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Environmental DNA and Tissue-Derived COI Metabarcoding Reveal Complementary Zooplankton Detection Patterns in Southern Malaysian Coastal Waters: A Preliminary Survey

(DNA Persekitaran dan Pengekodan Metabar COI Diperoleh daripada Tisu Mendedahkan Corak Pengesanan Zooplankton Pelengkap di Perairan Pantai Selatan Malaysia: Satu Tinjauan Awal)

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ABSTRACT

This preliminary study explored the use of mitochondrial COI metabarcoding to compare broad taxonomic detection patterns obtained from pooled environmental DNA (eDNA) and tissue-derived zooplankton DNA (T-DNA) samples collected from three Malaysian coastal locations in Johor: Pulau Kukup, Pengerang, and Pulau Besar. Water samples from three depths (surface, middle, and bottom) were pooled for eDNA extraction, while zooplankton samples were collected using a 140 µm plankton net for T-DNA analysis. Sequencing was performed using the Illumina MiSeq platform. Overall, T-DNA recovered more sequence reads and a higher proportion of taxonomically assigned amplicon sequence variants (ASVs), whereas eDNA and T-DNA generated distinct community profiles, indicating that the two approaches captured complementary biological signals. Site-specific variation in taxonomic diversity was also observed. However, these findings should be interpreted cautiously because samples were pooled prior to sequencing, technical replicates were not included, and sampling was conducted during different monsoon periods. Despite these limitations, this study provides important preliminary baseline data and methodological insights supporting the complementary use of eDNA and T-DNA metabarcoding for future biodiversity monitoring in tropical Malaysian coastal ecosystems.

Keywords: COI metabarcoding; eDNA; Malaysian coastal waters; tissue-derived DNA; zooplankton

ABSTRAK

Kajian awal ini meneroka penggunaan pengekodan metabar COI mitokondria untuk membandingkan corak pengesanan taksonomi luas yang diperoleh daripada DNA persekitaran terkumpul (eDNA) dan sampel DNA zooplankton (T-DNA) yang berasal daripada tisu yang dikumpulkan dari tiga lokasi pantai Malaysia di Johor: Pulau Kukup, Pengerang dan Pulau Besar. Sampel air dari kedalaman yang berbeza; lapisan permukaan, tengah dan bawah dikumpulkan untuk pengekstrakan eDNA manakala sampel zooplankton dikumpulkan menggunakan jaring plankton bersaiz 140 µm untuk analisis T-DNA. Penjujukan dilakukan menggunakan platform Illumina MiSeq. Secara keseluruhan, T-DNA memperoleh lebih banyak bacaan jujukan dan perkadaran varian jujukan amplikon (ASV) yang diberikan secara taksonomi yang lebih tinggi, manakala eDNA dan T-DNA menghasilkan profil komuniti yang berbeza, menunjukkan bahawa kedua-dua pendekatan tersebut mendapat isyarat biologi yang saling melengkapi antara satu sama lain. Variasi khusus tapak dalam kepelbagaian taksonomi juga diperhatikan. Walau bagaimanapun, penemuan ini harus ditafsirkan dengan berhati-hati kerana sampel dikumpulkan sebelum penjujukan, replikasi teknikal tidak dimasukkan dan persampelan dijalankan semasa tempoh monsun yang berbeza. Walaupun terbatas, kajian ini menyediakan data asas awal yang penting dan pandangan metodologi yang menyokong penggunaan eDNA dan pengekodan metabar T-DNA yang saling melengkapi untuk pemantauan kepelbagaian bio masa hadapan dalam ekosistem pantai tropika Malaysia.

Kata kunci: DNA persekitaran; DNA terbitan tisu; pengekodan metabar COI; perairan pesisir Malaysia; zooplankton

INTRODUCTION

Zooplankton communities serve as ideal targets for bio-monitoring, acting as sensitive indicators of marine ecosystem health. These organisms play a critical role in trophic dynamics, facilitating energy transfer from primary producers to higher trophic levels. Their abundance and diversity reflect environmental changes such as climate fluctuations, ocean acidification, and pollution

(Turner 2004). In Southeast Asian marine ecosystems, despite their high biodiversity value, monitoring efforts remain limited due to resource constraints. Thus, the development of efficient zooplankton assessment methods is particularly crucial.

Traditional approaches relying on morphological identification are often constrained by labor intensity, prolonged processing time, and reliance on specialized taxonomic expertise - resources increasingly scarce in the region. To overcome these limitations, molecular approaches such as tissue-derived DNA (T-DNA) metabarcoding have been introduced. This method involves extracting DNA directly from net-towed zooplankton samples, followed by high-throughput sequencing and taxonomic assignment via reference databases (De Vargas et al. 2015). Environmental DNA (eDNA) metabarcoding offers a complementary, non-invasive alternative by analyzing genetic material shed into the water through skin, mucus, feces, and other biological residues. This enables species detection without the need for direct sampling, particularly valuable for elusive or fragile taxa (Ruppert, Kline & Rahman 2019; Thomsen & Willerslev 2015). The complementary characteristics of T-DNA and eDNA suggest that integrating both approaches may provide a more comprehensive assessment of marine biodiversity.

This study focuses on three ecologically distinct Malaysian coastal sites representing different marine environments commonly found along the southern coast of Peninsular Malaysia. Pulau Kukup is situated in the southern Strait of Malacca, one of the world's busiest shipping routes (Jagerroos 2016), experiencing significant anthropogenic pressures. Pengerang connects the Johor Strait with the South China Sea and experiences strong monsoon influences typical of many Southeast Asian coastal zones. Pulau Besar, located within the Sultan Iskandar Marine Park (SIMP), represents a protected area with diverse habitats including coral reefs and seagrass beds. This selection enables evaluation of methodological approaches across varying environmental conditions that characterize the broader Southeast Asian marine landscape. Previous studies on zooplankton distribution in Malaysian waters have established important baseline knowledge, including research in the Malacca Strait by Rezai et al. (2009) and along the east coast in locations such as the Sultan Iskandar Marine Park (Shafie, Juneng & Abdul Rahim 2023). Similar research in neighboring Southeast Asian waters, including Singapore (Schmoker et al. 2014), has created a regional context for zooplankton studies. However, few have compared different DNA metabarcoding approaches for zooplankton assessment in these waters, representing an important knowledge gap for advancing regional marine monitoring.

Although eDNA metabarcoding is increasingly applied in marine biodiversity assessment, its implementation in Southeast Asia remains limited by tropical environmental conditions, incomplete regional reference databases, and limited methodological standardisation (Côté et al. 2023; Ip et al. 2021; Lamb et al. 2022; McCartin et al. 2022). In Malaysia, comparative information on eDNA and tissue-derived DNA metabarcoding for zooplankton assessment remains scarce, despite the ecological importance of zooplankton in tropical coastal ecosystems (Rezai et al. 2009; Shafie, Juneng & Abdul Rahim 2023). Preliminary studies are therefore needed to evaluate detection patterns, database limitations, and practical considerations for future replicated monitoring (Bucklin et al. 2022; Choi, Kim & Kim 2024; Deiner et al. 2017).

This preliminary study aimed to address this gap by: (i) comparing broad taxonomic detection patterns obtained from pooled tissue-derived DNA and eDNA samples using COI metabarcoding; and (ii) describing site-level differences in detected zooplankton-associated assemblages across three Malaysian coastal locations. By positioning the findings as a preliminary survey, this study provides baseline information for improving regional sampling design, reference database development, and future standardization of molecular biodiversity monitoring in Malaysia.

METHODS

STUDY SITES AND SAMPLING

Samplings was conducted at three coastal areas in Johor, Malaysia (Figure 1): Pulau Kukup (PK), Pengerang (PG), and Pulau Besar (PB). Samples were collected from multiple sites at each location (six in PK and PG; four in PB), based on the following timing: PK in March 2020 (Northeast Monsoon), PG in October 2020, and PB in October 2021 (Southwest Monsoon). The sites represent diverse coastal ecosystems: PK in the southern Malacca Strait experiences anthropogenic influence from nearby Tanjung Pelepas Port; PG connects Johor Strait with the South China Sea near Johor Port; and PB is located within the protected Sultan Iskandar Marine Park (SIMP), encompassing diverse habitats including coral reefs and seagrass beds. Because the locations were not sampled at the same time, site-level comparisons were interpreted descriptively and cautiously.

These sampling locations experience distinct monsoon influences, with the Northeast Monsoon affecting Pulau Kukup during our March 2020 sampling, while the Southwest Monsoon influenced Pengerang and Pulau Besar during October sampling (Shafie, Juneng & Abdul Rahim 2023). These seasonal patterns drive water circulation between the South China Sea and the Straits of Malacca, potentially affecting zooplankton community composition and distribution. Environmental parameters including temperature, salinity, pH, and

chlorophyll-*a* concentration were measured at each sampling site using a multiparameter water quality meter (Aquaread water monitoring system–AP-2000) as described in Shafie, Juneng and Abdul Rahim (2023), who conducted parallel conventional zooplankton sampling at the same locations.

For each site, dual sampling approaches were employed (Figure 2). Seawater samples (3 L) were collected using a Niskin bottle at three depths (surface, middle, and bottom) for environmental DNA analysis and stored at 4 °C. Zooplankton samples were collected using a plankton net (140 µm mesh) deployed vertically. Samples for morphological analysis were preserved with 4% formalin (Shafie, Juneng & Abdul Rahim 2023), while those for molecular analysis were preserved in absolute ethanol and stored at -20 °C. Samples were pooled prior to sequencing to generate composite location-level profiles. Therefore, the resulting data represent pooled detection profiles for each location and sample type rather than independent biological or technical replicates. Statistical and ecological interpretations were treated as descriptive rather than inferential.

DNA EXTRACTION AND PROCESSING

eDNA extraction

The environmental DNA (eDNA) extraction protocol was optimized for processing tropical marine samples, in which DNA degradation can occur rapidly due to higher water temperatures. For each sampling site, the 3 L seawater samples from different depths were filtered using 0.45 µm pore-size nitrocellulose membrane filters (Millipore, USA). Two filter papers were used for each 3 L sample to prevent clogging, a common issue when processing turbid Southeast Asian coastal waters. Filter papers were stored at -20 °C until extraction to preserve DNA integrity. Environmental DNA extraction employed the CTAB method, which has shown effective DNA recovery from marine samples in tropical environments. The filter papers were cut into smaller pieces and homogenized in 20 mL CTAB extraction buffer using an IKA homogenizer (Germany). The mixture was incubated at 60 °C for 30 min with inversion every 10 min, then centrifuged at 5,000 rpm for at least 5 min to separate liquid from filter debris. The liquid mixture was transferred to SS34 centrifuge tubes and processed through a phenol-chloroform (1:1) extraction, followed by centrifugation at 10,000 rpm for 10 min at 4 °C. The aqueous layer was transferred to a new SS34 tube and mixed with an equal volume of chloroform-isoamyl alcohol (49:1), then centrifuged again at 10,000 rpm for 10 min at 4 °C. DNA precipitation was performed by adding two volumes of cold absolute ethanol and 0.1 volume of sodium acetate to the aqueous layer. After centrifugation at 13,000 rpm for 30 min at 4 °C, the DNA pellet was washed with 70% ethanol, dried, and dissolved in 500 µL of elution buffer. DNA quantity was

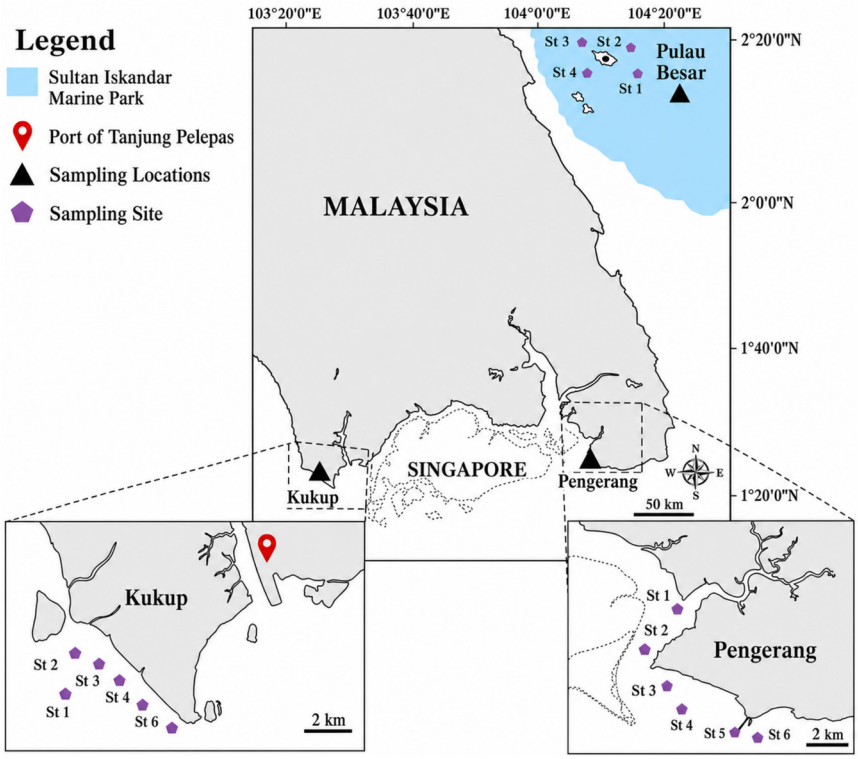


FIGURE 1. Map and coordinates of the sampling area in Johor at the south of Peninsular Malaysia

determined using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA). DNA samples from surface, middle, and bottom seawater were pooled to represent each site, and then site samples were pooled to represent each location, labeled as eDNA-PK (Pulau Kukup), eDNA-PG (Pengerang), and eDNA-PB (Pulau Besar).

Tissue-Derived DNA Extraction

For tissue-derived DNA extraction, the standard DNeasy Blood Tissue Kit (Qiagen, USA) protocol was modified to improve DNA yield from chitinous zooplankton exoskeletons, a critical adaptation for processing diverse tropical marine samples. Prior to extraction, ethanol-preserved zooplankton samples were dried to remove the preservative. To enhance lysis efficiency, a bead-beating step was incorporated using 0.1 mm glass beads (Sigma, USA) with zooplankton tissue in 360 µL ATL buffer. Samples were vortexed with a bead-beater adapter at maximum speed for 30 min, followed by addition of 40 µL proteinase K and incubation at 56 °C for 3 h with shaking. After centrifugation at 5000 rpm for 2 min to remove glass beads, the lysate was transferred to a new 1.5 mL microtube with 400 µL AL and 400 µL absolute ethanol. Subsequent steps followed the manufacturer's protocol, with final DNA elution in 100 µL buffer. Zooplankton samples from different sites within each location were pooled and labeled as T-DNA-PK, T-DNA-PG, and T-DNA-PB.

PCR AMPLIFICATION AND NEXT-GENERATION SEQUENCING

For both environmental and tissue-derived DNA samples, we targeted the COI gene region using the primer set jgHCO2198 (5'-TAIACYTCIGGGRTGICCRAARAAY CA-3') and mlCOIintF (5'-GGWACWGGWTGAACWGTW TAYCCYCC-3') (Wangensteen et al. 2018). This primer combination has shown effective amplification across diverse marine invertebrate taxa in tropical waters. We employed a two-round PCR approach to improve amplification success from potentially degraded or low-concentration eDNA samples. The second round PCR used the same primers but with added Illumina overhang adapter sequences: Adapter_ jgHCO2198 *CGTGGGCTCGGAGATGTGTATAAGAGACAG* TAIACYTCIGGR TGICCRAARAAYCA-3') and Adapter_ mICOIintF(5'-*TCGTCGGCAGCGTCAGATGTGTATAAGA GACAG*GGWACWGGWTGAACWG TWTAYCCYCC-3'). PCR reactions contained 1 µL DNA template, 5 µL 5X PCR buffer, 0.5 µL 10 mM dNTPs, 1.25 µL each of forward and reverse primers (10 µM), 0.25 µL thermostable DNA polymerase (2U/µL), and 15.75 µL nuclease water, for a

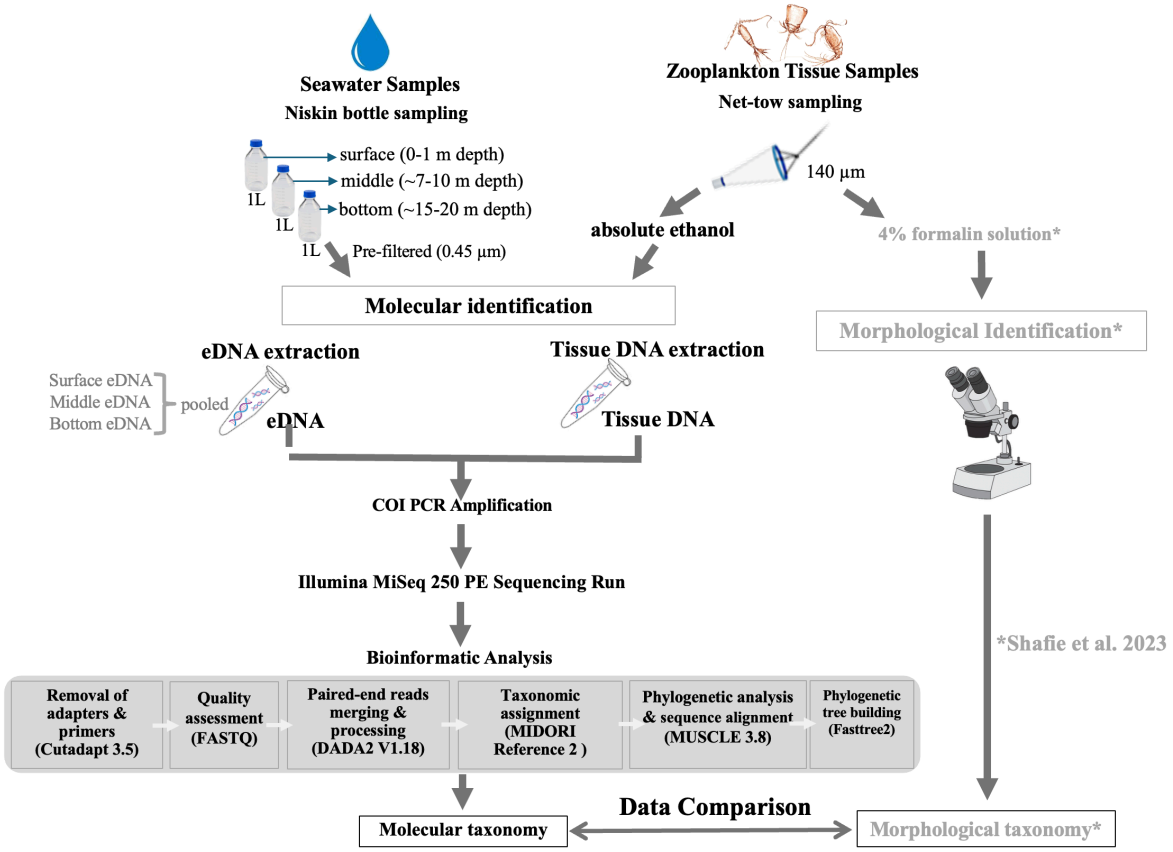


FIGURE 2. Flowchart of research methodology

final volume of 25 µL. The PCR thermal cycling program consisted of initial denaturation at 94 °C for 2 min; 5 cycles of 94 °C for 30 s, 45 °C for 40 s, and 65 °C for 1 min; 30 cycles of 94 °C for 30 s, 49 °C for 40 s, and 65 °C for 1 min; and final extension at 65 °C for 10 min. PCR products were visualized using 1.7% agarose gel electrophoresis. Successful amplicons were sequenced using the Illumina MiSeq PE 300 platform by Apical Scientific Sdn. Bhd., providing the high-throughput sequence data necessary for comprehensive biodiversity assessment.

BIOINFORMATIC ANALYSIS

Next-generation sequencing data were processed to generate COI metabarcoding profiles for eDNA and T-DNA samples. Quality assessment of raw reads was performed using FastQC, followed by primer/adaptor removal using Cutadapt 3.5. High-quality paired-end reads were processed to generate amplicon sequence variants (ASVs), and chimeric or low-quality sequences were removed prior to downstream analysis. Because the target marker was mitochondrial COI, taxonomic assignment was performed using a COI-compatible reference database, MIDORI Reference 2 (https://www.reference-midori.info/download.php.). The final ASV abundance table was used to summarize read recovery, taxonomic composition, ASV overlap, and alpha diversity patterns. For phylogenetic analysis, sequence alignment was performed using MUSCLE 3.8, followed by phylogenetic tree construction using FastTree2. All downstream summaries and visualizations were conducted using R package V3.6.1, with particular attention to community composition differences between sampling locations and detection methods.

RESULTS AND DISCUSSION

SEQUENCING PERFORMANCE

DNA metabarcoding yielded markedly different sequencing outputs across both methods and sites (Table 1). In this pooled dataset, T-DNA produced higher raw and filtered read counts than eDNA across all locations. For instance, at Pulau Besar (PB), T-DNA yielded 106,439 raw reads and 105,062 filtered reads, compared to 17,068 raw reads and 10,187 filtered reads from eDNA. Similarly, at PK, T-DNA retained over 69,000 filtered reads with minimal quality loss, whereas eDNA retained 38,742 reads from 53,853 raw reads. These observations indicate differences in sequence recovery between sample types, but they should be interpreted descriptively because technical replicates were not included. Sequence data have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1298409 (https://www.ncbi.nlm.nih.gov/sra/PRJNA1298409).

TAXONOMIC RESOLUTION

Across all samples, a total of 12,456 amplicon sequence variants (ASVs) were detected. T-DNA generated more ASVs than eDNA at PB and PK and also showed higher taxonomic assignment rates in several locations. At PK, 72.6% of T-DNA ASVs were assigned taxonomically, compared with 2.86% of eDNA ASVs. At PG, 47.9% of T-DNA ASVs were assigned, compared with 14.2% from eDNA. These patterns suggest that tissue-derived DNA provided more taxonomically resolved profiles in this dataset, whereas eDNA assignment was more limited, particularly in turbid or reference-poor tropical coastal samples.

Within this pooled dataset, T-DNA recovered more sequence reads and taxonomically assigned ASVs than eDNA. Because biological and technical replication was not performed, these observations are descriptive and should not be interpreted as evidence of superior analytical performance. This observation is consistent with the biological characteristics of tissue-derived zooplankton samples, where DNA is extracted directly from organisms retained by net sampling. In contrast, eDNA from seawater represents a more complex environmental signal that may include extracellular DNA, shed cells, larvae, gametes, faecal material, degraded DNA, and DNA transported by water

TABLE 1. Summary of sequence data processing

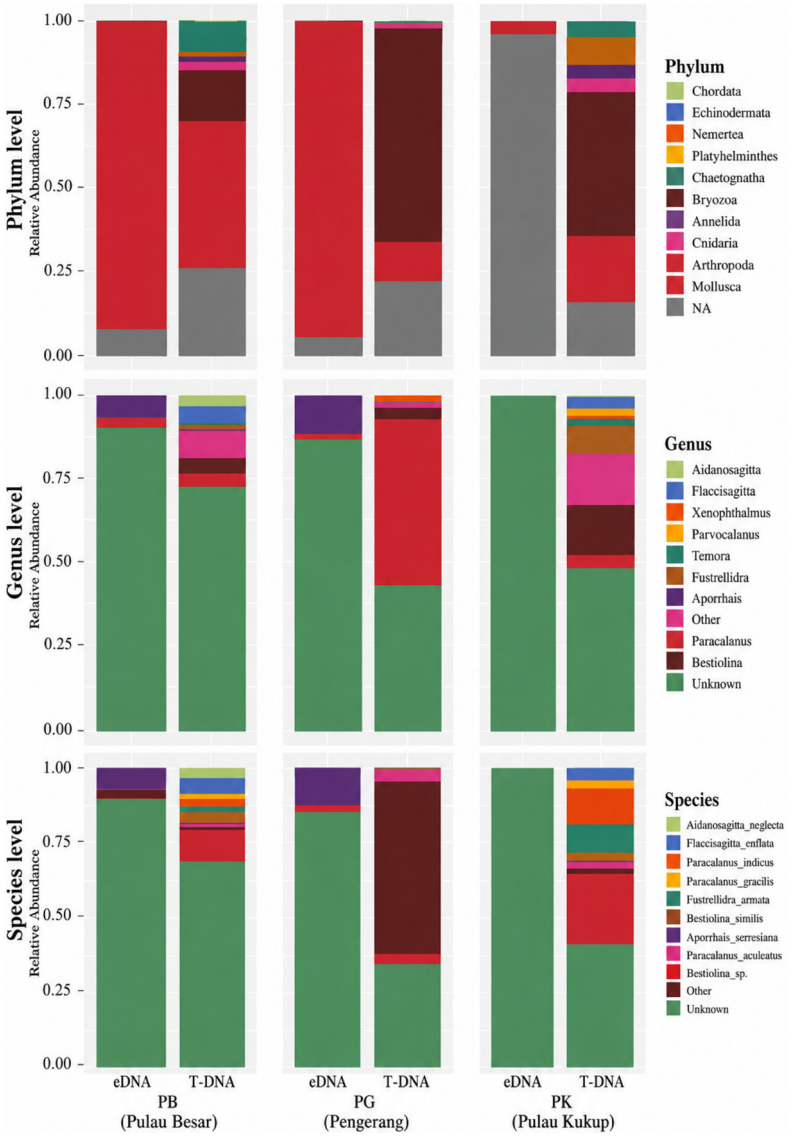
	PB		PG		PK	
	eDNA	T-DNA	eDNA	T-DNA	eDNA	T-DNA
Total raw reads	17,068	106,439	75,096	117,435	53,853	71,301
Total filtered reads	10,187	105,062	70,478	116,649	38,742	69,312
Total ASVs	697	3775	4515	763	1505	1201
Total annotated ASVs	131	826	641	368	43	872
% identified ASVs	18.8	21.9	14.2	47.9	2.9	72.6

movement. Therefore, the observed differences are more appropriately attributed to differences in sampling matrix and DNA source than to inherent differences in method performance.

The low assignment rate in some eDNA profiles also highlights the continuing limitation of COI reference databases for tropical Southeast Asian marine taxa. Because many local zooplankton and marine invertebrates remain underrepresented in public barcode databases, some ASVs may represent real biological diversity but cannot be confidently assigned to genus or species level. This reinforces the need for local COI reference libraries based on morphologically identified voucher specimens.

TAXONOMIC COMPOSITION AND COMPLEMENTARY DETECTION PATTERNS

Taxonomic classification showed distinct detection patterns between the two molecular approaches (Figure 3). Environmental DNA (eDNA) profiles were characterised by a predominance of Mollusca-assigned reads in Pulau Besar (PB; approximately 80%) and Pengerang (PG; approximately 85%). In contrast, tissue-derived DNA (T-DNA) profiles included different taxonomic compositions of zooplankton-associated phyla, including Arthropoda, Cnidaria, and Chaetognatha in PB, and Chordata and Echinodermata in PG. Within the pooled dataset, a greater number of taxa were assigned from T-DNA than from eDNA at finer taxonomic levels.



Bar plot of top ten ASVs at the Phylum, Genus and Species level. (PB: Pulau Besar; PG: Pengerang; PK: Pulau Kukup)

FIGURE 3. Relative abundance represents the proportion of taxonomically assigned ASVs attributed to each taxon within each pooled sample and does not represent organism abundance or biomass. Scientific names are italicised. Taxa designated as *sp.* indicate assignments resolved confidently to genus level only

These differences suggest that eDNA and T-DNA capture partly different biological signals rather than interchangeable community profiles.

Among the three locations, the PB T-DNA profile showed relatively high observed taxonomic diversity, which may be consistent with its protected status within the Sultan Iskandar Marine Park. However, this interpretation remains preliminary because the study was based on pooled samples and locations were sampled during different monsoon periods. The molecular findings complement conventional net-based sampling conducted at the same sites (Shafie, Juneng & Abdul Rahim 2023), which recorded the highest zooplankton abundance in Pengerang (256.157 ind/m^3), followed by Kukup (132.412 ind/m^3) and Pulau Besar (54.066 ind/m^3). Based on Shafie, Juneng and Abdul Rahim (2023), seven dominant copepod species identified via conventional microscopy: *Acrocalanus gracilis*, *Bestiolina similis*, *Euterpina acutifrons*, *Oithona nana*, *Oithona similis*, *Paracalanus aculeatus*, and *Paracalanus denudatus* were also detected in this molecular dataset, although with differing relative abundances.

The distinct taxonomic composition of eDNA and T-DNA supports their complementary value for preliminary biodiversity screening. T-DNA primarily reflects DNA obtained from organisms physically retained as intact zooplankton biomass, whereas eDNA represents a broader environmental signal from organisms present in, near, or hydrodynamically connected to the sampled water mass. Similar complementarity between eDNA, bulk or tissue-derived DNA, and conventional sampling has been reported in marine biodiversity and zooplankton studies (Bucklin et al. 2022; Choi, Kim & Kim 2024; Côté et al. 2023; Deiner et al. 2017). Thus, eDNA should be viewed as a complementary biodiversity signal rather than a direct one-to-one substitute for net-based zooplankton sampling.

SPECIES-LEVEL RELATIVE ABUNDANCE PATTERNS IN eDNA AND T-DNA PROFILES

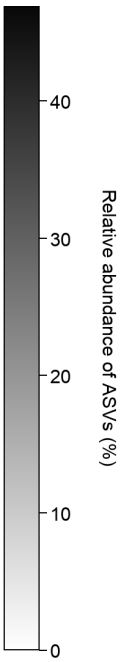
At the species level, eDNA and T-DNA produced markedly different relative abundance profiles, further supporting the complementary nature of the two sampling approaches (Tables 2 & 3). In the T-DNA dataset, a greater number of species-level assignments of zooplankton was detected across locations, with several copepod and other planktonic taxa represented in more than one site. The most prominent signal was observed for *Bestiolina* sp. in Pengerang, which accounted for 46.9% of the relative abundance of ASVs in the T-DNA profile. Other recurrent taxa included *Paracalanus gracilis*, *Paracalanus aculeatus*, *Bestiolina similis*, *Temora turbinata*, and *Flaccisagitta enflata* indicating that the T-DNA approach included several zooplankton taxa that were detected across multiple sampling locations. This pattern is consistent with previous studies showing that COI metabarcoding of bulk or tissue-derived zooplankton samples can provide useful species-level resolution, although detection remains influenced by primer bias, taxonomic coverage of reference databases, and the composition of the sampled biomass (Bucklin et al. 2022; Choi, Kim & Kim 2024). COI metabarcoding is particularly useful for metazoan diversity assessment, but incomplete barcode representation remains a recurring limitation for many marine invertebrate groups (Bucklin et al. 2022; Wangensteen et al. 2018).

In contrast, the eDNA species-level profile was represented by fewer detected taxa and showed a more restricted taxonomic signal. *Aporrhais serresiana* was the dominant assigned species in both Pulau Besar and Pengerang eDNA profiles, contributing 6.1% and 10.8% of the relative abundance of ASVs, respectively, while no species-level assignment was shown for Pulau Kukup in the eDNA graphical table. Other eDNA-detected taxa included *Bestiolina* sp., *Alpheus bellulus*, *Paracalanus aculeatus*, *Mesonerilla roscovita*, *Boccardia pugettensis*, *Xenophora* sp., *Pleistacantha kannu*, *Portunus pelagicus*, *Pupa strigosa*, and *Syndesmis* sp., mostly at low relative abundance. This different taxonomic composition may reflect several non-exclusive factors, including low DNA concentration, dilution, degradation, environmental transport, PCR bias, and limited taxonomic assignment due to incomplete COI reference databases. Environmental DNA in marine systems can be affected by water movement, temperature, salinity, suspended particles, and degradation processes, and eDNA decay may be faster under warmer conditions and in marine water compared with freshwater systems (Collins et al. 2018; Lamb et al. 2022; McCartin et al. 2022). These factors are especially relevant in tropical coastal waters, where turbidity, high temperature, and hydrodynamic mixing may reduce the consistency of species-level detection.

The differences observed between the two graphical summaries should be interpreted cautiously because the analyses were based on pooled samples without biological or technical replication. Within the present dataset, T-DNA yielded a greater number of species-level assignments, whereas eDNA reflected a different subset of assigned taxa. These observations most likely reflect differences in sample matrix and DNA source rather than inherent differences in analytical performance. T-DNA represents DNA extracted from organisms physically collected during plankton net sampling, whereas eDNA represents DNA suspended within the surrounding water column and may originate from local organisms as well as transported or degraded biological material. Accordingly, the species-level results indicate that eDNA and T-DNA provide complementary biodiversity information rather than directly interchangeable representations of the same zooplankton community. Similar studies comparing eDNA and bulk-DNA metabarcoding have likewise shown that the two approaches recover overlapping

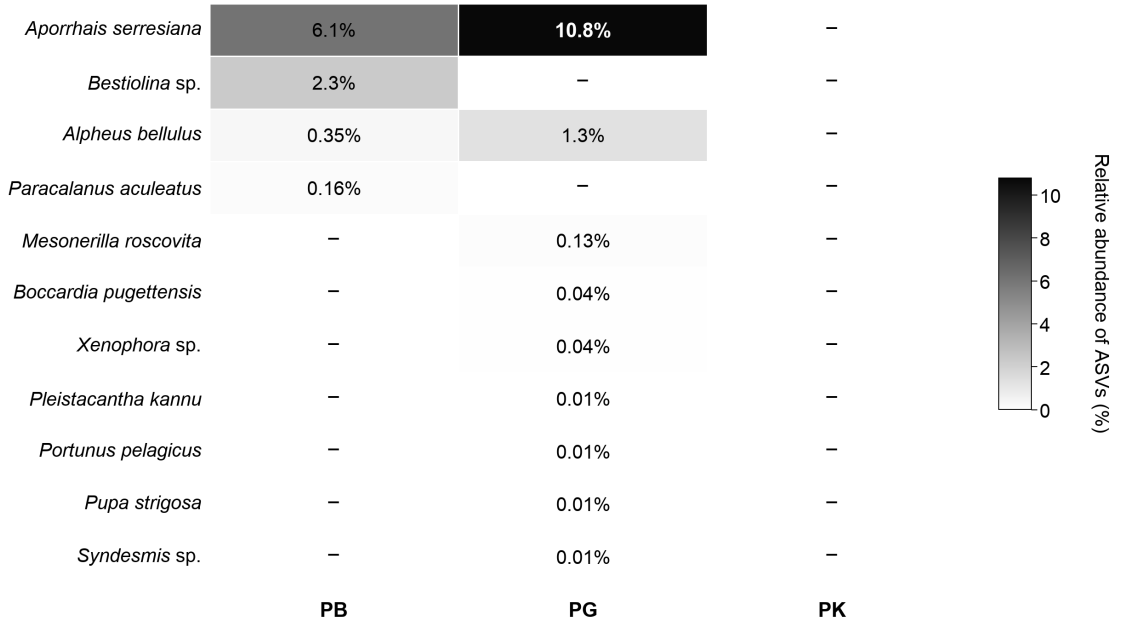
TABLE 2. Species-level graphical table of tissue-derived DNA (T-DNA) COI metabarcoding profiles showing the top 40 species based on maximum ASV relative abundance across Pulau Besar (PB), Pengerang (PG), and Pulau Kukup (PK)

	PB	PG	PK
Bestiolina sp.	0.69%	**46.9%**	1.5%
Paracalanus gracilis	2.2%	0.17%	9.8%
Flustrellidra armata	1.4%	0.32%	7.7%
Flaccisagitta enflata	4.3%	–	3.5%
Paracalanus aculeatus	1.0%	3.1%	1.9%
Aidanosagitta neglecta	3.0%	–	0.28%
Bestiolina similis	2.9%	0.40%	2.0%
Paracalanus indicus	1.2%	–	2.3%
Xenophthalmus pinotheroides	–	0.16%	2.0%
Temora turbinata	0.65%	0.10%	1.9%
Striatobalanus amaryllis	–	0.15%	1.6%
Parvocalanus crassirostris	0.01%	1.2%	0.97%
Canthocalanus pauper	0.30%	–	1.1%
Sphaeronectes koellikeri	1.1%	–	–
Lucifer sp.	0.14%	–	0.86%
Amphibalanus reticulatus	–	0.17%	0.77%
Candacia bradyi	0.06%	–	0.67%
Chloeia sp.	0.67%	–	0.05%
Subeucalanus subcrassus	0.44%	–	0.64%
Sarmatium germaini	–	–	0.63%
Calanopia elliptica	0.05%	–	0.56%
Sagitta sp.	0.55%	–	0.13%
Clytia sp.	–	0.01%	0.49%
Macrostella gracilis	–	–	0.48%
Penilia avirostris	0.45%	–	–
Acartia odontacantia pacifica	0.33%	0.01%	0.37%
Branchiostoma belcheri	0.37%	–	–
Lupocycloporus minutus	–	–	0.37%
Aporrhais serresiana	0.31%	0.08%	0.19%
Acartia Odontacartia erythraea	0.31%	–	0.08%
Zonosagitta bedoti	0.08%	0.29%	0.06%
Mierspenaeopsis hardwickii	–	–	0.28%
Temora discaudata	0.27%	–	–
Tortanus sp.	0.26%	–	0.24%
Centropages furcatus	0.25%	–	0.05%
Centropages orsinii	0.23%	–	0.04%
Diacavolinia sp.	0.22%	–	–
Pseudoevadne tergestina	0.22%	–	–
Liomera venosa	–	–	0.19%
Liriope sp.	0.19%	–	–



PB = Pulau Besar; PG = Pengerang; PK = Pulau Kukup. Scientific binomials are italicised; taxa designated as sp. were resolved confidently to genus level only; '-' indicates not detected.

TABLE 3. Species-level graphical table of environmental DNA (eDNA) COI metabarcoding profiles showing all detected species based on ASV relative abundance across Pulau Besar (PB), Pengerang (PG), and Pulau Kukup (PK)



PB = Pulau Besar; PG = Pengerang; PK = Pulau Kukup. Scientific binomials are italicised; taxa designated as sp. were resolved confidently to genus level only; '-' indicates not detected.

but non-identical components of zooplankton diversity, highlighting the value of integrating multiple sampling approaches together with appropriate sampling design and, where possible, morphological validation (Choi, Kim & Kim 2024; Côté et al. 2023).

A further consideration is that the species names presented in these profiles should be regarded as taxonomic assignments rather than confirmed species records, particularly for low-abundance ASVs. Species-level assignments derived from short COI metabarcoding reads depend strongly on the completeness, accuracy, and geographic relevance of available reference databases. In Southeast Asian waters, many marine zooplankton and invertebrate taxa remain underrepresented in public barcode databases, contributing to unassigned ASVs and uncertain taxonomic assignments. Consequently, these graphical summaries are best interpreted as descriptions of dominant assigned ASV patterns rather than complete species inventories. Future studies incorporating biological and technical replication, together with morphological identification and voucher-based COI barcoding of locally collected specimens, will further strengthen species-level interpretation.

COMMUNITY OVERLAP AND PRELIMINARY SITE-LEVEL SIGNALS

Community-level comparisons showed distinct ASV distribution patterns among the pooled location-level profiles (Figure 4). Within the eDNA dataset, only two ASVs were shared across all three sampling locations, representing a small proportion of the taxonomically assigned diversity. Most ASVs were unique to individual locations, with Pengerang (PG; 577 ASVs) containing the highest number of location-specific ASVs, followed by Pulau Besar (PB; 66 ASVs) and Pulau Kukup (PK; 36 ASVs). In the T-DNA dataset, 53 ASVs were shared across all three locations, while PB (667 ASVs), PK (654 ASVs), and PG (247 ASVs) each contained substantial numbers of location-specific ASVs. These observations indicate that the pooled profiles differed among sampling locations. However, because biological and technical replication was not included, these patterns should be regarded as preliminary observations rather than definitive evidence of spatial differences.

The limited overlap of ASVs among locations is consistent with the possibility of spatial variation in zooplankton-associated biodiversity across southern Malaysian coastal waters. However, the observed patterns should be interpreted cautiously because samples were pooled by location and collected during different monsoon periods. Consequently, the differences observed among locations may reflect a combination of biological variation, seasonal conditions, hydrodynamic processes, sampling matrix, and methodological factors, rather than location-specific ecological differences alone.

Within the present dataset, the PB T-DNA profile exhibited relatively high observed diversity metrics, whereas the Pengerang and Pulau Kukup profiles showed different taxonomic compositions. These observations may be associated with local environmental characteristics, including habitat conditions, water

circulation, turbidity, or anthropogenic influences. However, the present study was not designed to evaluate the contribution of these environmental drivers, and causal interpretations, including the effects of marine protected status, industrial activities, or local ecological resilience, require replicated sampling together with environmental covariate analyses. Previous studies have likewise reported that zooplankton communities in Malaysian coastal waters vary according to spatial and temporal environmental conditions, including circulation patterns and local habitat characteristics (Rezai et al. 2009; Shafie, Juneng & Abdul Rahim 2023).

DIVERSITY METRICS AND METHODOLOGICAL CONSIDERATIONS

Figure 5 presents normalized diversity indices (Observed, Chao1, ACE, Shannon, Simpson, and Inverse Simpson) using min–max scaling to facilitate visual comparison among the pooled profiles. Within each diversity index, values closer to 1 indicate relatively higher diversity or richness relative to the other pooled profiles included in the dataset. The PK-TDNA profile exhibited relatively high normalized values across several diversity indices, whereas the PB-TDNA profile also showed relatively high values, particularly for the Shannon, Simpson, and Chao1 indices. In contrast, the PK-eDNA profile was characterized by comparatively lower normalized values across the diversity indices. These normalized values provide a descriptive summary of diversity patterns within the pooled dataset and are intended solely for visual interpretation. Because the analyses were based on pooled samples without biological or technical replication, these patterns should not be interpreted as statistically supported differences among sampling locations or between molecular approaches.

Several methodological limitations must be considered when interpreting the diversity patterns. The main limitation of this study is the absence of technical and biological replication at the sequencing level. Because samples were pooled before sequencing, variation among depths, sampling stations, DNA extractions, PCRs and sequencing libraries could not be evaluated. Therefore, diversity metrics, ASV overlap and taxonomic composition patterns are best interpreted as preliminary location-level profiles rather than statistically tested differences among sites or methods.

The sampling design also influences the biological signal detected by each method. The 0.45 µm filter may capture a mixture of intracellular and extracellular DNA fractions, while the 140 µm plankton net is biased toward larger zooplankton and may underrepresent smaller life stages or microzooplankton. Tropical coastal waters can also accelerate DNA degradation and often contain suspended particles, inhibitors or high turbidity that affect filtration efficiency and DNA recovery (Ip et al. 2021; Lamb et al. 2022; McCartin et al. 2022).

Future work should build on this preliminary dataset by incorporating field replicates, filtration replicates, extraction replicates, PCR replicates and independent sequencing libraries. Sampling should ideally be synchronized across locations and repeated across seasons to separate spatial and temporal effects. Comparative testing of filter pore size, water volume, net mesh size and preservation methods would also help optimize protocols for turbid tropical coastal waters. In parallel, targeted COI barcoding of morphologically identified Southeast Asian zooplankton specimens would improve regional reference databases and strengthen species-level assignments. Overall, the findings support the value of integrating eDNA and T-DNA approaches for preliminary biodiversity screening, while also

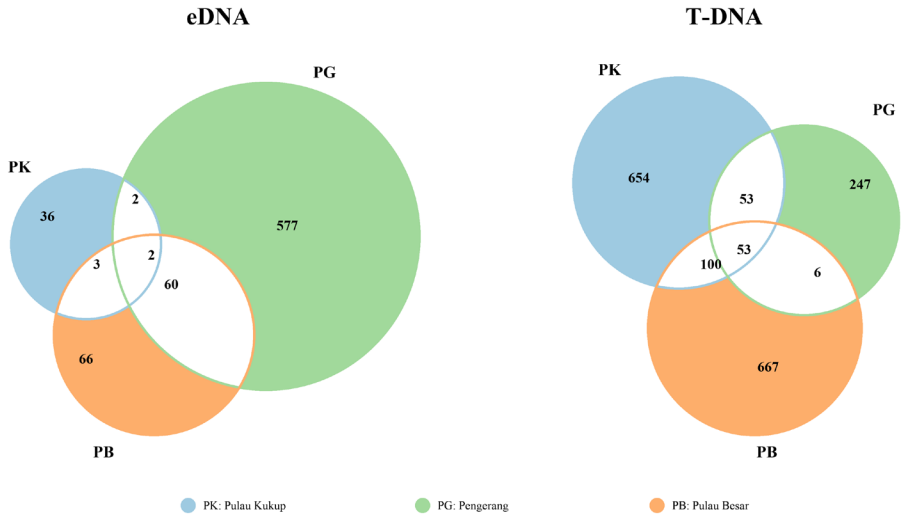


FIGURE 4. Venn diagram of taxonomically assigned ASVs distribution between Pulau Kukup (PK), Pulau Besar (PB) and Pengerang (PG) for eDNA and T-DNA. Unclassified or unknown ASVs were excluded

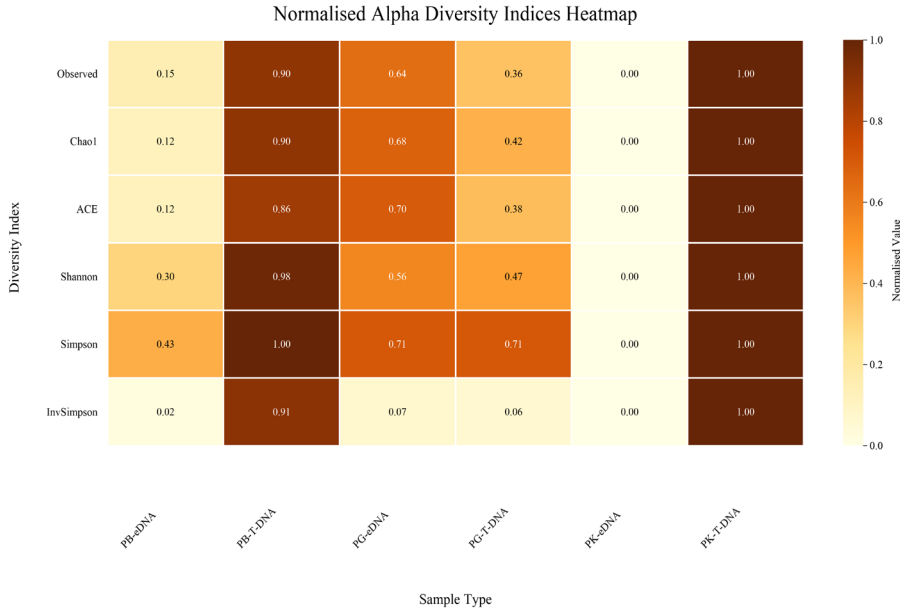


FIGURE 5. Normalized alpha diversity indices across sampling sites (PB, PG and PK) and DNA sampling types (eDNA vs T-DNA). Each index (Observed, Chao1, ACE, Shannon, Simpson, Inverse Simpson) was normalized using min-max scaling to enable visual comparison. Higher values (closer to 1) represent relatively higher diversity or richness within that specific index

demonstrating the need for careful sampling design and database development before these methods can be used for routine ecological assessment or conservation decision-making in tropical marine systems.

CONCLUSION

This preliminary study compared pooled environmental DNA and tissue-derived zooplankton DNA samples using COI metabarcoding across three Malaysian coastal locations. Within the pooled dataset, T-DNA recovered more sequence reads and taxonomically assigned ASVs than eDNA. Site-level differences were observed, suggesting potential spatial structuring of zooplankton-associated biodiversity in southern Malaysian coastal waters. The high proportion of unassigned ASVs further highlights the need for improved regional COI reference databases for Southeast Asian marine taxa. Overall, this study provides useful preliminary baseline data and methodological insights to guide future replicated eDNA and T-DNA metabarcoding surveys in tropical coastal ecosystems.

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