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A single degenerated primer significantly improves COX1 barcoding performance in soil nematode community profiling

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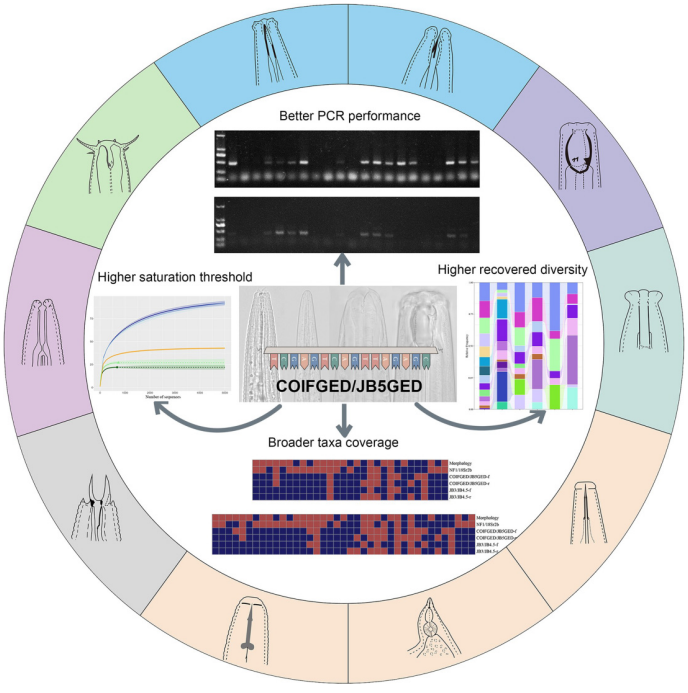
ABSTRACT

• A new COX1 primer for soil nematode metabarcoding was designed, and this primer outperforms other commonly used COX1 primer pairs in species recovery and quantity of PCR products.
• The lack of reference database is the main reason that led to the low species recovery in COX1 metabarcoding.
• We expanded current NCBI database by adding 51 newly generated COX1 reference sequences.

Microscopic nematodes play important roles in soil ecosystems and often serve as bioindicators of soil health. The identification of soil nematodes is often difficult due to their limited diagnostic characters and high phenotypic plasticity. DNA barcoding and metabarcoding techniques are promising but lack universal primers, especially for mitochondrial COX1 gene. In this study a degenerated COX1 forward primer COIFGED was developed. The primer pair (COIFGED/JB5GED) outperforms other four commonly used COX1 primer pairs in species recovery and quantity of polymerase chain reaction (PCR) products. In metabarcoding analysis, the reads obtained from the new primer pair had the highest sequencing saturation threshold and amplicon sequence variant (ASV) diversity in comparison to other COX1 as well as 18S rRNA primers. The annotation of ASVs suggested the new primer pair initially recovered 9 and 6 out of 25 genera from mock communities, respectively, outperformed other COX1 primers, but underperformed the widely used 18S NF1/18Sr2b primers (16 out of 25 genera). By supplementing the COX1 database with our reference sequences, we recovered an additional 6 mock community species bringing the tally closer to that obtained with 18S primers. In summary, our newly designed COX1 primers significantly improved species recovery and thus can be supplementary or alternative to the conventional 18S metabarcoding.



Keywords degenerated primers, DNA metabarcoding, mitochondrial cytochrome oxidase *c* subunit I gene, phylogeny, ribosomal RNA gene, soil nematodes

1 Introduction

Nematodes are considered the most ubiquitous and abundant metazoans on Earth (De Ley et al., 2005; Bardgett and van der Putten, 2014). With abundance ranging between 10^5 and 10^8 per square meter in marine and terrestrial domains (Lambshead and Boucher, 2003), nematodes account for ~80% of all animal individuals (Lorenzen, 1994). In soil ecosystems, nematodes display a range of feeding habits including bacterivorous, fungivorous, plant-parasitic, omnivorous and predatory behaviors (Yeates et al., 1993).

Although some species threaten the health of plants, most are beneficial to soil health (Neher, 2001; Yi et al., 2021), primarily by driving the turnover of soil microbial community composition and biomass (Bardgett et al., 1999; Ekschmitt et al., 2001) and hence influencing ecosystem processes such as decomposition and nutrient mineralization (Wang et al., 2004). Moreover, since shifts in nematode community diversity and composition can reflect shifts in soil texture (Griffin, 1996), nutrient inputs (Ferreira et al., 2015), natural and anthropogenic disturbances (Rosli et al., 2018), or climate (Duyck et al., 2012), they are often used as bioindicators of soil conditions or health (Neher, 2001; De Mesel et al., 2004).

Historically, nematode community characterization has been based on the use of morphology and microscopy. However, morphological identification is time-consuming and usually involves a significant learning curve through years of training (Floyd et al., 2002; De Ley et al., 2005). More importantly, with the presence of high phenotypic plasticity, nematode species often lack morphological identifying characters (Derycke et al., 2008) while morphological species concepts are too abstract (Coomans, 2002; Nadler, 2002).

To overcome the limitations of traditional morphologically-based identification for assessing biodiversity, molecular approaches involving DNA barcoding have been proposed and applied (Hebert et al., 2003; Floyd et al., 2010). This approach was further integrated with high-throughput sequencing technology as DNA metabarcoding, and has gained great attention in soil nematode community profiling (Porazinska et al., 2009; Kawanobe et al., 2021). Despite its advantages, nematode metabarcoding presents its own challenges, which are largely defined by the difficulty of selecting the most appropriate barcoding genes and/or designing universal primers (Prosser et al., 2013; Aivelo and Medlar, 2018; Ahmed et al., 2019). Currently, driven by a broad taxon representation in databases, 18S rRNA has been the most commonly used barcoding gene marker (Porazinska et al., 2009; Nassonova et al., 2010; Nakacwa et al., 2013). However, 18S rRNA gene is highly conserved and lacks the resolution required to distinguish among closely-related lineages (Kiewnick et al., 2014; Schenk et al., 2020). In addition, the 18S rRNA gene contains a high level of intra-genomic polymorphism potentially reducing the reliability of nematode species identification (Qing et al., 2020). As a result, the operational taxonomic units (OTUs) obtained from 18S rRNA metabarcoding are typically only annotated to the genus level (Porazinska et al., 2010).

Arguably, mitochondrial genes can partly circumvent these challenges due to their uni-parental inheritance and high mutation rate (Gissi et al., 2008; Pagan et al., 2015). For example, the mitochondrial cytochrome oxidase *c* subunit I (COX1) gene has become a popular marker for population genetics and phylogeographic studies across the animal kingdom (Avise, 1994) and an effective metabarcoding marker for resolving animal communities such as insects at the species level (Ritter et al., 2019; Liu et al., 2020; Svenningsen et al., 2021). Unfortunately, unlike other animals, the Nematoda phylum is characterized by substantial COX1 gene variation (Sikder et al., 2020) limiting the discovery/design of universal primers (Ahmed et al., 2019; Waeyenberge et al., 2019; Macheriotou et al., 2019). The most popular primer pair JB3/JB4.5 (l3-M11 partition of COX1 gene) has poor amplification performance with a strong bias toward a few species while the general species recovery rate is low (Macheriotou et al., 2019; Sikder et al., 2020). Although attempts to modify the Folmer primers (M1−M6) (Bowles et al., 1992) for marine nematodes have been made (Derycke et al., 2010), their performance remains unsatisfactory and untested for soil nematodes.

To alleviate some of these issues, in this study we: (1) designed and tested the performance of a new degenerate COX1 primer pair, and (2) compared the new primer set with the commonly used primer sets for 18S rRNA gene (NF1/18Sr2b) and COX1 gene (JB5GED/JB3/JB4.5).

2 Materials and methods

2.1 Sample preparation and DNA extraction

We tested our primers on nematode species recovered from soil samples collected from Jiangsu, China. Nematode specimens extracted with the modified Baermann tray method (Whitehead and Hemming, 1965) were extracted by hand and each was individually mounted on a temporary slide in a drop of water. Nematodes were preliminarily identified to a genus level using morphological characters, photographed using an Olympus BX51 microscope equipped with an Olympus DP72 camera (Olympus Corporation, Tokyo, Japan). All identified specimens with their origins are listed in the Supplementary Tables S1, S2.

In addition to soil-extracted nematodes, seven species from pure cultures were used (Table S2). Fungivorous *Aphelenchoides* sp. and *Bursaphelenchus xylophilus* were cultured on *Botrytis cinerea* maintained on PDA medium (Cheng et al., 2018). Plant-parasitic *Pratylenchus* sp. and *Ditylenchus* sp. were cultured on carrot callus discs (Reise et al., 1987). Bacterivorous *Caenorhabditis elegans*, *Cephalobus* sp., and *Pseudodiplogasteroides* sp. were cultured on *Escherichia coli* strain OP50 and maintained on Nematode Growth Medium (Cheng et al., 2016).

A total of 18 nematode species were used (2 for Dorylaimida, 2 for Enoplida, 1 for Mononchida, 1 for Plectida, and 12 for Rhabditida; for details see Table S1) to evaluate the performances of four COX1 primer pairs in molecular

barcoding. For the metabarcoding analysis, the mock community was constructed using 130 individuals belonging to 25 species (19 families, 4 orders). These nematodes represented major trophic groups including plant-parasites, fungivores, bacterivores, omnivores, and predators (Table S2).

For DNA extraction, the individual specimens mounted on temporary glass slides were immediately processed for DNA extraction by transferring them into a drop of sterilized ddH_2O placed on a sterilized glass slide and cutting them into pieces by a scalpel. These pieces were then transferred to 20 µL of worm lysis buffer (50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 0.45% Tween 20 and 0.05% gelatin) (Williams et al., 1992). Genomic DNA was extracted by adding 1 µL proteinase K (10 mg mL^{-1}), followed by incubation at 60°C for 1 h and another 15 min at 95°C (Williams et al., 1992).

2.2 PCR amplification and sequencing

The new primers were designed with Primer3 in the Geneious platform (Kearse et al., 2012) using nematode COX1 sequences available in GenBank as reference. We designed three degenerated sites in primer with two Ns (ambiguous nucleotides) in hyper-variable sites. A total of six primer pairs were used in this study (Table 1) including: (1) a newly designed forward primer COIFGED targeting *c.a.* 450 bp region of the COX1 gene, together with a degenerated reverse primer JB5GED (Bowles et al., 1992); (2) a similar but without degeneration primer JB3/JB5GED (Bowles et al., 1992); (3) the universal COX1 primers JB3/JB4.5 for nematodes barcoding (Bowles et al., 1992); (4) the COIF/COIR amplifying the COX1 gene (Lazarova et al., 2006); (5) 18S rRNA metabarcoding primers NF1/18Sr2b (Porazinska et al., 2009); (6) 28S rRNA amplify the D2-D3 segment of 28S rRNA gene (Nunn, 1992).

All four COX1 primers were tested for molecular barcoding using DNA templates of 18 nematode species (see below). The polymerase chain reaction (PCR) was performed in a 25 µL mixture containing 2 µL primer (10 µM), 1 µL DNA template, 12.5 µL Ex Taq DNA polymerase (Bioscience, Shanghai, China) and 7.5 µL sterilized ddH_2O. The PCR program for COX1 primers was 94°C for 4 min, following 35 cycles of 94°C for 30 s, 45°C for 30 s and 72°C for 45 s and a final extension at 72°C for 10 min.

The newly designed primer pair COIFGED/JB5GED was also evaluated against the popular 18S primer pair NF1/18Sr2b and COX1 primer JB3/JB4.5 for metabarcoding using the mock communities (130 nematode individuals). To minimize bias introduced during the PCR, three independent amplifications were conducted for each treatment, and the products were pooled as a single sample. The PCR conditions for the COX1 were the same as for molecular barcoding, while PCR conditions for 18S was 95°C for 5 min, followed by 35 cycles of 95°C for 60 s, 54°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 10 min.

To expand the NCBI COX1 database we generated 51 COX1 reference sequences (Table S3) using the newly designed primers and pre-existing nematode samples from various studies conducted in our laboratory. The identities of newly obtained reference sequences were first assigned by morphology and confirmed by 28S (Table 1) using primer D2A/D3B with PCR conditions the same as those of 18S.

The PCR products were purified using the Gel Extraction Kit (DSLAB Biotech, Nanjing, China), and quantified using the Qubit® 1× dsDNA HS Detect Kit (Yeasen Biotech, Shanghai, China) on the Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, USA). The genomic library preparation was performed using the Illumina TruSeq DNA Sample Preparation Kit (Illumina Inc, USA). The paired-end sequencing was performed on Illumina MiSeq 2× 250-bp platform at the Personalgene Corporation (Personalbio, Shanghai, China).

Table 1 Primer sets used in this study.

Target gene	Primer name	Nucleotide sequence (5′−3′)	Fragment length (bp)	Reference
COX1	COIFGED	CCTTTGGGCATCC**N**GA**R**GT**N**TAT	450	This study
	JB5GED	ACCTAAACTTA**RW**AC**R**TA**R**TGAAAATG		Bowles et al., 1992
COX1	JB3	TTTTTTGGGCATCCTGAGGTTTAT	420	Bowles et al., 1992
	JB5GED	ACCTAAACTTA**RW**AC**R**TA**R**TGAAAATG		
COX1	JB3	TTTTTTGGGCATCCTGAGGTTTAT	442	Bowles et al., 1992
	JB4.5	TAAAGAAAGAACATA ATGAAAATG		
COX1	COIF	GATTTTTTGGKCATCCWGARG	400	Lazarova et al., 2006
	COIR	CWACATAATAAGTATCATG		
18S	NF1	GGTGGTGCATGGCCGTTCTTAGTT	380	Porazinska et al., 2009
	18Sr2b	TACAAAGGGCAGGGACGTAAT		
28S	D2A	ACAAGTACCGTGAGGGAAAGT	750	Nunn, 1992
	D3B	TGCGAAGGAACCAGCTACTA		

2.3 Bioinformatics analyses

The raw sequencing metabarcoding data were processed in QIIME 2 pipeline (Bolyen et al., 2019). The adapters were identified and removed using cutadapt (Martin, 2011), allowing up to 20% mismatch across the lengths of the primers. Subsequently, the sequences were quality filtered, error corrected, and chimera were removed using DADA2 (Callahan et al., 2016). Forward and reverse 18S sequences were merged into single amplicon sequence variants, ASVs, while both sequences were used separately for COX1 data sets using the denoise-single plugin in QIIME 2, as no read overlap was found. The taxonomy of ASVs was assigned using a BLAST search against the NCBI nucleotide non-redundant database in SEED2 (Vetrovský et al., 2018). The ASV assignments with sequence similarity < 85% for 18S and < 80% for COI and coverage < 70% were categorized as "unidentified".

Using the forward data set of the new primer pair as an example, we further examined the identities for "unidentified" ASVs by phylogenetic analysis. The maximum likelihood phylogeny was reconstructed using "unidentified" ASVs together with our newly sequenced COX1 references. The ASVs placed outside of Nematoda were removed after initial tree reconstruction, and only those nested within the nematode clade were retained. Phylogenetic analysis was performed using RAxML (Stamatakis et al. 2008) based on MAFFT alignment with G-INS-i algorithm (Katoh and Standley, 2013).

To compare the levels of species recovery by each primer, saturation thresholds of each metabarcoding data set were examined by rarefaction implemented in R package "iNEXT" (Hsieh et al., 2016). To evaluate the effect of primers on community composition, the community compositions recovered by metabarcoding and morphological identification were visualized by ggplot2 package (Ito and Murphy, 2013). To evaluate the alpha diversity for all obtained ASVs as well as those limited to Nematoda, the Shannon index was calculated using the R packages "Vegan" (Oksanen et al., 2007). To visualize the shared families and genera between different approaches, heatmaps at the level of family and of genus were created by R package "pheatmap" (see the website: github.com).

3 Results

3.1 The performance of COI primer pairs in molecular barcoding

A total of 36 genera representing 6 orders (Tables S1, S2) were morphologically identified from soil, and subsequently used for molecular barcoding and metabarcoding. The anterior body region of examples is shown in Fig. 1. Among 18 tested taxa (Table S1), our newly designed primer COIFGED/JB5GED outperformed other COX1 primers with 14 out of 18 positive results (Fig. 2A), followed by JB3/JB4.5 with 10 out of 18 positive results (Fig. 2B), and 6 out of 18 (one third) using JB3/JB5GED and COIF/COIR (Fig. 2C and D). In addition, the newly designed primer produced brighter bands than faint bands produced by others. In respect to identities, four species i.e., *Aphelenchoides* sp., *Labrys* sp., *Pratylenchus* sp. and *Xiphinema brevicollum* produced clear bands using both COIFGED/JB5GED and JB3/JB4.5 primers. Conversely, five species including *Alaimus* sp., *Acrobeloides* sp., *Prionchulus* sp., *Pristionchus* sp. and *Tripylina* sp. were successfully amplified with the COIFGED/JB5GED primer, but only weak or no bands were observed for the JB3/JB4.5 primer. Several taxa (e.g., *Ditylenchus destructor*, *Merlinius brevidens*, and *Plectus* sp.) produced no bands regardless of the used primer pair.

3.2 The abundance and diversity of recovered ASVs

The 18S data set produced 10 362 ASVs with an average length of 322 bp. For COX1 primers, the COIFGED/JB5GED produced 11 028 forward and 11 865 reverse ASVs with average lengths of 228 and 224 bp, respectively, and the JB3/JB4.5 produced 686 forward and 736 reverse ASVs with an average length of 219 bp in either direction. The rarefaction analysis suggested that the newly designed COX1 primer pair had the highest saturation threshold at > 5 000 sequences, followed by the 18S primer at > 2000, and the JB3/JB4.5 primer at < 1 000 sequences (Fig. 3A). As expected, all-included ASVs data sets had a higher overall diversity than the Nematoda-only data sets. The newly designed COIFGED/JB5GED primer recovered a similar diversity in comparison to that of the 18S primer in Nematoda-only data set, but a higher diversity in all-ASVs included data set. The lowest diversity was found for the JB3/JB4.5 primer (Fig. 3B), in both all-included and Nematoda-only data sets.

3.3 The primers performance in taxa recovery

The performance of newly designed primer was evaluated using a mock community. At phylum level, the 18S primer NF1/18Sr2b recovered the highest percentage of Nematoda ASVs (95.5%), followed by the forward JB3/JB4.5 and COIFGED/JB5GED (92.2% and 80.6%, respectively). In contrast, ASVs of non-Nematoda origin were extensively (40%−60%) present in the reverse JB3/JB4.5 and COIFGED/JB5GED primers (Fig. 4A). Within Nematoda, the morphological and 18S identifications were closest in recovering community taxon composition and abundance, except for Enoplida which was absent in the mock community but nevertheless recovered with 18S metabarcoding. Although

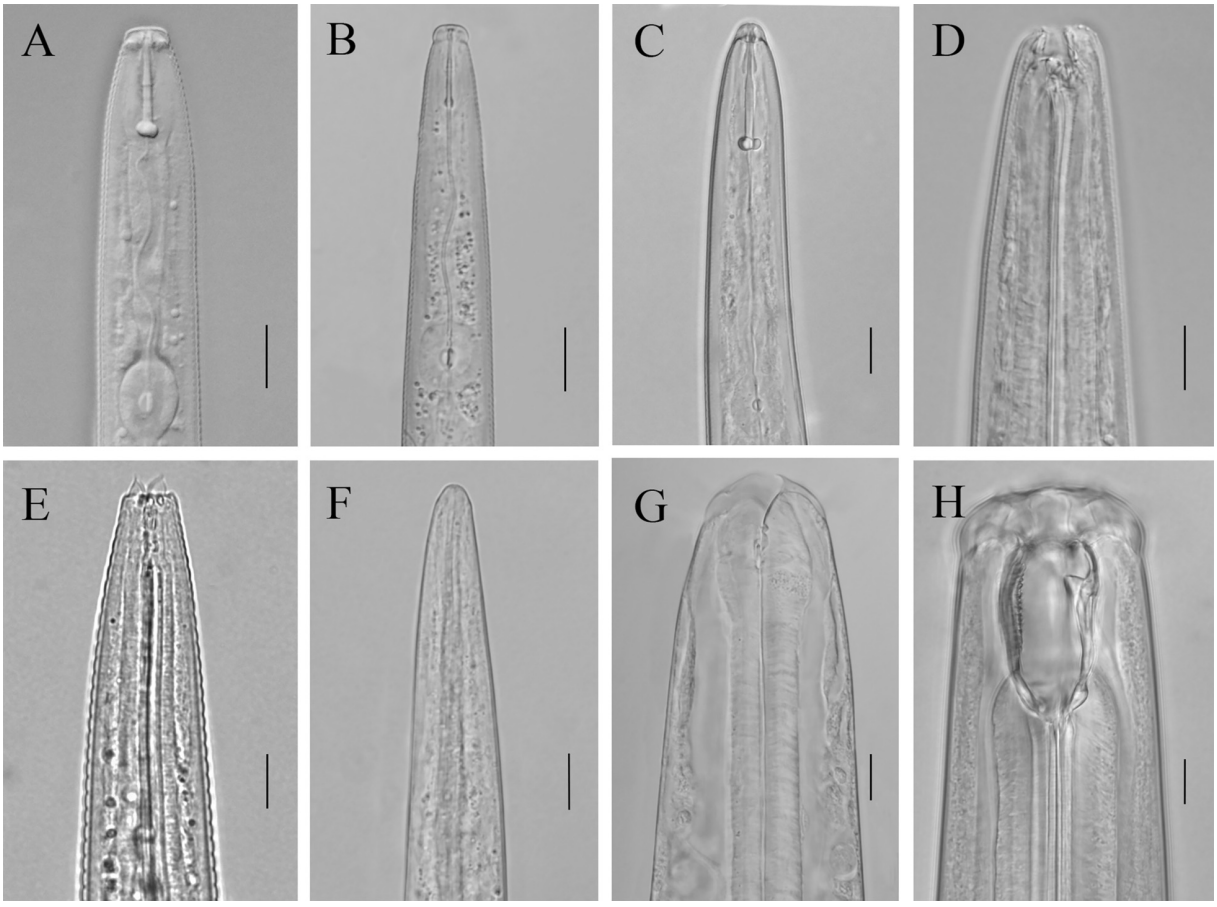


Fig. 1 Anterior body region of some of the nematode species used in this study. A: *Pratylenchus* sp. (Rhabditida); B: *Aphelenchoides* sp. (Rhabditida); C: *Helicotylenchus* sp. (Rhabditida); D: *Pristionchus* sp. (Rhabditida); E: *Acrobeloides* sp. (Rhabditida); F: *Alaimus* sp. (Enoplida); G: *Tripylina* sp. (Enoplida); H: *Prionchulus* sp. (Mononchida). (Scale bars: 10 μm).

the newly designed primer failed to amplify Triplonchida and Mononchida, it outperformed commonly used JB3/JB4.5 primers in profiling Dorylaimida, as this order was completely absent in results using the JB3/JB4.5 primer (Fig. 4B).

There were only 3 families consistently recovered across all primers and these included Aphelenchoididae, Hoplolaimidae, and Rhabditidae. The highest number of families shared with morphology was for 18S (13), followed by COIFGED/JB5GED (4), and JB3/JB4.5 (3) (Fig. 4C). The 18S NF1/18Sr2b primer recovered 18 families, 5 of which were unexpected (Alaimidae, Mononchidae, Neodiplogasteridae, Panagrolaimidae, Trichodoridae). The newly designed COIFGED/JB5GED primer recovered 8 families, with 4 expected (Longidoridae, Aphelenchoididae, Hoplolaimidae, Rhabditidae) and 4 unexpectedly presented in each of forward and reverse data sets (Panagrolaimidae, Strongyloididae, Dictyocaulidae, Filaroididae in forward data set and Panagrolaimidae, Heteroderidae, Angiostrongylidae, Filaroididae in reverse data set). Likewise, the universal primer JB3/JB4.5 recovered 5 families in forward with 2 unexpected families (Strongyloididae, Filaroididae), and 7 families in reverse data sets with 4 (Angiostrongylidae, Panagrolaimidae, Heteroderidae, Filaroididae) unexpected families.

At the genus level, the best performance was recovery of 23 out of 25 mock community species by 18S, followed by performance of COIFGED/JB5GED primers (14 and 12 for forward and reverse data sets, respectively), and that of JB3/JB4.5 primers (8 and 11 for the forward and reverse data sets, respectively). As with the family level annotations, not all recovered genera were present in the mock community. The 18S primer pair shared the highest number of genera with morphological identification (16), followed by newly designed COIFGED/JB5GED primers (with identification of 9 and 6 for forward and reverse data sets, respectively), and finally JB3/JB4.5 primers (6) (Fig. 4D). Among these commonly recovered genera, only *Bursaphelenchus*, *Caenorhabditis* and *Oscheius* were recovered in all tested primers. Six genera (*Mesodorylaimus*, *Dorylaimoides*, *Cephalobus*, *Pseudodiplogasteroides*, *Pratylenchus*, *Boleodorus*) were recovered by morphology but not metabarcoding.

3.4 The primers performance in abundance recovery

To evaluate the influence of abundance taxa recovery by different primers, the top 20 most abundant morphologically

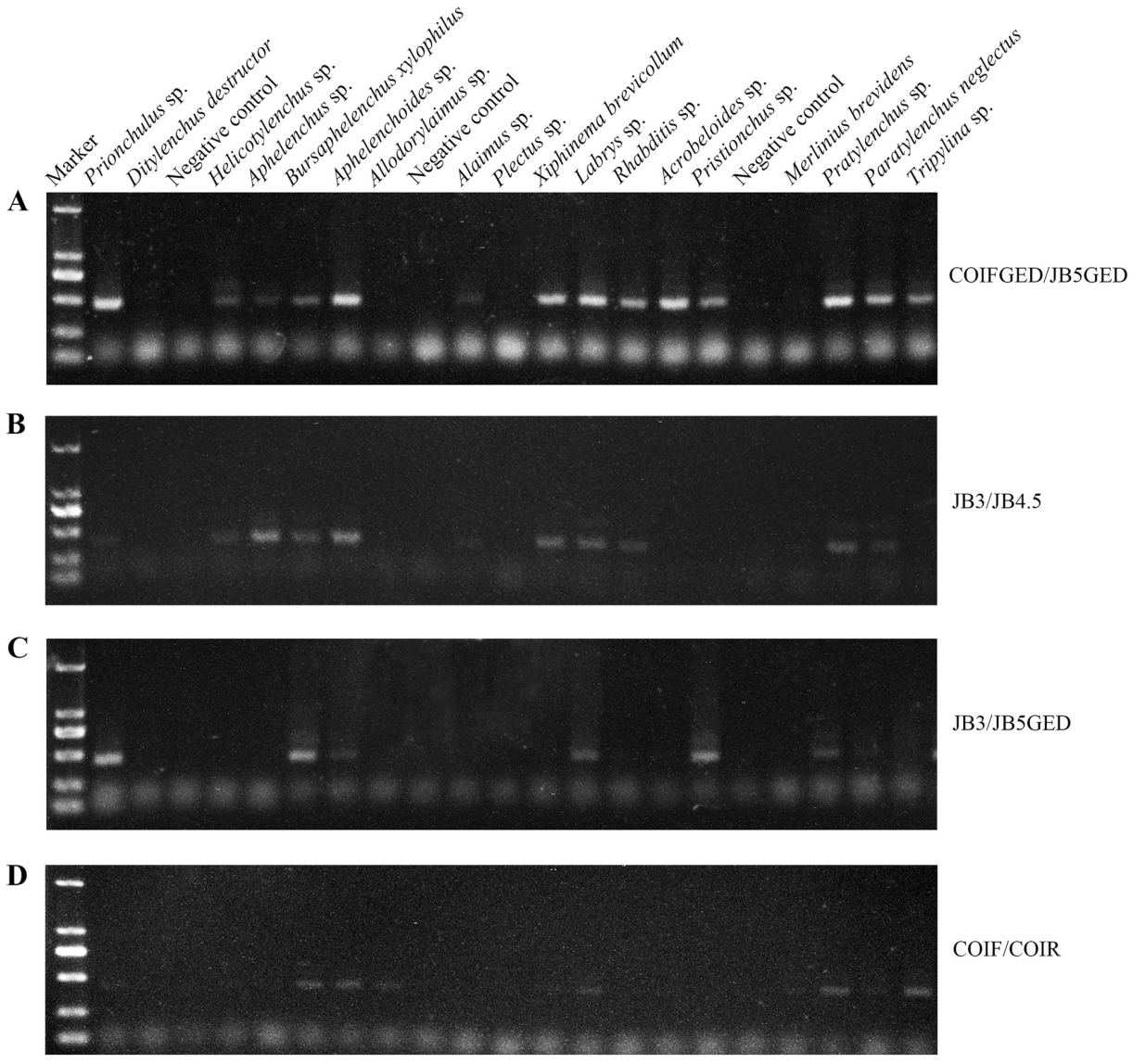


Fig. 2 Amplification products of 18 nematode species with COI primer pairs (A) COIFGED/JB5GED, (B) JB3/JB4.5, (C) JB3/JB5GED, and (D) COIF/COIR, respectively.

genera were examined (Fig. 5). In general, the relative abundances from morphological and metabarcoding data were not proportional, although 3 morphologically abundant genera remained abundant with metabarcoding (i.e., *Bursaphelenchus*, *Rotylenchus* and *Aphelenchoides*). In contrast, other genera deviated from the expected abundance. For example, *Eudorylaimus* and *Mylonchulus,* although abundant by morphology, represented only minor proportions in metabarcoding data sets, while the abundance of *Ditylenchus* and *Caenorhabditis* was exaggerated.

3.5 The analysis of COX1 problematic ASVs

At phylum level, two main issues with COX1 generated ASVs were encountered. First, among the non-Nematoda ASVs, the majority were classified as 'unidentified' (Fig. 4A). Secondly, several ASVs were classified as unexpected animal parasites (e.g., *Strongyloides*, *Perostrongylus* and *Dictyocaulus*) but with low similarity values (< 91%) suggesting potential errors or database coverage limitations.

We examined theses ASVs by checking their phylogenetic placements. Using the newly designed primers, we first generated 51 COI reference sequences (Table S3) covering 32 genera in 4 orders commonly encountered alongside soil nematode taxa. Seventeen of the previously 'unidentified' ASVs were sister to a known nematode reference or nested within a well-supported clade containing a known reference. Interestingly, the majority of these closely related references represented our mock species (*Eudorylaimus*, *Aporcelaimellus*, *Axonchium*, *Mylonchulus*, *Miconchus*, and *Aphelnchus)* confirming their presence in metabarcoding data but not recovered due to a limited COX1 reference database.

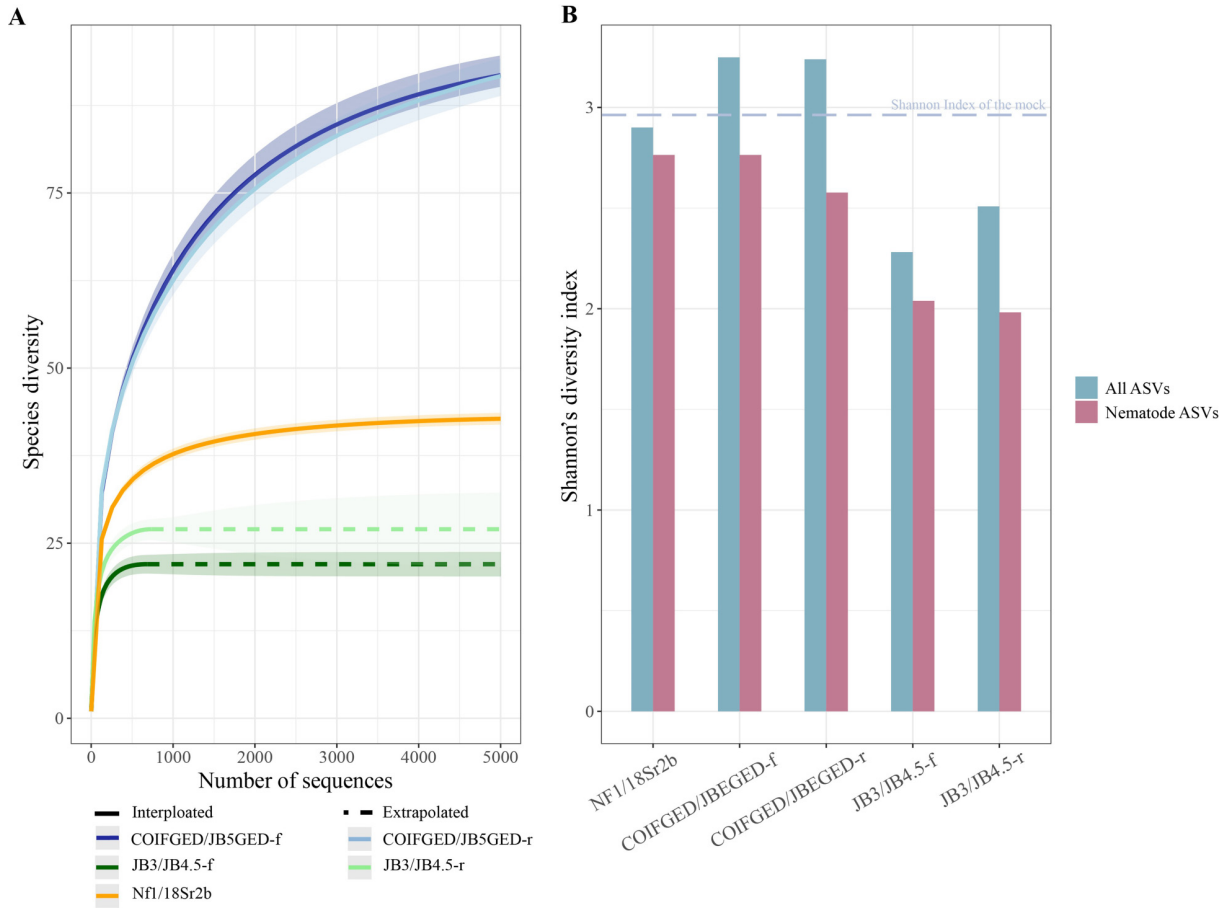


Fig. 3 Abundance and diversity of recovered ASVs of the mock community obtained by different primers. A: Rarefaction analysis for ASVs obtained by different primers with -r and -f indicating forward and reverse sequences. B: The comparison of ASVs Shannon diversity recovered by different primers from all-included ASVs data sets (all ASVs) and the Nematoda-only data sets (nematode ASVs). The dashed line represents the value of the Shannon index for this mock community.

Therefore, the new COX1 primer had a comparable performance to the 18S marker (16 out of 25 vs. 15 and 12 out of 25).

4 Discussion

The COI primer and molecular barcoding

The ability of a marker to recover as many taxa as possible could easily be one of the main benchmarks for determining its suitability for molecular barcoding. In this respect, the hyper-variable mitochondrial COX1 gene is a suitable gene to use for discriminating among closely related species or even phylogeographic groups within a single species (Cox and Hebert, 2001; Hebert et al., 2003). Despite the great advantage of COX1 based taxonomic profiling, its application has been largely limited due to the lack of available universal primer (Ahmed et al., 2019; Waeyenberge et al., 2019; Macheriotou et al., 2019). In this study, we introduced four degenerated sites in forward primer COIFGED, aiming to improve the primer binding efficiency for the hyper-variable COI gene. Together with existing reverse primer JB5GED (Derycke et al., 2007), the new primer pair outperforms the commonly used JB3/JB4.5 primers both in broader taxon recovery and higher amplicon production. The poor representation of COX1 sequences at the NCBI for Triplonchida (9 species), Mononchida (1) and Cephalobidae (1) can be partially attributed to the lack of appropriate primers. Our newly designed COX1 primer successfully amplified taxa within these orders (*Tripylina*, *Prionchulus*, and *Acrobeloides*) and thus offers a better option for future studies of some difficult taxa. Unfortunately, other taxa, including *Ditylenchus* and *Merlinius*, failed to be amplified irrespective of our new primers. These two genera have been problematic in other studies. For example, to amplify *Ditylenchus* either species-specific primers (Skwiercz et al., 2017) or targeted longer fragment (615 bp) rather than the most common I3-M11 partition of COX1 gene have been used (Vovlas et al., 2011). Similar difficulty has been observed for *Merlinius,* resulting in not even a single COX1 sequence available in the NCBI database regardless of its worldwide distribution and importance as a plant parasite. Consequently, special

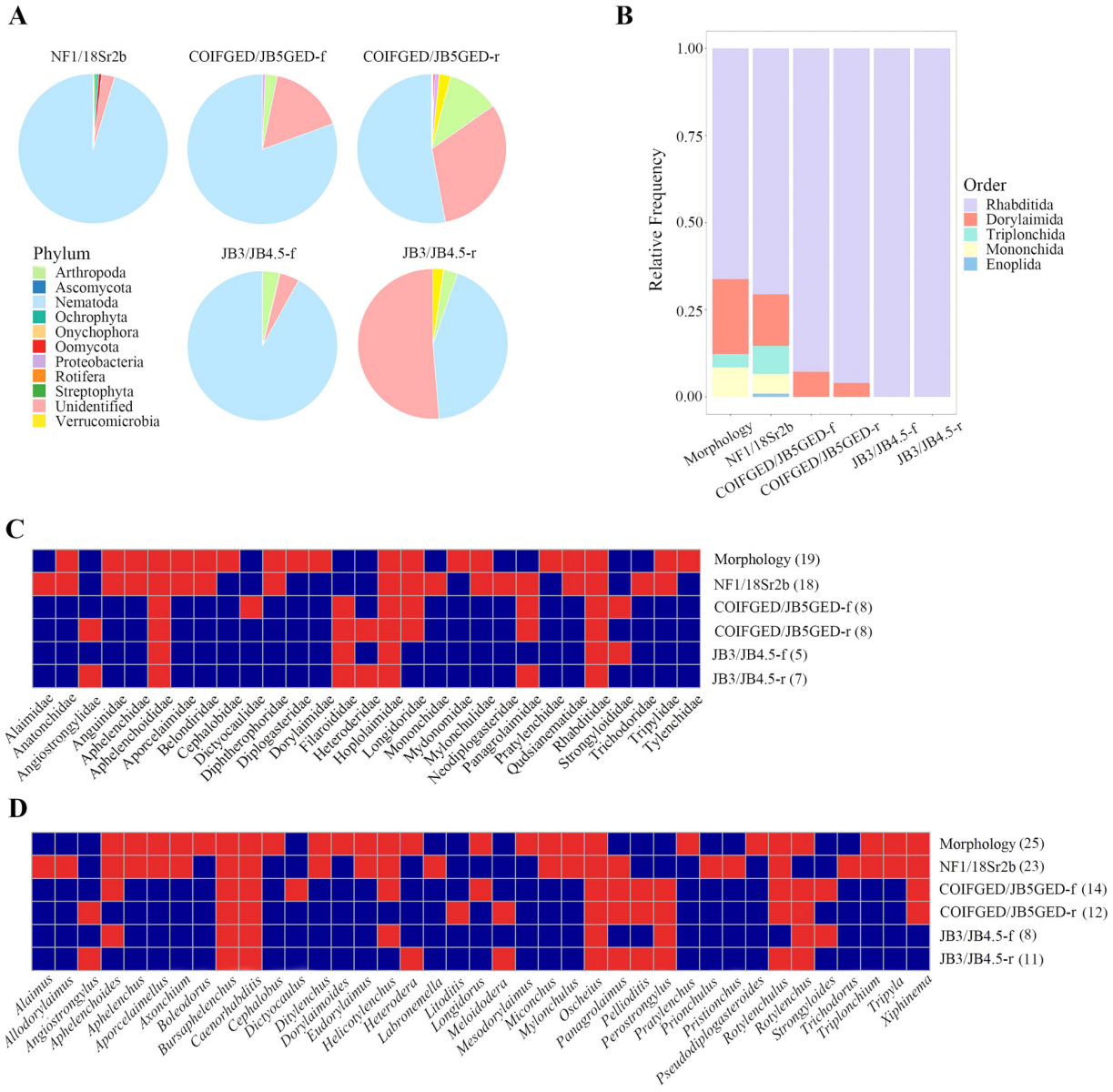


Fig. 4 Taxonomic profiles of the mock community obtained by different primer pairs. A: composition of all obtained ASVs annotated at the phylum level; B: relative abundance Nematoda-only ASVs annotated at the order level; C: identity profile of the recovered mock community at the family; D: genus levels. Red and blue colors indicate presence and absence, respectively. Notes on C and D: morphology indicates taxonomic profile based on morphological identification; the ASVs from COI primer are indicated with suffix-r for forward and -f for reverse direction. The numbers in brackets represent the number of nematode individuals included in the mock community.

efforts are needed to design efficient primers for *Ditylenchus* and *Melinius*, or the Dolichodoridae family in general.

The COI primer and metabarcoding

With the rapidly declining cost of high-throughput sequencing technology, metabarcoding has become an important tool in soil nematode community profiling (Porazinska et al., 2009; Kawanobe et al., 2021). These studies predominantly use 18S rRNA markers, as this allows development of universal primers that target a wide range of the nematode phylogenetic spectrum. However, 18S rRNA has been shown to provide limited taxonomic resolution, especially at species level (Kiewnick et al., 2014; Schenk et al., 2020) or even genus level for some nematode groups (e.g., dorylaims) (Holterman et al., 2008,Heydari et al., 2020). Alternatively, although research toward developing and testing universal COX1 primers JB3/JB4.5 has been conducted, low taxon recovery remains a major problem (Roelfsema et al., 2016). Indeed, high mutation rate and the lack of universal primers are among main challenges for successful amplification of the COX1 gene (Creer et al., 2010). However, the

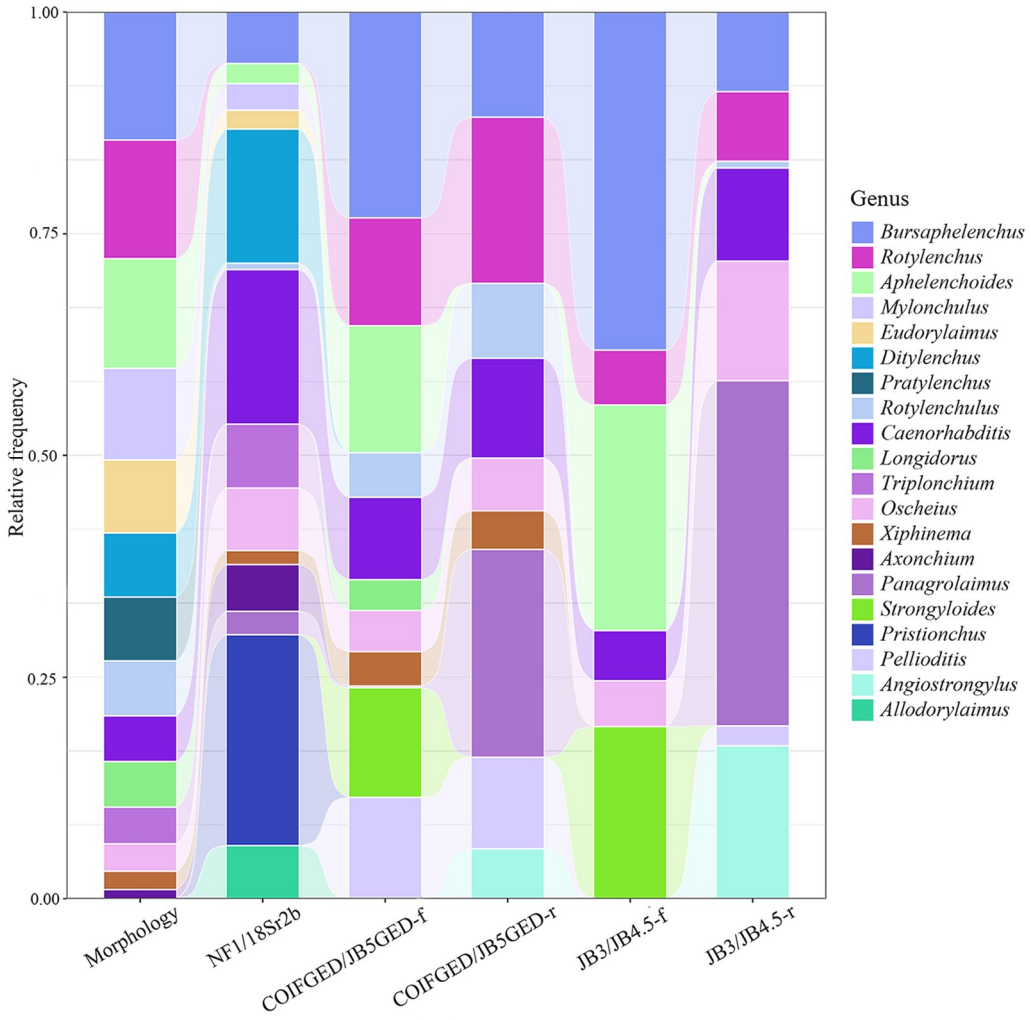


Fig. 5 The community profiles of 20 genera with highest overall morphological abundance. The shadow bands linking different bars indicate same genus recovered by different primers.

application of highly degenerated primers has been shown to improve taxonomic coverage and resolution (Elbrecht and Leese, 2017). In the present study, our COX1 primers still underperformed in comparison to the 18S primers in terms of taxon recovery, suggesting that further primer improvement may be difficult and/or limited. Consequently, PCR-free methods such as mitochondrial metagenomics could be more promising (Gendron et al., 2023). Nevertheless, in comparison to the commonly used JB3/JB4.5 primers, our newly designed COX1 primer significantly improved binding efficiency, and thus can be supplementary to the conventional 18S metabarcoding.

Overall, the relative abundances recovered from metabarcoding data were not proportional to morphological observation. Genera *Eudorylaimus* and *Mylonchulus* are huge in physical size and frequently presented in mock, but only represented a small proportion in metabarcoding data sets. Conversely, genera like *Ditylenchus* and *Caenorhabditis* have small to medium physical size but their relative abundances are exaggerated in metabarcoding. This result is contrary to the 18S based nematode metabarcoding which found relative read abundances well correlated with species biomass (Schenk et al., 2019). The variability in these recovered abundance values can be attributed to primer biases, as some nematode taxa lack an adequate primer binding site due to mutations in the primer binding regions, especially for the COX1 genes (Schenk et al., 2020).

Apart from primer binding difficulties, the major constraint for COX1-based metabarcoding is the extremely narrow scope of taxon coverage within available COI reference databases (Holovachov et al., 2017; Ahmed et al., 2019; Gendron et al. 2023), often resulting in a high number of unassignable sequences (e.g., Ahmed et al., 2019; Sikder et al., 2020). In this study, failures in recovery of taxa without COX1 sequences in the NCBI database (e.g., many representatives of Dorylaimida and Mononchida) were expected. Indeed, while we observed a significant proportion of unidentified ASVs, others were assigned unexpected taxa resulting in many mock community taxa being unrecovered. Based on a phylogenetic reconstruction, we confirmed that

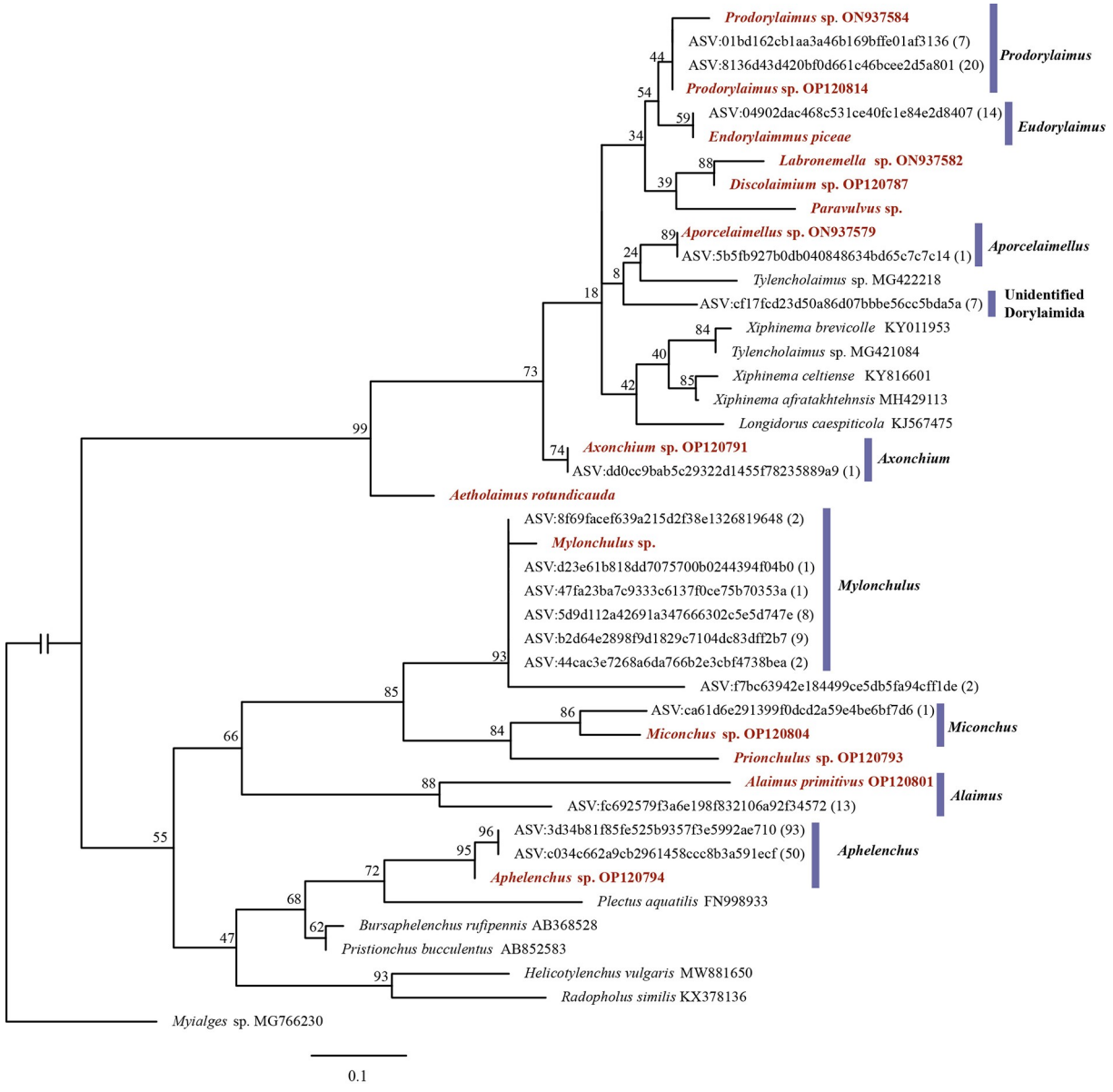


Fig. 6 The placements of unidentified ASVs in the COX1 tree inferred from the maximum likelihood phylogenetic analysis. The newly generated reference sequences are indicated in bold brown font. The numbers in brackets represent the number of reads within respective ASVs. The values at clade nodes indicate bootstrap support.

many of the unidentified ASVs had Nematoda origin emphasizing the importance of database coverage, and comprehensive work that will need to take place toward nematode COX1 database expansion before the COX1 gene can be used as a metabarcoding marker. A proportion of the non-Nematoda ASVs were assigned to soil eukaryotes such as Arthropoda and Ascomycota. These organisms may represent a part of nematode gut microbiomes (McQueen et al., 2022) as soil nematodes feed on a large variety of food sources (Waeyenberge et al., 2019), alternatively their degraded debris can be carried by nematode cuticles (McQueen et al., 2023).

The Illumina Miseq is the most popular platform for amplicon sequencing. Since this PE 250 platform generates 250 bp pair-end reads, the ideal amplicon should be less than 400 bp, excluding adapters. Our newly designed primer targeting c.a. 450 bp fragment of the COX1 gene is longer than other metabarcoding primers like NF1/18Sr2b for 18S (c.a. 380 bp) and D3A/D3B for D3 region of 28S (c.a. 315 bp) (Lehmitz and Decker, 2017). As a result, reads from two primer directions hardly overlap, causing difficulties in the process of merging reads. Given that 200 bp of the COX1 gene can carry substantial information, this constraint can be easily overcome by using single-end reads for annotation. Indeed, we demonstrated that these short COX1 sequences can be successfully annotated to species level when references are available. Therefore, the single-end reads obtained from the newly designed primer can serve as the

"minibarcode" in insects (Meusnier et al., 2008). Alternatively, the 450 bp fragment or even full-length COX1 amplicons can be sequenced through Illumina Miseq PE300 platform or long-read sequencing platforms like Oxford Nanopore and PacBio (Tedersoo et al., 2018; Matsuo et al., 2021).

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Conflict of interest

The authors declare no competing interests.

Electronic supplementary material

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