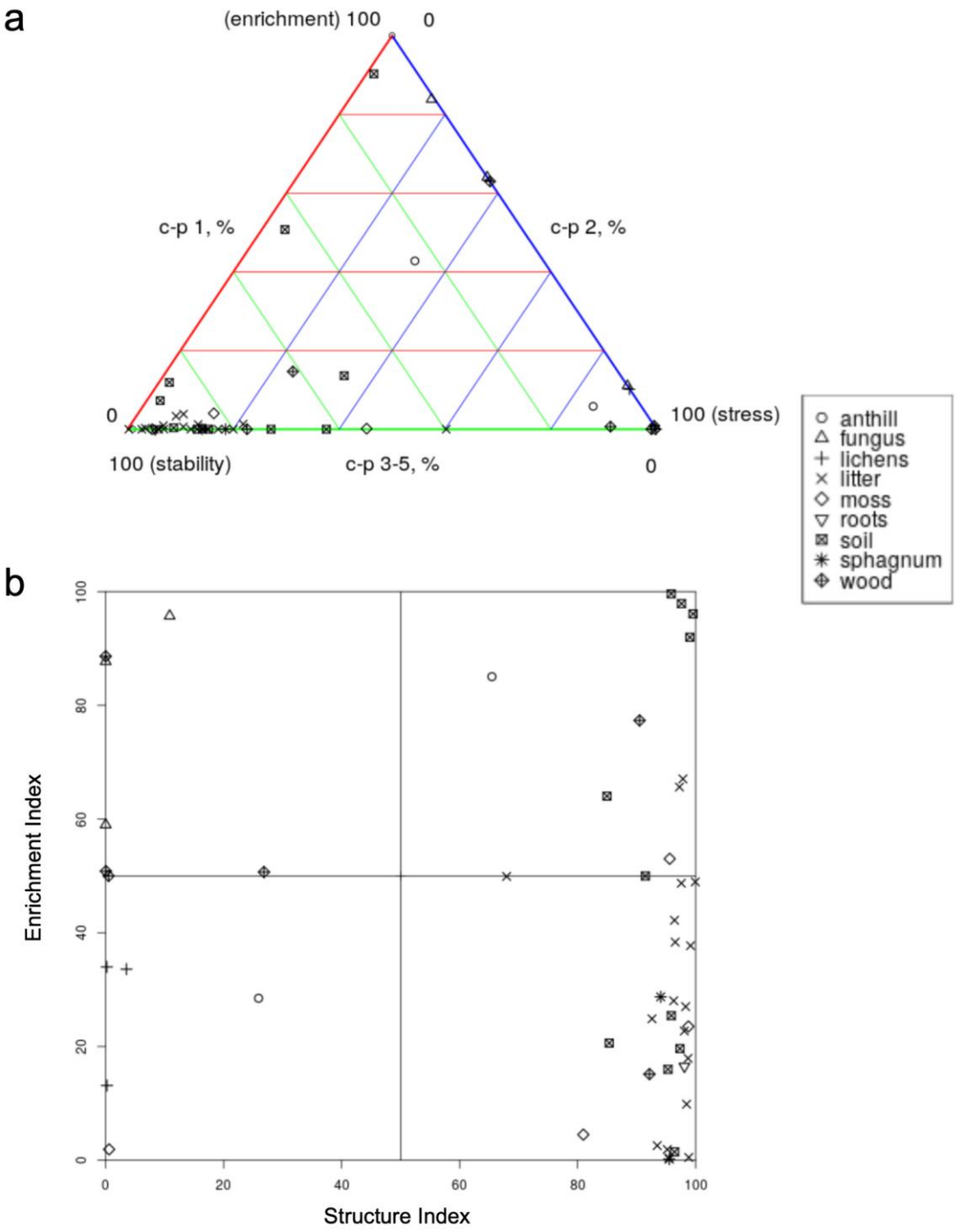

Figure 10. CP triangle and food web analysis of the different medium types. CP triangles (a) depict the stability of the communities. Food web analysis plots (b) depict the maturity of the food webs within the communities. a) CP triangle showing samples categorised under different medium types. b) Food web analysis showing samples categorised under different medium types.

Discussion

Nematode suspension after extraction from substrates such as soil and litter often contain other metazoans. Among the most frequently encountered groups in such nematode extracts are terrestrial tardigrades and some micro-arthropods. It was therefore not unexpected that almost a tenth of the total ZOTUs were assigned to these two groups combined, especially considering the universal nature of the primers used. Our effort to minimise the amplification of other non-targets such as fungi by extracting nematodes first from the substrate and reducing the volume of the suspension down to 50µl was to some extent successful. But despite that, fungal and unidentified ZOTUs (most likely Archaean) together constituted almost 38% of the total ZOTUs. Primer combinations exist that can address this through preferential amplification of nematode 18S rDNA (Sapkota & Nicolaisen 2015; Waeyenberge et al. 2019; Kawanobe et al. 2021), thus limiting the amplification of non-nematode DNA. Kawanobe et al. (2021) demonstrated through in silico analysis the high tendency of NF1/18Sr2b primers to amplify non-nematode eukaryotes. Their analysis identified three primer combinations that showed better coverage and specificity to nematodes. Our goal for this study was not to exclude all other eukaryotes. Therefore, using any of the nematode-specific primers would have limited the detection of other metazoans recovered in this study. For most use cases however, these nematode-specific primers can be better alternatives to the widely used NF1/18Sr2b.

Our analysis recovered a massive diversity of nematodes, with a total of 47 nematode families identified representing 10 different orders. We identified 76 nematode genera in total across all samples, 46 of which were identified to species level. Even though reads associated with family Qudsianematidae were the most dominant, species or genus assignments of ZOTUs belonging to Qudsianematidae were not supported according to the sintax assignment method. Similar to the RDP naïve Bayesian classifier, the sintax attaches posterior probability scores to each rank classification (Wang et al. 2007; Edgar 2016). Any rank classification receiving a support value below the set threshold (0.8/1 in this study) was considered not supported enough. For some taxa a blast search against the Genbank reference database could resolve the assignment. For orders such as Dorylaimida and Rhabditida, although more so for the former, many ZOTUs could not be identified further beyond the rank of an order, even with BLAST search. In the case of Qudsianematidae, the ZOTUs could only be assigned to the rank of family (Figs 7; 8). A possible explanation for this is the conserved nature of the 18S rDNA region for delineating some species. This is particularly widespread among species of most free-living Dorylaimida of which Qudsianematidae is a member. With this group, it has been shown that even the full-length 18S rDNA sequence is extremely conserved (Holterman et al. 2006; 2008).

Litter and soil were the two most sampled habitats. For the majority of the samples, these two medium types shared similar taxonomic composition (Figs 6a, 6b). However, in some samples there were observable differences in the community structure. For example some soil samples appeared to be uniquely dominated by Rhabditidae which were not observed with such high

abundance in the litter samples. Other than these two medium types which were generally similar in their community structure, the rest of the medium types produced unique taxonomic distributions (Fig. 6a). At the species level, some taxa showed exclusive association with certain medium types (Figure 11). In general, it appears that had the sampling effort been limited only to mineral soil samples, the majority of the observed nematode families would still have been recovered. However, at the species level, many taxa would have been missing, specifically those associated with litter, decomposing wood, sphagnum, lichens and moss samples. Soil sampling alone does not adequately reflect the diversity of nematodes, and depending on the geographic location, the degree to which taxa are missed as a result of this sampling strategy can vary (Powers et al. 2009; Porazinska et al. 2010b; 2012). Even though the collection of samples beyond the mineral soils has in fact been practised by authors in the past (Yeates 1972; Sohlenius and Boström 2001), and that the importance of collecting litter and other materials above the mineral soil was understood then, some biodiversity studies still focus only on soil samples. The current study concurs with previous studies in demonstrating the importance of sampling not just the mineral soil, but other habitats as well (Powers et al. 2009; Porazinska et al. 2010b; 2012). This must be taken into consideration when attempting to create a baseline metabarcoding datasets for different biomes to be used as reference points in the future monitoring of ecosystem changes. Such baseline reference datasets for complex land use type (forest) can not be based on standard soil+litter samples. As we have shown above, a considerable percentage of diversity was found in media types other than mineral soil and litter. Thus, in order to establish comprehensive metabarcoding baseline for a given biome, all possible microhabitats where the target taxon may occur, must be included in sequencing.

In spite of the inability of the barcode marker to identify the Qudsianematid ZOTUs beyond the family level, the 76 genera recovered is quite remarkable, especially considering the fact that the current study was based on a one-time sampling. Comparing this study with others carried out within Sweden or regions with similar climatic conditions clearly shows a higher recovery of nematode taxa. For example, over the course of three sampling series spanning over a period of 25 years, in which 156 soil samples from Scots pine forest in Sweden were analysed, Sohlenius and Boström (2001) identified 36 unique nematode taxa. Of these, 31 were identified to the genus level. The majority of the genera identified in that study were recovered here as well, with the exception of *Geocenamus*, *Acrobeles*, *Cervidellus*, *Achromadora*, *Wilsonema*, *Eudorylaimus*, *Microdorylaimus* and *Thonus*. In contrast, over 50 of the genera identified in this study were missing in their taxa list. Compared to the current study, Yeates (1972) also identified fewer unique genera (41) from an 85-year old Danish beech forest studied over a period of 12 months. Another study on the metazoan microfauna of a mire in northern Sweden sampled over a period of four months also identified 24 taxa representing 17 unique genera (Sohlenius et al. 1997). One of the likely reasons for this is that metabarcoding allows to identify immature individuals and eggs, which morphology-based approaches are unable to do.

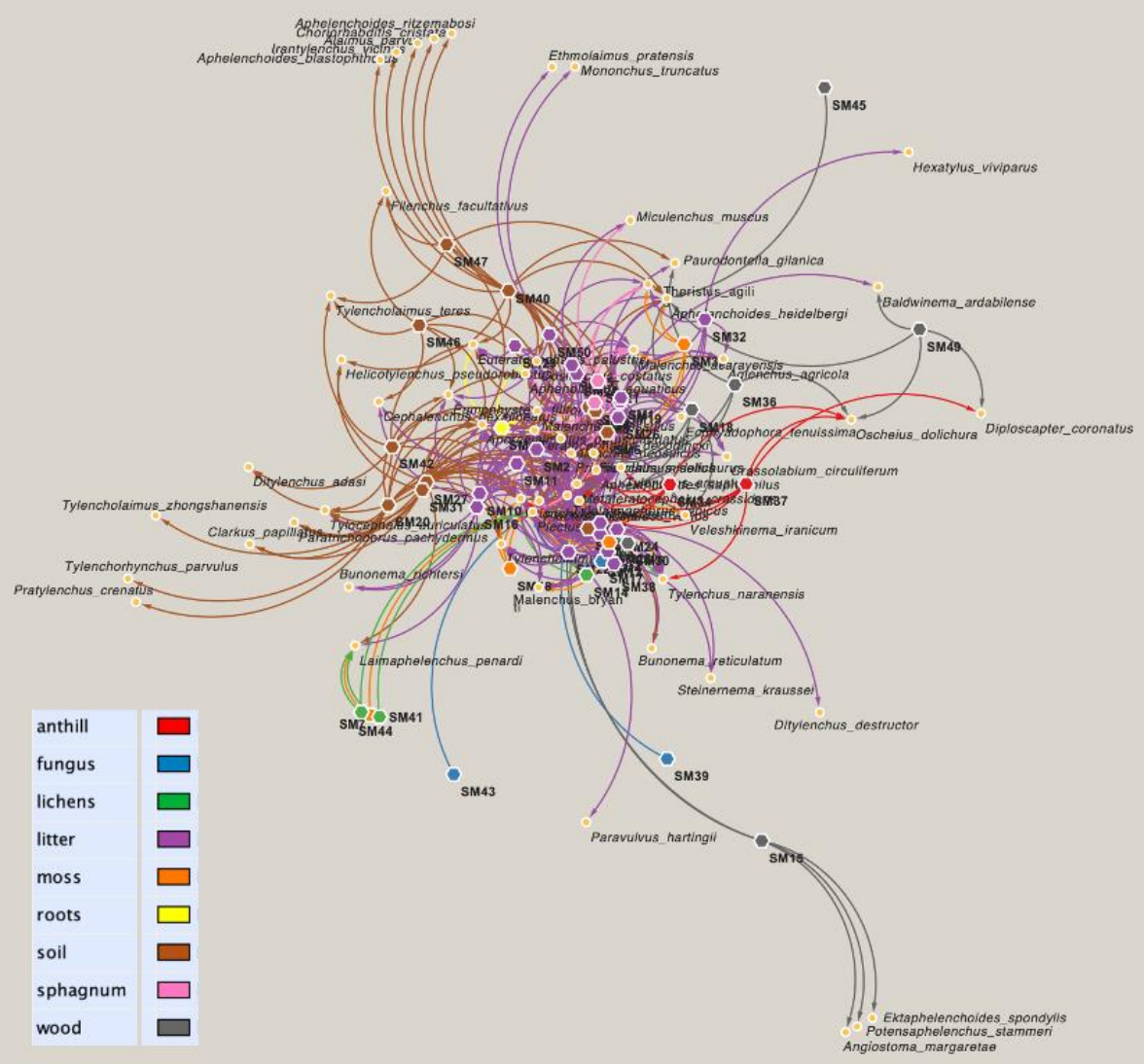

Figure 11. Species network showing association between taxa and samples. Samples represented by hexagonal nodes; taxa represented by circular nodes. Sample-taxon association depicted by arrows extending from the sample to the taxon. Medium types are represented by different colours. Both the nodes representing a sample and edge extending from it are colour to depict the medium type it belongs to.

Nematode distribution appeared to show no association with vegetation types. It appeared nematode community structure was influenced more by the medium type that was collected. This was expected given that most nematode taxa will be more associated with certain habitats than others regardless of what the local vegetation cover of the habitat is. Across the different vegetations, none of the community structures stood out as unique (Fig. 6b). Due to the strong influence the medium type has on the community, a better comparison of communities under the different vegetation types would be one that is restricted to only one type of sample medium. This

could not be done for any of the medium types because none of the medium types was represented across all five vegetation types.

Indices used in this study that describe the structure and maturity of the community are heavily dependent on abundance data. And since sequence read abundance does not always directly correlate with the abundance of taxa in a typical metabarcoding analysis, there is constraint in the inferences that can be made about the condition of the samples based on these indices (Waeyenberge et al. 2019). Nevertheless, the largely stable conditions depicted by our analysis are expected especially given the pristine nature of the Store Mosse National Park.

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