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Diversity and phylogeny of free-living marine nematodes from Singapore, including 20 new genus records for the Strait of Singapore

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Abstract. Free-living marine nematodes are small ubiquitous roundworms that inhabit nearly every ecosystem but are not well studied in several parts of the world. We collected sediment samples from two locations in the Strait of Singapore and analysed the nematode diversity with microscopy and molecular methods. The study revealed the presence of 20 genera previously unrecorded in the Singapore Strait, and two new records from Southeast Asian sediments, as well as an unusual abundance of epistrate-feeders.

Key words. meiofauna, roundworms, soft bottoms, diversity, phylogeny

INTRODUCTION

Free-living marine nematodes (Phylum Nematoda) are small ubiquitous roundworms that inhabit bottom sediments or fouling communities on soft and hard substrates (Tchesunov & Ivanenko, 2022). While Appeltans et al. (2012) estimated a total of 6,900 species of free-living marine nematodes existing globally (Tchesunov & Ivanenko, 2022), this phylum is poorly studied in many areas of the world, and the true number of species is likely much larger than current estimates. Marine free-living nematodes in Southeast Asia are known to have a high diversity, as demonstrated via both morphological and molecular methods (Shabdin et al., 2013; Armenteros et al., 2014; Macheriotou et al., 2019). In one study (Shabdin et al., 2013), a total of 111 species, included in 47 genera and 20 families, were identified from 24 sites along the coastal waters of Sarawak, and several new species have been described from Vietnam, Southeast China, Malaysia, and the Philippines (Rho & Kim, 2005; Shabdin et al., 2013; Tchesunov, 2013; Lu et al., 2022). Molecular analyses using 18S rDNA, 28S rDNA and cytochrome oxidase subunit I (COI) have demonstrated key phylogenetic patterns, such as the monophyly of the order Enoplida, sister of the order Chromadorida (Armenteros et al., 2014). At the family level, the main taxa found have been Desmodoridae, Comesomatidae, and Xyalidae (Armenteros et al., 2014), and at the subfamily level Stilbonematinae, Desmodorinae, and Spiriniinae (Tchesunov, 2013; Armenteros et al., 2014). Genera described from the region include *Catanema*, *Stilmbonema*, *Elzalia*, *Minolaimus*, *Setoplectus*, and *Tenuidraconema* (Rho & Kim, 2005; Tchesunov, 2013; Lu et al., 2022). These studies have revealed complex evolutionary relationships and potentially significant hidden diversity of free-living marine nematodes in Southeast Asian waters.

In this geographic area, only a few studies on free-living marine nematode diversity have been conducted in Singapore. The Singapore Strait is a relatively shallow and narrow waterway located inside the Sunda Shelf between the South China Sea and the Strait of Malacca (Tkalich et al., 2013). The nation of Singapore is one of the busiest ports in the world where the intense marine traffic leads to significant anthropic pressure and possibly disturbance for marine biodiversity (Chou, 2006; Sheppard, 2019). Although scarce, there is some information about nematodes in Singapore waters. For instance, one previous study (Neves et al., 2016) about the meiofaunal community in the area reported that common meiofaunal taxa, such as Copepoda and Nematoda, were found in all sites investigated, but the focus of the study was on Tardigrada, Kinorhyncha, and Rhabditophora. Other studies have been dedicated to describing new nematode species. Free-living *Setosabatieria singaporensis* was discovered from Chek Jawa by Chen & Long (2015), and two more species, *Prooncholaimus tani* and *Acanthonchus singaporensis*, were described from an intertidal sandy-rocky shore on Pulau Ubin by Chen et al. (2015). Finally, Ip et al. (2023) analysed cryptobiome diversity on Autonomous Reef Monitoring Structures deployed at four reef sites from 2016 to 2018. They identified nematodes as some of the rare taxa responsible for observed changes between sites because of their high levels of specialisation.

Knowing meiofaunal diversity is an essential element needed to understand the ecological relationships existing between species (Neves et al., 2016), in this paper, we

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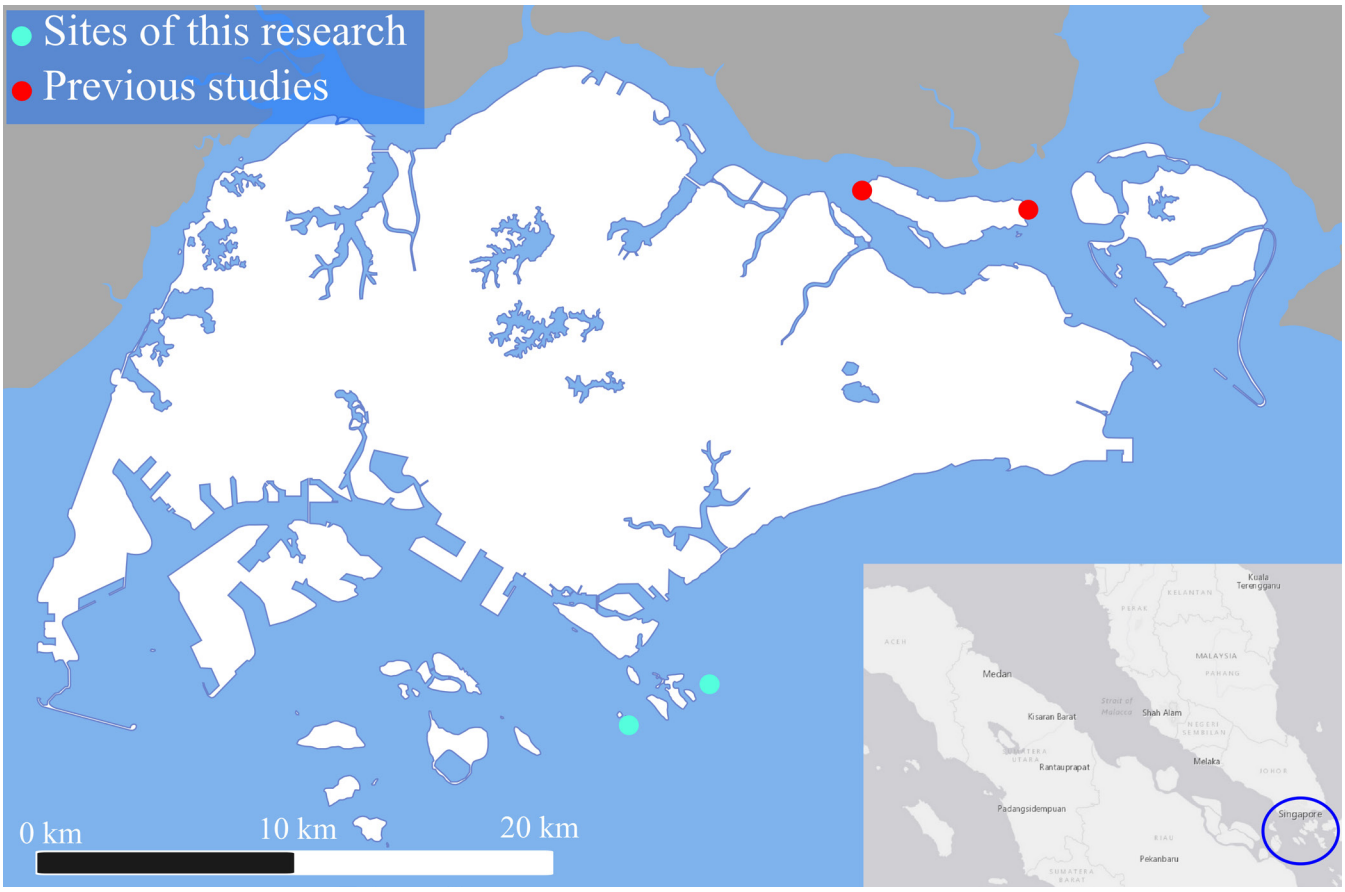

Fig. 1. Map of Singapore showing previous records of marine free-living nematodes (red), and the sites of this study (blue). Outline of Singapore. From "Singapore Outline.svg" by Seloloving, 2014, Wikimedia Commons.

analysed 15 replicates from two sites in the Singapore Strait through morphological and molecular methods. We provide a qualitative assessment of nematode richness from these subsamples of coastal sediment in an under-studied region, and provide new information on the diversity, distribution, and phylogenetic relationships among free-living marine nematodes in the Singapore Strait based on specimens obtained from a recent collection.

MATERIAL AND METHODS

Specimen collection. Five sediment samples were collected on 25 and 26 June 2023 from the Singapore Strait at depths between 3 m and 11 m: two from Pulau Subar Laut (1°12′44.92″N, 103°50′10.36″E) and three from Kusu Island (1°13′32.45″N, 103°51′35.65″E) (Fig. 1, Table 1; Seloloving, 2014). Sediment samples were collected while SCUBA diving by pushing a manual corer (50 ml, 10 cm H) vertically into the sediment for a total of 15 replicates. Each of these sediment samples consisted of three replicates of a volume of 50 ml each, the sediment was immediately fixed in 70% ethanol and stored at −4 °C. The sediment was then filtered through a 2 mm mesh sieve to eliminate coral rubbles and rocks. Each sample was divided and transferred into four Falcon® tubes that were filled with a mix of 2 parts of Ludox® (Burgess, 2001) and 3 parts of distilled water, centrifuged at 3,000 g for 10 minutes and washed again. Finally, the supernatant was filtered with a 45 µm sieve. This process was repeated three times to maximise the number of nematode specimens collected.

Specimen identification (trophic groups, colonisers-persisters). All nematode specimens were picked up from all replicates, counted, and identified at the lowest taxonomic level possible under a stereomicroscope (LEICA S8AP0) using temporary slides. The nematodes were first identified at the family or genus-level using the pictorial keys from Platt & Warwick (1983), Platt & Warwick (1988), Warwick et al. (1998), and the World Database of Nematodes (Nemys, 2025https://nemys.ugent.be/) and delimitated into Operational Taxonomic Units (OTUs). All nematodes were then categorised into four trophic groups according to the division made by Wieser (1953): selective deposit feeders (1A), non-selective deposit feeders (groups 1B), epistrate feeders (2A), and predators/omnivores (groups 2B), and divided based on the coloniser–persister (c-p) scale (Bongers, 1990). The c-p scale includes species from colonisers/r-strategist nematodes (level 1) to persisters/K-strategist nematodes (level 5), which reflect the life strategies and sensitivity to disturbance of each species (Bongers, 1990; Bongers & Bongers, 1998; Ferris et al., 2001).

Phylogenetic analyses. Next, we performed molecular analyses on the morphologically identified specimens to confirm their identity and infer phylogenetic relationships

Table 1. List of environmental variables of each sampling location. Temperature and salinity were not measured on site and were obtained from Sheppard (2019) and Sin et al. (2016).

Sample	Coordinates	T (°C)	S (ppt)	Depth (m)	Sediment	No. nematodes	No. genera
Kusu Island 1	1.2257° N, 103.8599° E	30.5	24	3.7	Silt	34	9
Kusu Island 2	1.2257° N, 103.8599° E	30.5	24	10.2	Silt	19	4
Kusu Island 3	1.2257° N, 103.8599° E	30.5	24	6.3	Silt	11	2
Pulau Subar Laut 1	1.2125° N, 103.8362° E	30.5	24	3.6	Silt	47	9
Pulau Subar Laut 2	1.2125° N, 103.8362° E	30.5	24	5.1	Silt	38	2

among families and genera. The specimens were transferred into 1.5 ml tubes containing 180 µl of ATL buffer from a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), and we followed the manufacturer's DNA extraction instructions until the final elution. The final elution was repeated twice for a final volume of 50 µl.

The successfully extracted samples were amplified using three markers: nuclear 18S ribosomal DNA (18S) and 28S ribosomal rDNA (28S), and mitochondrial cytochrome c oxidase subunit I (COI). These three markers are commonly used for nematode identification and diversity studies. 18S is universal in all eukaryotes (Kounosu et al., 2019; Knot et al., 2020; Nisa et al., 2022) and contains both conserved and variable regions (Ahmed et al., 2019; Knot et al., 2020). 28S is a standard phylogenetic marker for plant-parasite nematodes and provides more resolution than the 18S (Kounosu et al., 2019; Schenk et al., 2020; Nisa et al., 2022). COI was chosen because it has high species-level resolution for nematodes (Derycke et al., 2010; Knot et al., 2020; Nisa et al., 2022) and accordingly is a standard marker for DNA barcoding of several metazoan taxa (Derycke et al., 2010).

To amplify nuclear 18S, we used the primer pair Nem18SF (5′-ATTCCGATAACGARCGAGAC-3′)/Nem18SR(5′-CCGCTKRTCCCTCTAAGAAGT-3′) (Wood et al., 2013) and for 28S, D2A (5′-ACAAGTACCGTGAGGGAAAGT-3′) and D3B (5′-TGCGAAGGAACCAGCTACTA-3′) (Nunn, 1992). The optimised PCR cycling conditions for 18S and 28S were an initial denaturation of 5 min at 94°C, followed by 40 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1 min, then followed by a final extension of 10 min at 72°C. Additionally, COI fragments were amplified using JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′)/JB5 (5′-AGCACCTAAACTTAAAACATAATGAAAATG-3′) (Hu et al., 2002). Furthermore, the optimised PCR cycling conditions for COI were an initial denaturation of 5 min at 94°C, followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1 min, then followed by a final extension of 10 min at 72°C. Each 25 µl PCR reaction for the three markers consisted of 2.5 µl of 10X Standard Taq Reaction Buffer, 0.5 µl of 10 mM dNTPs, 0.5 µl of forward primer, 0.5 µl of reverse primer, 0.125 µl of Taq DNA polymerase, 1.0 µl of MgCl, 17.875/18.375 µl of ultra-pure RNA-free water, and 2.0/1.5 µl of raw DNA. PCR products were visualised on a 1.2% agarose gel, and successful amplifications were purified using Exonuclease I- Shrimp Alkaline Phosphatase (ExoSAP) digestion and sent for Sanger sequencing in both directions to Fasmac (Kanagawa, Japan).

The quality of the obtained sequences was assessed in Geneious Prime 2023.2 (Kearse et al., 2012); short sequences (<600 bp for 18S, 28S, and <300 bp for COI) and/or of low sequence quality (<80%) were discarded. Forward and reverse sequences were assembled and trimmed using the same software. We obtained an initial identification of the species by BLAST (Basic Local Alignment Search Tool; Altschul et al., 1990) to match our sequence data against GenBank sequences and compared the results with our preliminary fast morphological identification. MEGA11 (Molecular Evolutionary Genetics Analysis version 11; Tamura et al., 2021) was used to create a reference database of the taxa identified. The sequences were chosen based on correspondence to the ones obtained with this study and preliminary morphological examination, on their length (>700 bp for 18S and 28S, >400 bp for COI), presence of clear and valid taxonomic annotation, and with the exclusion of redundant sequences. Next, the sequences were aligned using MUSCLE (MUltiple Sequence Comparison by Log-Expectation; Edgar, 2004) in MEGA11 with default parameters and the ends of the resulting alignments were trimmed to the same length and concatenated. In addition, previously reported 18S rDNA (n=21), 28S rDNA (n=21), COI mtDNA (n=10), and concatenated (n=10) sequences of marine nematodes and 3 non-nematode species as an outgroup from GenBank were included in our respective databases (S1).

The best substitution model for each gene (GTR + G) was selected with the final alignment, and a Maximum Likelihood tree was reconstructed with a 1,000 bootstrap substitution rate, using as parameters 'complete deletion' by using R software (R Core Team, 2021). We obtained a tree for each primer set separately and one for the concatenated sequences. The obtained trees were visualised with FigTree version 1.4.4 (Rambaut et al., 2018) and edited with Inkscape version 1.3.2 (Inkscape Project, https://inkscape.org/).

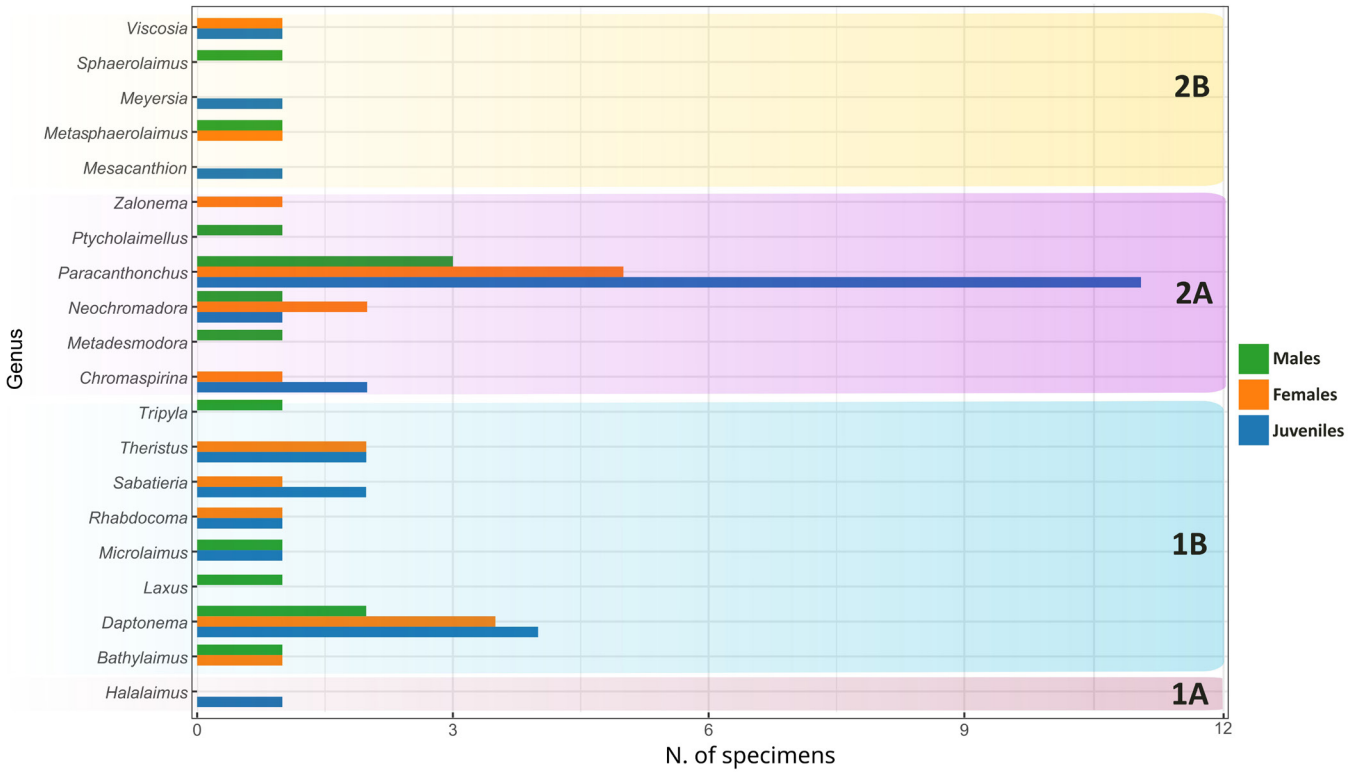

Fig. 2. Histogram showing genera found in this study, divided based on feeding strategy (2B=omnivores/predators, 2A=epistrate-feeders, 1B=non-selective deposit-feeders, and 1A=selective deposit-feeders), coloniser-persisters value (1, 2=colonisers, 3=intermediates, 4=persisters).

RESULTS

As a result of the sorting, a total of 149 nematodes were retrieved from all the samples: 47 and 38 individuals from the two Pulau Subar Laut samples, and 34, 19, and 11 individuals from the three Kusu Island samples, respectively (Table 1). 118 specimens were morphologically identified to the family or genus level, while we were unable to determine the identity of 31 specimens due to their small size or poor conditions. We proceeded to extract the DNA of each of the 118 specimens and analysed them using the 18S, 28S, and COI marker sequences. The 18S final alignment consisted of 107 sequences, of which 93 were newly obtained from this study (25 sequences could not be amplified) and from 25 specimens) resulting in 56 Molecular Operational Taxonomic Unit (MOTUs; Tables S1, S2). The 28S final alignment consisted of 117 sequences of which 93 were sequences from this study (again sequences could not be amplified from 25 specimens) resulting in 63 MOTUs (Tables S1, S2), and the COI final alignment consisted of 98 sequences, of which 77 were sequences from this study (41 specimens not amplified) and 50 MOTUs (Tables S1, S2).

Among these, we were able to identify 63 specimens both morphologically and with the three genetic markers (Fig. 2, Table 1). 86 specimens in total were not included in the final tree, of which 55 specimens were excluded because we could not obtain sequences from all three markers. Of the specimens identified both morphologically and with the three genetic markers, juveniles were most common (28 specimens), followed by females (21), and males (14). Regarding feeding, epistrate-feeders were more frequent (29 specimens), followed by non-selective deposit feeders (26) and omnivores/predators (7), with only 1 selective feeder specimen observed (Table 1).

The final concatenated alignment (18S, 28S and COI) consisted of 99 sequences (71 newly obtained sequences and 28 NCBI sequences of each marker), 63 taxa (50 from this study and 13 from the NCBI database;), and it was composed of 42 MOTUs (accession numbers 18S=PV748047–PV748088, PV778078–PV778080; 28S= PV753920–PV753958, PV778719–PV778721; COI= PV785693–PV785729, PV770015–PV770016, PV771502–PV771504; Tables S1, S2). Among the 99 specimens that we were able to identify with both morphological and molecular methods (Table 1, Fig. 2), the main families found were Cyatholaimidae (n=19, MOTUs=9), Xyalidae (n=18, MOTUs=9), and Desmodoridae (n=6, MOTUs=6), and the three most common genera found were *Paracanthonchus* (Cyatholaimidae, 20 specimens, MOTUs=9), *Daptonema* (Xyalidae, 14 specimens, MOTUs=6), and *Theristus* (Xyalidae)/*Neochromadora* (Chromadoridae)—(4 specimens each, MOTUs=3; 2 respectively).

13 genera were found in Kusu Island, and 10 in Pulau Subar Laut (Fig. 2, Table 1). The genus *Paracanthonchus* (Cyatholaimidae) was the most common overall, and it was present on both sites, as well as *Sabatieria* (Comesomatidae) and *Theristus* (Xyalidae).

The three individual gene trees (Figs. S3, S4, S5) and the concatenated phylogenetic tree (Fig. 3) showed low support values (<70%) for most of the nodes, although at

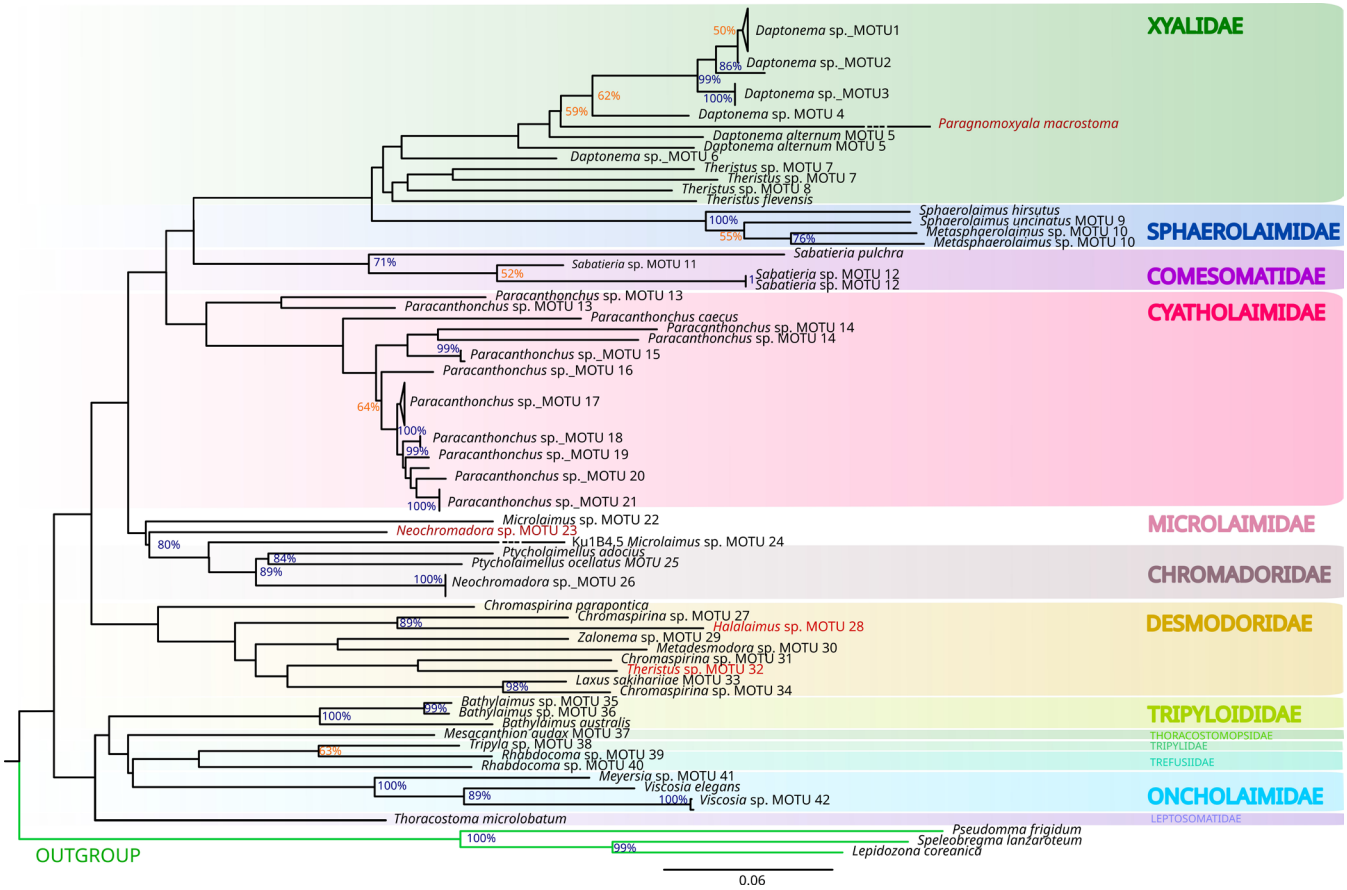

Fig. 3. Phylogenetic tree for the concatenated datasets (18S, 28S and COI), generated by Maximum Likelihood. Numbers at the branches represent bootstrap values. Values between 70 and 100% are shown in blue. Values between 50 and 69% are shown in orange. Values below 50% are not shown.

the genus-level many nodes were well supported (>75%). Although not well supported, the concatenated maximum likelihood tree (Fig. 3) revealed that *Daptonema* and *Theristus* formed a clade together, while specimens of the family Sphaerolaimidae (*Sphaerolaimus* and *Metasphaerolaimus*) constituted a sister group. *Daptonema* included at least three well supported MOTUs. Some families only included one genus: Comesomatidae (*Sabatieria*), Tripyloididae (*Bathylaimus*), Thoracostomopsidae (*Mesacanthion*), Tripylidae (*Tripyla*), Trefusiidae (*Rhabdocoma*), and Leptosomatidae (*Thoracostoma*). The families Microlaimidae and Chormadoridae were polyphyletic but with low support values, and *Neochromadora* appeared in both families. The family Desmodoridae was one of the most diverse with four genera recorded (*Chromaspirina, Zalonema, Metadesmodora*, and *Laxus*). Finally, the family Oncholaimidae had two genera observed in our specimens: *Meyersia* and *Viscosia*.

DISCUSSION

This study represents a preliminary attempt to record and understand the free-living marine nematode community structure of subtidal sites in the Singapore Strait, using a combination of morphological and molecular tools.

Phylogenetically, both the individual gene trees (Figs. S3, S4, S5) and concatenated tree (Fig. 3) returned results similar to previous studies (Bik et al., 2010a, b; Derycke et al., 2010; Pereira et al., 2010; Armenteros et al., 2014; Kumar et al., 2015; Avó et al., 2017), and the topology of the resulting trees distinguished the two classes: Enoplea and Chromadorea (Blaxter et al., 1998; Smythe et al., 2019; Shimada et al., 2023; Lim et al., 2024). In this study, the phylogenetic relationships within genera were well supported (with bootstrap values mostly >95%). On the other hand, most other support values were globally low (~70% or lower), especially at family level and above, indicating uncertainty of the phylogenetic relationships among these groups (Wiesemüller & Rothe, 2006). Possible causes of the low support of these phylogenetic signals could be insufficient number of sequences and/or conflicting character patterns in our datasets (Soltis & Soltis, 2003). Increasing the number of informative characters and genes (Soltis & Soltis, 2003) and enhancing the number of specimens would improve support values in future analyses. For these reasons, the relationships at the family level and above recovered here should be considered tentative, and more data are necessary to clarify the phylogenetic patterns among free-living marine nematodes.

The tree showed four cases of species nested in clades where they were not expected to belong (*Paragnomoxyala macrostoma*, *Neochromadora* sp., *Halalaimus* sp., and *Theristus* sp.). This unexpected topology likely reflects either evolutionary complexity and convergent evolution within

Table 2. List of Operational Taxonomic Units (OTUs). J=Juveniles, F=females, M=males, FS=Feeding strategy (1A=selective deposit-feeder, 1B=non-selective deposit-feeder, 2A=epistrate-feeder, 2B=omnivorous/predators). C-P=coloniser-persister scale (from 1 to 5). Ku=Kusu Island; Pu=Pulau Subar Laut.

MOTUs	Name	Family	J	F	M	FS	CP	Specimens
MOTU1	*Daptonema* sp.	Xyalidae	2	2	1	1B	2	Ku1B4.8, Ku1B3.3, Ku2B2.1, Ku2B3.4, Ku2B3.6
MOTU2	*Daptonema* sp.	Xyalidae	0	1	0	1B	2	Ku1B4.2
MOTU3	*Daptonema* sp.	Xyalidae	1	0	0	1B	2	Ku1B4.12
MOTU4	*Daptonema* sp.	Xyalidae	1	0	0	1B	2	Ku1B2.8
MOTU5	*Daptonema alternum*	Xyalidae	1	0	1	1B	2	Ku1B4.13B, Ku2B2.6
MOTU6	*Daptonema* sp.	Xyalidae	0	1	0	1B	2	Ku1B3.9
MOTU7	*Theristus* sp.	Xyalidae	1	1	0	1B	2	Pu1B3.13, Ku1B4.7
MOTU8	*Theristus* sp.	Xyalidae	1	0	0	1B	2	Pu1B1.15
MOTU9	*Sphaerolaimus uncinatus*	Sphaerolaimidae	0	0	1	2B	4	Ku1B3.7
MOTU10	*Metashaerolaimus* sp.	Sphaerolaimidae	0	1	1	2B	4	Ku1B2.2, Ku1B2.1
MOTU11	*Sabatieria* sp.	Comesomatidae	0	1	0	1B	2	Pu1B1.16
MOTU12	*Sabatieria* sp.	Comesomatidae	1	1	0	1B	2	Pu1B1.6, Ku1B2.3
MOTU13	*Paracanthonchus* sp.	Cyatholaimidae	2	0	0	2A	3	Pu2B1.11, Pu1B1.8
MOTU14	*Paracanthonchus* sp.	Cyatholaimidae	1	1	0	2A	3	Pu2B2.6, Pu1B3.3B
MOTU15	*Paracanthonchus* sp.	Cyatholaimidae	0	1	1	2A	3	Pu1B1.14, Pu1B1.7
MOTU16	*Paracanthonchus* sp.	Cyatholaimidae	1	0	0	2A	3	Ku1B4.9
MOTU17	*Paracanthonchus* sp.	Cyatholaimidae	2	2	1	2A	3	Pu1B1.13, Ku1B3.5, Pu1B2.2, Pu1B3.2, Pu2B3.1
MOTU18	*Paracanthonchus* sp.	Cyatholaimidae	2	0	0	2A	3	Pu1B2.11, Pu1B2.1
MOTU19	*Paracanthonchus* sp.	Cyatholaimidae	1	0	1	2A	3	Pu2B2.16B, Pu2B2.2B
MOTU20	*Paracanthonchus* sp.	Cyatholaimidae	1	0	0	2A	3	Pu1B2.4
MOTU21	*Paracanthonchus* sp.	Cyatholaimidae	1	1	0	2A	3	Pu1B3.2B, Pu1B1.14
MOTU22	*Microlaimus* sp.	Microlaimidae	0	0	1	1B	2	Ku3B2.2
MOTU23	*Neochromadora* sp.	Chromadoridae	0	1	0	2A	3	Ku3B1.2
MOTU24	*Microlaimus* sp.	Microlaimidae	1	0	0	1B	2	Ku1B4.5
MOTU25	*Ptycholaimellus ocellatus*	Chromadoridae	0	0	1	2A	3	Ku2B2.2
MOTU26	*Neochromadora* sp.	Chromadoridae	1	1	1	2A	3	Ku3B3.2, Ku3B2.4, Ku3B2.3
MOTU27	*Chromaspirina* sp.	Chromadoridae	0	1	0	2A	3	Pu1B3.4B
MOTU28	*Halalaimus* sp.	Halalaimidae	1	0	0	1A	1	Pu1B3.14
MOTU29	*Zalonema* sp.	Desmodoridae	0	1	0	2A	3	Pu1B3.11
MOTU30	*Metadesmodora* sp.	Desmodoridae	0	0	1	2A	3	Ku1B4.11
MOTU31	*Chromaspirina* sp.	Desmodoridae	1	0	0	2A	3	Pu2B2.10
MOTU32	*Theristus* sp.	Xyalidae	0	1	0	1B	2	Pu1B3.16B
MOTU33	*Laxus sakihariiae*	Desmodoridae	0	0	1	1B	2	Pu1B3.12
MOTU34	*Chromaspirina* sp.	Desmodoridae	1	0	0	2A	3	Pu1B2.5
MOTU35	*Bathylaimus* sp.	Tripyloididae	0	0	1	1B	2	Ku2B2.4
MOTU36	*Bathylaimus* sp.	Tripyloididae	0	1	0	1B	2	Ku1B4.14

MOTUs	Name	Family	J	F	M	FS	CP	Specimens
MOTU37	*Mesacanthion audax*	Thoracostomopsidae	1	0	0	2B	4	Pu1B2.3
MOTU38	*Tripyla* sp.	Tripylidae	0	0	1	1B	2	Pu1B2.14
MOTU39	*Rhabdocoma* sp.	Trefusidae	0	1	0	1B	2	Pu1B1.1B
MOTU40	*Rhabdocoma* sp.	Trefusidae	1	0	0	1B	2	Pu1B1.1
MOTU41	*Meyersia* sp.	Oncholaimidae	1	0	0	2B	3	Ku1B4.15
MOTU42	*Viscosia* sp.	Oncholaimidae	1	1	0	2B	3	Ku2B1.7, Ku1B3.6

the phylum Nematoda (van Megen et al., 2009) and/or phylogenetic inference artefacts like limited marker coverage or incomplete sorting (Holterman et al., 2006), suggesting that the topology might not reflect true taxonomic relationships.

The family Desmodoridae appears to be paraphyletic based on several lines of molecular evidence (Leduc et al., 2018; Ahmed et al., 2022). Furthermore, taxa that are morphologically distinct and traditionally assigned to different families may appear phylogenetically closer and cluster within the same family (van Megen et al., 2009; Armenteros et al., 2014; Leduc & Zhao, 2016). Convergent evolution is a particularly important factor and has been described as one of the most relevant phenomena observed in phylum-wide analyses (van Megen et al., 2009; Armenteros et al., 2014; Leduc et al., 2018). This suggests that morphological features traditionally used to define family-level distinctions may have evolved independently multiple times (Armenteros et al., 2014), leading to misidentifications (Blaxter et al., 1998; Tchesunov, 2013) or cases where taxa that are morphologically very distinct share a common ancestry (Leduc & Zhao, 2016). Similarly, the presence of *Paragnomoxyala macrostoma* without the genus *Daptonema* can be explained by the documented paraphyly of the genus itself (Neres et al., 2010) known for synonymising related genera within the family Xyalidae (e.g., *Trichotheristus*; Arythaka & Kito, 2018). On the other hand, *Neochromadora* has consistently been placed in the family Chromadoridae (e.g., Russo & Eard, 2012; Venekey et al., 2019) which is phylogenetically distinct from Microlaimidae, suggesting the odd placement here may be an artefact (Holterman et al., 2006).

Moreover, when comparing our morphological and molecular results, we found that both methods when applied to these unstudied areas resulted in similar identifications. However, due to the low number of free-living marine nematode sequences publicly available, and the consequent low percentages of similarity obtained after blasting the sequences (Table S2), morphological examination was still a necessary step for identification. Juveniles and females were generally more frequent than males, which also further hindered accurate morphological identification to the species level. Although females are generally more common in many species, male features such as spicules, gubernaculum, and pre-cloacal supplements are more useful and sometimes diagnostic because of their high interspecific morphological variability in nematodes (Mokievsky et al., 2024).

As a practical compromise for environmental monitoring, identification at the genus level is standard practice as it is considered to be sufficient for assessing community structure and functional diversity (De Ley et al., 2005; Bredtmann et al., 2017; Schenk et al., 2020). For detailed taxonomic work, such as identifying cryptic and pathogenic species, accurate species-level identification is required (De Ley et al., 2005; Bredtmann et al., 2017; Schenk et al., 2020). Nevertheless, especially in biodiverse and little studied regions, even broad examination at the genus level is a first step in documenting the existing and undescribed species of the area, as this provides morphological and molecular directions for future efforts (Vicario et al., 2024).

In our study, the most abundant genus was *Paracanthonchus* (Table 2; Fig. 2), which is cosmopolitan and has been reported in association with different substrates, mostly in shallow water areas including brackish waters and lagoons (Gourbault, 1980; Miljutina & Miljutin, 2015). The high morphological variances within *Paracanthonchus* (Miljutina & Miljutin, 2015), as well as its overlapping traits with other genera in the same family (Miljutina & Miljutin, 2015) makes identification challenging and suggests that this genus may not be monophyletic (Miljutina & Miljutin, 2015; Kim et al., 2023). In this study, 19 *Paracanthonchus* specimens were identified through morphology mainly due to their long size (adults >1.5 mm), cylindrical pharynx and thin bulb, conical tail presenting sparse somatic setae, punctuated cuticle with longitudinal rows, fovea spiraled in at least five turns, and six lips forming a cup-shaped buccal cavity armed with five teeth, of which one was a prominent large dorsal tooth (Nemys, 2025; https://nemys.ugent.be/). The specimens corresponded to nine *Paracanthonchus* MOTUs, with three of them well supported in our molecular phylogenetic trees. In East Asia, the *Paracanthonchus* has been recorded previously only in Hong Kong (Kim et al., 2023).

The second-most abundant genus was *Daptonema* (Table 2, Fig. 2). This genus is common worldwide, but in Southeast Asia, has been recorded only from Thai mangroves and mudflat areas of the river Samut Sakhon (Sawangarreruks et al., 2011) and from muddy and sandy intertidal zones in Malaysia (Turpeenniemi et al., 2001; Long & Ross, 2008). As a deposit feeder, this genus is particularly abundant in the first layers of sediment, where organic matter is more concentrated (Soetaert & Heip, 1995). The genus *Theristus* (Table 2) is also frequently recorded in benthic environments,

from intertidal zones to the deep sea (Soetaert & Heip, 1995; Baia & Venekey, 2019). The genus *Neochromadora* (Table 2) has previously been recovered from inland water bodies of Central and Southern Vietnam (Tran et al., 2017). All the 20 genera found in this study represent new records for Singapore, and *Mesacanthion* and *Tripyla* are new records from Southeast Asia.

The previously reported nematodes in Singapore (*Setosabatieria singaporensis*, *Prooncholaimus tani*, and *Acanthonchus singaporensis*) were not found in this study. Possible causes of their absence might be related to a combination of different factors between this and the previous studies such as microhabitats (seagrass/sandy-rocky bottom vs. sandy soft bottom; Ndaro & Ólafsson, 1999; Netto et al., 1999; Armenteros & Ruiz-Abierno, 2015; Ruiz-Abierno & Armenteros, 2016; Song et al., 2022), location (North vs. South of Singapore; Corinaldesi et al., 2022; Grassi et al., 2023), depths (intertidal vs. sublittoral; Semprucci et al., 2018; Grassi et al., 2023;) and seasonality (October/November vs. June; Yodnarasri et al., 2008). For instance, *Setosabatieria singaporensis* was found at an intertidal seagrass area in Chek Jawa (1°24′44.88″N, 103°59′42.66″E), Pulau Ubin, on 17 October 2012 (Chen & Long, 2015). *Prooncholaimus tani* and *Acanthonchus singaporensis* were collected from a small intertidal sandy-rocky beach at Tanjung Tajam (1°25′24.48″N, 103°55′35.34″E; Outward Bound School), Pulau Ubin, from 15 October to 2 November 2012 (Chen et al., 2015).

Most of the nematodes found in this study were epistrate-feeders (due to the high abundance of *Paracanthonchus*) and non-selective deposit feeders (Fig. 2, Tables 1, 2). This is unusual and unexpected because epistrate-feeders are often reported to have low abundances compared to other trophic groups because of their specialised diets (Tietjen, 1989; Kang, 2021). Deposit feeders, especially non-selective ones, often dominate benthic environments because of their ability to exploit wide food sources (Pape et al., 2013; Zotz & Traunspurger, 2016; Kang, 2021). On the other hand, epistrate-feeders traditionally have been considered to mostly feed by piercing microalgae to absorb their content or scraping off microalgal turf from hard substrates (Schmidt-Rhaesa, 2014), suggesting the presence of a habitat with high primary productivity and rich in diatoms (Pape et al., 2013). However, this is not always true because epistrate-feeders may also rely on bacterial food (Mokievsky et al., 2014), making the correlation between this feeding strategy and the surrounding habitat not always linear. Seasonal shifts in microalgal blooms may also cause a temporary increase in epistrate-feeders (Dung, 2020). Given that this study covered only two sites at a single time point, we refrain from inferring the causes of the high epistrate-feeder abundances. Potential contributing factors are discussed below.

Although no major algal bloom was observed in Singapore during our June 2023 survey, the high abundance of epistrate-feeders may be explained by the presence of fine-grained sand (Wieser, 1959; Jansson, 1967; Boucher & Chamroux, 1976; Franzo & Del Negro, 2019), heterogeneous substrates (Tietjen, 1984; Jensen, 1988; Franzo & Del Negro, 2019) and lack of major hydrodynamic processes (Jensen, 1988; Franzo & Del Negro, 2019), which could affect sediment stability in the area. Moreover, most of the epistrate-feeders were specimens of the genus *Paracanthonchus,* which is known to thrive in sediments rich in organic matter and nutrients (Corinaldesi et al., 2022), and in areas of reduced sediment disturbance (Franzo & Del Negro, 2019). Such conditions could be reflected in the highly sedimented Singapore marine environment (Browne et al., 2015; Morgan et al., 2020; Ridall & Ingels, 2021).

CONCLUSIONS

In this study we investigated free-living marine nematode diversity with morphological and molecular methods in two sites from Singapore based on a limited amount of sediment and across a narrow depth range. The phylogenetic analysis aligned with results from previous studies as the classes Enoplea and Chromadorea and the main families were distinct, although relationships above the family level had low support due to limited data availability. Because of the scarcity of nematode sequences available, morphological identification remains necessary but can be hindered by an abundance of juveniles and females lacking important diagnostic features. In our study, identification to the species level was not possible due to the lack of male specimens. Still, even with generic-level identification, we found two new records (*Mesacanthion* and *Tripyla*) from Southeast Asian sediments and 20 new generic records for Singapore, possibly because of differences in term of microhabitats, location, depth and season among this study and the previous studies. Most nematodes recovered were epistrate-feeders or non-selective deposit feeders, an unusual dominance that can be potentially linked to sediment properties and the high level of nutrients, conditions favourable for the genus *Paracanthonchus*. Molecular data suggested the genus may be polyphyletic because of its complex taxonomy and presence of many species.

Because of the limited sampling size and paucity of sufficient previous records, we caution against making definitive conclusions on nematode assemblages and distribution in Singapore and suggest more focused future research is needed to further explore the topic. Such studies should generate a more complete specimen and sequence reference collection, thus also aiding future environmental DNA and metabarcoding surveys, and leading to a better understanding of marine nematode diversity in this understudied region.

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