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From fish to invertebrates: multi-marker eDNA metabarcoding for monitoring wetland biodiversity and non-indigenous species in Macao SAR China

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Abstract

Urban wetlands provide crucial ecosystem services but are increasingly threatened by urbanization. In Macao, a densely populated city on the Pearl River Estuary, roughly half of the historical wetland habitats have been lost, yet they remain vital for the East Asian–Australasian Flyway. To assess biodiversity in the remaining wetlands, this study applied environmental DNA (eDNA) metabarcoding targeting 12S rRNA, 18S rRNA, and COI genes with a primary focus on fish and invertebrates. The results revealed 85 fish, 298 invertebrate and 9 non-fish chordate species, including 18 non-indigenous fish and several invertebrates. The communities were highly site-specific, showing clear distinctions between inland and coastal wetlands, but non-indigenous fish were widespread, reflecting strong anthropogenic pressure. Moreover, while not observed in fish, coastal invertebrate communities showed strong seasonal turnover. Nevertheless, 56% of COI-derived ESVs could only be assigned to higher taxonomic levels, suggesting substantial diversity remains uncharacterized due to incomplete reference databases. Collectively, these findings demonstrate how fragmentation and seasonal dynamics shape biodiversity differently across taxonomic groups. This study establishes the first comprehensive eDNA baseline for Macao’s wetlands, highlighting the need to expand local reference databases and integrate molecular techniques with traditional surveys to improve monitoring and conservation of urban ecosystems.

Keywords

eDNA metabarcoding, COI-12S-18S markers, molecular biodiversity assessment, wetland biodiversity, freshwater and estuarine wetlands, Macao

Introduction

Urban wetlands are key components of urban biodiversity and ecosystem function, they moderate floods, improve water quality, sequester carbon, support fisheries and recreation, provide habitat and migratory stopovers, help manage stormwater, offer refuge for threatened and migratory fauna, contribute to water conservation, and create opportunities for community education and connection with nature[1-7]. Yet, they are among the fastest declining habitats globally, with losses driven by land conversion, pollution, species invasion and hydrological change[8-10]. Since 1970, the world has lost an estimated 22% of its wetland area with an ongoing annual decline of 0.52%[9]. This trend is also evident in the Pearl River Delta's Greater Bay Area (Guangdong, China), where wetland area decreased by 12.13% between 2000 and 2020[11]. Wetlands in this region are primarily concentrated along the west bank of the Pearl River, while inner-city wetlands are scarce[12,13]. Notably, ponds and rivers on the west bank serve as key hotspots for wetland biodiversity[14].

On the western bank of the Pearl River Estuary, this global pattern is also reflected in Macao, a highly populated city where its coastal environment has been naturally shaped by the dynamic interplay of freshwater discharge and the saline tides of the South China Sea[15]. As a result, shallow, sediment-rich estuarine waters were developed, with depths generally ranging from one to three meters and an irregular semi-diurnal tidal regime[16]. The diverse hydrographic condition historically supported extensive wetland habitats across separate islands[17,18]. Nevertheless, this natural setting has now been fundamentally reshaped by rapid urbanization process, which reached 98% urbanization rate by 1975 followed by more recent subsequent large-scale land reclamation, such as the Cotai Strip[13]. As a result, the territory has lost approximately half of its historically vital wetland habitats, which once sustained the crucial East Asian-Australasian Flyway migratory bird populations[13,14]. Today, under the immense pressure from a population density exceeding 20,600 people per square kilometer[19], Macao's remaining wetlands are governed by the Macao Urban Master Plan

(2020-2040), the first plan to officially delineate boundaries between urban construction and ecological protection[20]. Consequently, assessing the biodiversity within these fragmented habitats is crucial for their effective management and conservation.

Conventional biodiversity assessment has relied on field sampling techniques such as electrofishing, netting or camera traps[21-23]. While these methods provide direct and strong evidence, they are often labour intensive, invasive, costly, and limited in their ability to detect rare, cryptic, or migratory species[24-27]. Environmental DNA metabarcoding (eDNA) analysis, on the other hand, offers a non-invasive, highly sensitive, and cost-effective alternative[25,28-30]. For instance, a large-scale study on 109 urban water bodies in Beijing utilized eDNA to identify 126 vertebrate species, uncovering 40 fish species that have not been recorded by traditional surveys[31]. Similarly, more fish species and rare species that were missed entirely by the traditional surveys were detected by eDNA metabarcoding in China’s Lake Taihu[32]. By detecting genetic material shed by organisms, eDNA metabarcoding allows the simultaneous characterization of entire communities[33]. This approach is particularly beneficial for detecting spatiotemporal ecological patterns.

Although eDNA techniques for biodiversity surveys are now well established and widely applied globally, particularly for aquatic communities, such as fish assemblages[34] and invertebrates[35], their application in Macao has remained unexplored. To date, biodiversity monitoring in the region still relied primarily on traditional methods[36]. These methods limit visual-based surveys in Macao, especially for underwater organisms, due to high water turbidity. This study therefore pioneers the first application of eDNA metabarcoding in Macao’s wetlands, targeting fish and invertebrates as key functional component of wetland ecosystem to provide a comprehensive assessment of aquatic biodiversity in the region. These groups also serve as vital bioindicators for ecosystem health and provide critical information on food web structure and ecosystem function[37,38].

To better understand how communities shift along gradients of environmental factors and spatial connectivity, water samples were analyzed from a range of wetland sites in Macao. Our hypotheses were (1) fish and invertebrate community composition is strongly influenced by hydrological connectivity and habitat fragmentation, and (2) coastal communities exhibit stronger seasonal variation than inland sites due to seasonal fluctuations of salinity and tidal level. Overall, this study aimed to establish a baseline for future biodiversity monitoring and contribute to more effective management of Macao’s fragmented wetland ecosystems.

Results

Performance of the primers and overall biodiversity

To characterize the aquatic biodiversity of Macao, water samples were collected from 9 wetland sites ranging from inland freshwater to coastal open wetland system across summer and winter. From these samples, we obtained 41 amplicon libraries for each of COI and 12S markers, and 50 for the 18S marker. After quality filtering, a total of 17,794,351 sequence reads were retained across all markers: 6,587,648 for 12S, 6,073,363 for COI, and 6,298,956 for 18S (Supplementary Table S1). Taxonomically, the COI and 12S primers collectively detected 290 chordate exact sequence variants (ESVs), while the COI and 18S primers detected 5631 invertebrate ESVs.

Taxonomic coverage of fish and other chordate species

Of the 290 chordate ESVs, 224 were fish (Actinopterygii) while 30 were non-fish chordates and the remaining 36 assigned only at phylum level. Collectively, these taxa encompassed 32 orders, 50 families, and 73 genera across 6 classes, with Non-fish taxa detected only by the COI marker. The majority of these ESV records could be confidently assigned to species level, but 9 were manually reduced to higher level classification due to database inconsistencies or geographic implausibility.

Both COI and 12S primers detected a relatively broad range of fish species, with 59 and 67 species identified by each primer respectively, of which 32 fish species were shared between the two markers. In addition to fish, 9 non-fish species were also detected and the total Chordate taxa detection at family level is illustrated in Figure 2 (Fig. 2).

The most represented order from the fish profile was Gobiiformes, accounting for 55 ESV occurrences (18.9%) across 12S and COI libraries, including 22 species from 15 genera. Other dominant orders included Cypriniformes (10%), followed by the non-indigenous order Cichliformes, contributing approximately 8.6% of detections. Of the identified fish species, salinity tolerance categories based on existing literature revealed that majority (76 species, 89.4%) were associated with brackish environments, while only 9 species (10.5%) were strictly freshwater specialists. Those that can adapt in brackish environment could be further defined into three groups, including 36 euryhaline species capable of tolerating the full salinity spectrum, 28 freshwater-brackish species primarily occurring in lower salinity ranges, and 12 brackish-marine species that are more frequently recorded in higher salinity environments. In contrast, non-fish detections (9 species) were predominantly terrestrial or freshwater-associated, with only one species (*Nycticorax nycticorax*) potentially utilizing marine habitats. A full list of the detected Chordate species is provided in supplementary Table S2.

Invertebrate taxonomic detection and coverage

The invertebrate communities encompassed substantial taxonomic diversity, including 19 phyla, 43 classes, 133 orders, 291 families, and 324 genera. Due to unreliable species-level matches, 25 ESVs were reassigned to higher taxonomic levels. As shown in Fig. 3, the two molecular markers exhibited distinct profiles in both assignment depth and detection breadth.

The 18S marker produced a smaller dataset (738 ESVs) but with greater assignment depth: all detections were resolved to at least the order level, with a high proportion assigned to family (92.4%), genus (63.8%), and species (20.3%). In contrast, the COI marker demonstrated greater detection breadth (4,893 ESVs), but with shallower assignment depth. Most of its assignments reached only the phylum level (65.5%), with fewer resolved to class (34.5%), order (26.9%), family (19.6%), genus (10.5%), and species (8.9%). Consequently, while COI had a lower species-level assignment rate, its broader detection scale resulted in a higher number identifications at species level (435 ESVs) compared with 18S (151 ESVs).

COI and 18S markers revealed complementary detection patterns. The majority of ESVs were detected using the COI marker, with Arthropoda being the most dominant group, including 295 Insecta ESVs (118 species) and 120 Hexanauplia ESVs (46 species), COI also contributed most detections in Rotifera, Mollusca, Cnidaria, and Annelida. In contrast, 18S recovered fewer ESVs overall but outperformed COI in certain groups, detecting greater diversity in Nematoda, Hexanauplia, and deeper genus-level resolution in Gastrotricha, Annelida, and Nematoda.

Together, the two largely non-overlapping community profiles revealed a total of 298 distinct species, with only 11 species shared between the two profiles. Of these, 210 species were uniquely detected by the COI marker, while the 18S marker detected 77 unique species. The shared species, including several copepods such as *Pseudodiaptomus annandalei*, *Parvocalanus crassirostris*, and *Acartia pacifica*, are listed in supplementary Table S3, along all other species with their corresponding site and habitat information.

Fish community composition across sites and seasons

Site conditions during sampling were consistent with previous baselines. Summer water temperatures were higher than in winter (28–32 °C vs. 17–21 °C). pH was generally alkaline, peaking at 9.9 in Ká Hó Reservoir (winter) and ranging 7.5–8.5 in coastal sites. Salinity was lowest in inland wetlands (0–0.5 ppt) and highest in coastal sites, increasing from 2–9 ppt in summer to 16.5–20 ppt in winter.

To mitigate the effects of primer bias and limited amplification of non-target taxa, community analyses was restricted to fish assemblages. Fish community composition varied across sites. NMDS ordination based on Jaccard distance (presence/absence data) revealed clear clustering by site and partial separation by season (Fig. 4). PERMANOVA confirmed significant differences among sites (PERMANOVA $F = 4.47$, $R^2 = 0.47$, $p = 0.001$), with no evidence of heterogeneous dispersion among groups (PERMDISP: $F = 1.69$, $p = 0.21$). However, post hoc comparisons showed no significant difference between Cotai Ecological Protection Zone I and II. Given their high taxonomic overlap (almost 50% shared species) and the partial hydrological connectivity by a tunnel, the two sites were grouped as a single “Cotai Ecological Protection Zone (EI & EII)” in the NMDS plot and other fish community analysis. Among all locations, Ká Hó Reservoir Freshwater Wetland exhibited the most distinct community composition relative to other sites.

Analysis of community composition by habitat type revealed distinct assemblages between inland and coastal wetlands (illustrated with 80% confidence ellipses, PERMANOVA: $F = 4.7$, $R^2 = 0.10$, $p = 0.001$, Fig. 4), with no overall significant seasonal shift ($F = 1.21$, $R^2 = 0.03$, $p > 0.21$). However, ANOSIM showed strong seasonal structuring at both the Cotai Ecological Protection Zone ($R = 0.7$) and Taipa Mangrove ($R = 0.9$), indicating a strong effect size in seasonal shifts despite non significant p-values resulting from limited sample size. This pattern aligns with the NMDS ordination, which also shows clear seasonal separation at this site.

Fish species composition and non-indigenous fishes

Among all sites, Cotai Ecological Protection Zone I and II exhibited the highest fish species richness, with 52 species detected. A total of 45 species were exclusive to a single site across all wetlands. Ká Hó Reservoir Freshwater Wetland had the highest proportion of private species (4 of 6 species), followed by Cotai Ecological Protection Zone (19 of 52), Avenida da Praia Taipa Wetland (8 of 24), Taipa Mangrove Wetland (6 of 21), Sai Van Lake (6 of 28), and Seac Pai Van Park Wetland (2 of 14), while Coloane

Village Wetland (14 species) and Hac Sá Reservoir (3 species) had no private species (Fig. 5).

The overall detection rate of common species across sites was relatively high. The most commonly detected species, which is absent only from Ha Sac Reservoir, was the native goby species *Rhinogobius duospilus,* which can adapt to both freshwater and brackish water habitats. In addition, two other native species, *Mugilogobius abei* and *Scatophagus argus*, were also widespread, occurring in 6 and 5 out of the 8 sites, respectively.

Nonetheless, many of the species shared among sites were non-indigenous. In total, 18 non-indigenous species were identified, with their prevalence varied among sites. For example, Ká Hó Reservoir Freshwater Wetland consisted of only native species, Whereas Hac Sá Reservoir consisted entirely of non-indigenous species. Avenida da Praia Taipa Wetland had the second highest proportion of non-indigenous species, with 12 species accounting for 50% of the fish community at that site. In contrast, at Cotai Ecological Protection Zone, despite the highest species richness, contained only 9 non-indigenous species. Several non-indigenous species were more particularly widespread, this included *Coptodon zillii,* which were detected in 6 out of 8 sites, *Oreochromis mossambicus,* detected in 5 sites, and followed by those detected in 4 sites including *Gambusia affinis, O. aureus*, *O. niloticus*, and *Ctenopharyngodon idella*., as shown in Fig. 5.

Invertebrate community divergence by habitat and season

Similar to the fish community, assumptions of homogeneity were met for the invertebrate dataset (PERMDISP: F = 2.41, p = 0.12). The invertebrate composition was highly site specific, with the exception of Cotai Ecological Protection Zone I and II (PERMANOVA: $F_{7,40}$ = 3.06, R^2 = 0.349, p = 0.001, Fig. 6a), while also significantly influenced by habitat type and its interaction with season. This was evidenced by distinct groupings in the NMDS ordination, and a substantially higher overall taxa diversity in summer (474 taxa) than in winter (289 taxa) (Habitat type: F = 4.67, R^2 = 0.087, p = 0.001; Season: F = 2.99, R^2 = 0.056, p = 0.001; Habitat type X

Season F = 2.03, R^2 = 0.038, p = 0.002). In addition, visualized by 80% confidence ellipses, post-hoc tests showed seasonality varied across habitats (Fig. 6a). That is, the invertebrate assemblages in inland wetlands are relatively stable and did not differ significantly between seasons (post hoc p = 0.165), whereas coastal wetlands exhibited pronounced seasonal turnover (post hoc p = 0.001). Therefore, while site specific differences were strong, habitat type nested within sites emerged as a key driver of invertebrate community composition, with seasonal dynamics primarily observed in coastal ecosystems.

At the phylum level, ecological groups exhibited different associations with habitat type and season (Fig. 6b). The invertebrates can be broadly grouped into four functional categories, although no formal functional classification was applied. Macroinvertebrates, including Arthropoda, Mollusca, and Annelida, showed clear separation between inland and coastal wetlands (post hoc p = 0.001), with coastal community further diverging by season (post hoc p = 0.0012, 0.002 and 0.0015). This pattern was driven largely by mobile taxa such as insects, crustaceans, gastropods, and annelid worms. On the other hand, planktonic invertebrates, including Rotifera and Cnidaria both differentiated between inland and coastal wetlands (post hoc p = 0.006 and 0.001) but with only Rotifera showed weak seasonal turnover (post hoc p = 0.003). Sediment associated meiofauna, including Nematoda, Platyhelminthes and Gastrotricha, appeared relatively stable but still showed significant differences between inland and coastal wetland without seasonal turnover (post hoc p = 0.022, 0.018 and 0.001). Finally, sessile taxa, including Porifera and Bryozoa, were almost exclusively detected in coastal wetlands.

Hence, the effect of habitat types on invertebrate community composition is driven mainly by macroinvertebrates and planktonic taxa, while meiofaunal remain relatively stable in the sample sites and sessile group are more restricted to coastal habitats.

Database Coverage of observational survey species

To assess the availability of regional reference data coverage, species record from an annual observational survey conducted in Cotai Ecological Protection Zone I & II[38] were assessed. The assessment included 119 fish, 5 amphibians, 22 reptiles, 10 mammals, 198 birds and 667 invertebrate species.

Gap analysis revealed remarkedly different reference sequence coverage between taxonomic groups. For chordates, coverage of 12S and COI markers was very high, with approximately 83% of species records found in at least one database (Fig. 7a). In contrast, invertebrate coverage for COI and 18S markers was poor, with only 48.7% of species covered in reference databases. In addition, the invertebrate coverage was fragmented and unevenly distributed, GenBank contained the majority of unique records (36.4%), followed by SILVA (2.4%) and BOLD (0.4%), the overlap between databases was also very limited (Fig. 7a).

Comparison between eDNA detections and observational surveys

Despite relatively robust database coverage for fish and other chordates, comparison between eDNA metabarcoding detections and observational records revealed limited overlap. Only 76 species detected by the present eDNA study were previously reported in the surveys, comprising of 34 fish, 5 non-fish chordates, and 37 invertebrates (Fig. 7b), indicating a substantial disparity between molecular and traditional survey methods.

Discussion

This study demonstrated the use of eDNA metabarcoding for assessing biodiversity in wetland systems, focusing on both fish and invertebrate communities across multiple sites and seasons. The approach employed three primer sets, each targeting different taxonomic groups, with each performing robustly within its intended scope[39-42]. The mlCOIintF/LoboR1 (COI) primer targets the standard animal barcode region[43], and has a broad taxonomic range, proving highly effective for aquatic invertebrates[44,45]. The MiFish-U (12S) primer is very effective for detecting fish across diverse aquatic conditions.[46], while the TAReuk454FWD1/TAReukREV3 (18S V4) primer offers broad eukaryotic coverage, detecting groups from macroinvertebrates to picoeukaryotes and meiofauna[35].

By combining the three primer sets, our eDNA analysis provided a comprehensive view of Macao’s wetland biodiversity, capturing both diverse communities and locally significant species, such as the endemic *Nanhaipotamon macau* and the IUCN-vulnerable *Boleophthalmus pectinirostris*, providing not only record of species presence but also insight into ecological patterns. Subsequent NMDS ordination revealed consistent patterns across sites and seasons, reflecting differences in hydrological connectivity and management regimes. While fish and invertebrates both showed strong site specificity, most of the detected fish species were euryhaline, capable of living across varying salinities, leading to more shared species across sites. In contrast, invertebrates showed lower site overlap, with several extreme outliers at individual sites, consistent with the fine scale turnover reported for meiofauna over just a few meters [42].

The distinct site-specific patterns observed in fish and invertebrate communities was consistent with the status of wetlands in Macao, which are fragmented due to development and land reclamation[15,38,47]. For example, Ká Hó Reservoir Freshwater Wetland, Macao’s only restricted inland freshwater wetland, was found to host the most distinct fish assemblages with only a few taxa overlapping with other sites. However, its small size and fragmentation were associated with relatively low fish richness in contrast to larger or more connected habitat such as Avenida da Praia Taipa Wetland and Cotai Ecological Protection Zones, which supported much more diverse fish profile. This pattern reflects the well documented relationship between wetland size, fragmentation, and aquatic species richness, where small wetlands often host unique communities but are more vulnerable to environmental fluctuations and population pressures[48]. For invertebrates, such effect on species richness was less obvious, likely due to the microhabitat variability and fine scale environmental heterogeneity[49,50].

In addition to the spatial patterns of individual sites, distinct inland and coastal communities were identified for both fish and invertebrates, reflecting habitat differences such as varying hydrological connectivity, salinity regimes, nutrient factors and other water quality parameters[51]. These differences were more evident for invertebrates, which showed not only clear inland and coastal differentiation, but also strong seasonal turnover in coastal wetlands. For fish community, such seasonal difference was only partially observed at one coastal site.

The limited seasonal variation observed in fish communities in our study were contrasting with the clear seasonal patterns documented in previous regional studies**[52,53].** This discrepancy can be attributed to a combination of methodological and ecological factors. While the limited seasonal sampling may have constrained the detection of such temporal signals, the predominant explanation likely lies in the highly homogenized nature of fish assemblages, characterized by relatively low overall species richness. That is, a considerable number of fish species were shared across different sites, often regardless of inland freshwater or coastal environment. Many of these widely distributed species were non-indigenous species, indicating that the fish community in the region has likely been homogenized due to artificial release[54]. This included several nationally and globally recognized invasive species such as *O. niloticus* and *C. zillii*, which have been dominating across multiple sites in Macao over the years[55]. The ecological impact of these introduced species were well documented in the nearby areas. Shuai, et al. [56], for example, have found that *O. niloticus* simplified the food web, and reduced both the structural and functional complexity of the native fish community in a different tributary of the Pearl River, which is connected indirectly to Macao. Tsang and Dudgeon [57], on the other hand, found less impact from the invasive poecilids on freshwater streams in Hong Kong, though studies from the same group noted trophic niche overlaps with native species, suggesting potential competition [58,59].

Although information on the introduction pathways and ecological impacts of these non-indigenous species in Macao remains limited, most of the detected species, except 2 of them, were identified in the neighboring Pearl River estuary region including Hongkong[60-62]*.* Chan, et al. [62] have documented the introduction pathways of these species, which are primary linked to aquaculture escapes (e.g. tilapias such as *Oreochromis* spp. and *C. zillii*, etc.*),* the release of aquarium pets (e.g. ornamentals including *X. maculatus, Carassius auratus* and *Vieja* spp*.*, etc.), Buddhist release rituals (*Cyprinus carpio* and *X. maculatus*, etc.) and government introduction for biological control (*G. affinis*). Consistent with these patterns observed in neighboring areas, community analysis of the present study suggested that artificial release by public likely play an important role in introducing non-indigenous species. This was supported by the correlation between public access and the presence of non-indigenous species**.** While restricted sites like the Ká Hó Reservoir Freshwater Wetland[47] were free of non-indigenous species, recreational wetlands with 24-hour access exhibited much higher numbers and proportions of non-indigenous species. This was most obvious in confined inland wetland including Avenida da Praia Taipa Wetland and Hac Sá Reservoir, which receives substantially more visitors than other sites due to its popularity as a tourist destination, recreation site and birdwatching hotspot[47].

The establishment of non-indigenous fish species is complemented also by the detection of several well known non-indigenous invertebrates, including the golden apple snail (*Pomacea canaliculata*), the golden mussel

(*Limnoperna fortunei*), and false mussels (*Mytilopsis* spp.). Similar to the establishment of non-indigenous fish, these invertebrates pose substantial threats to the local ecosystem resulting from anthropogenic disturbance in Macao[63-65]. Nonetheless, the degree of biological homogenization observed in invertebrate communities was substantially lower than that for fish assemblages.

Indeed, in contrast to the comparatively stable fish communities, invertebrate assemblages showed much higher sensitivity to seasonal environmental changes, which heightened their well-documented capacity to reflect environmental fluctuation, particularly in dynamic estuarine systems[66]. The subtropical monsoon climate of Macao creates strong seasonal dynamics, where coastal wetlands experience substantial salinity fluctuations from river discharge and saltwater intrusion, while inland wetlands vary primarily in water level and temperature[67]. This seasonality was known to drive distinct shifts in invertebrate communities around the Pearl River Estuary, resulting in highest species richness of invertebrates during spring[68,69]. Such dynamics was also confirmed in the present study. More specifically, the previous studies has shown that mesozooplankton thrive during the wet season, with high abundance likely linked to increased nutrient availability[69]. While more stable high salinity conditions can support higher macrobenthos diversity and biomass during dry season[68].

Invertebrate communities are influenced by a complex of environmental factors which shift spatially and seasonally even within the same estuary[70]. These dynamic factors differentially affect taxonomic groups based on their ecological traits, and ultimately generating distinct seasonal patterns across different invertebrate group[66,68,71]. For example, mobile macroinvertebrates are strongly filtered by hydrological conditions[72], with their distribution and diversity shifting along salinity gradients and flow regimes according to the specific ecological niches, feeding strategies and connectivity[73], therefore more likely expressing distinct seasonal pattern[74]. Similarly, planktonic invertebrates, varying from micro to macro size, such as Rotifera and Cnidaria, are strongly shaped by salinity, hydrological connectivity and also nutrient availability, though their specific responses vary by species and system[75,76]. In contrast to these two groups, sediment associated meiofauna like nematodes are primarily structured by sediment stability and organic matter[77,78], which can be influenced by rainfall and river discharge[79]. While sessile taxa, such as Bivalvia, often integrate fluctuating environmental conditions through physiological responses as they cannot escape from the environment[68,80].

Beyond their role in natural ecosystem dynamics, the sensitivity of different invertebrate groups makes them excellent bioindicators for assessing aquatic ecosystem health[81]. Several taxa detected in the present study could serve this role in Macao's wetlands, each associated with different stressors. For example, Rotifers such as *Keratella* spp. and *Polyarthra* spp. are often linked to good water quality[82], while Calanoid copepods respond to chemical pollutants such as heavy metals and pesticides[83]. Among the benthos, the presence of certain organisms including Nematode genera (*Plectus*, *Caenorhabditis*, *Mononchus*, and *Xiphinema) and the* annelid worm *Limnodrilus hoffmeisteri* typically indicates organic enrichment, low dissolved oxygen levels, contamination level and pollutant exposure in the sediment[84-86]. And in a longer temporal scale, Molluscs can serve as long-term bioindicators due to their ability to bioaccumulate contaminants[87]. This has been examined by Lu, et al. [80], which reported using *Crassostrea hongkongensis* in monitoring metal pollution over an eight-year period in the region. The Asian clam *Corbicula fluminea*, on the other hand, has also shown potential as a metal indicator*[88].*

The present study demonstrates, for the first time in Macao, the use of eDNA metabarcoding to establish a comprehensive biodiversity baseline for the region’s wetlands. With a total detection of 85 fish, 9 non-fish chordate and 298 invertebrate species, only 76 were overlapping with previous conventional surveys. Such limited overlap between molecular and traditional methods is consistent with previous findings[89], including studies conducted in the Pearl River region[53]. This highlights that eDNA and conventional surveys are not contradictory but complementary, each capturing distinct components of biodiversity. It is important to note, however, that our results were derived solely from water-based eDNA, which is known to be highly effective for detecting fish species but less so for benthic taxa[44,90]. Nevertheless, this approach successfully recovered a portion of the known local benthic diversity, underscoring its utility for broad biodiversity assessment.

Overall, the three primers performed well, with each fulfilling its intended taxonomic role. The 12S primer provided high species-level resolution for fish, while the 18S V4 primer offered broad eukaryotic coverage and improved genus and order level resolution for invertebrates. Although the COI primer produced a larger proportion of higher-level (e.g., phylum-only) assignments, it captured the greatest taxonomic diversity, including unique metazoan taxa and additional fish species.

The combined use of these primers enhanced biodiversity detection by capturing complementary community components. Specifically, COI detected over five times more total ESVs and 2.5 times more species-level matches than 18S. Nevertheless, the COI marker faced annotation challenges due to incomplete reference databases. This issue varies widely across studies, with reported species-level identification rates ranging from 96.6%[91] to as low as 70.9% of clusters being assigned only at the phylum level[92]. The variation is likely driven by misidentification, gaps in reference databases, along with choice of primer[93].

The gap analysis of the present study confirmed the reference database deficiencies, with 51.3% of the locally known invertebrates missing, therefore leaving a large portion of the diversity undetectable via eDNA metabarcoding. These results highlight that, despite eDNA metabarcoding’s proficiency in detecting species presence and revealing community patterns, the method faces limitations such as primer bias, incomplete reference datasets, issues of taxonomic lumping[94], and relatively minor factors such as bioinformatic pipeline choices[95] and PCR artifacts[96]. Furthermore, eDNA-based approaches struggle to provide reliable abundance or biomass estimates due to species-specific DNA shedding rates and environmental variability, which limits their utility for interpreting result, for example, regarding to bioindicator species[97].

In conclusion, despite these limitations, our study revealed key ecological patterns in Macao's wetlands, including fish community homogenization and established non-indigenous populations. Yet, the presence of the locally endemic species has shown the conservation importance of these fragmented habitats as refugia for unique and imperiled biodiversity, highlighting the benefit of integrating eDNA with traditional surveys. For future studies, it is recommended to expand and validate primer sets to broaden the taxonomic detection range, sequence local species to improve reference databases and overall taxonomic detection range, and employ bulk DNA metabarcoding from sediments or artificial substrates to enhance benthic species detection[44]. Such enhancements will likely provide a more comprehensive picture and thereby supporting connectivity restoration, biosecurity, and long-term ecosystem management.

Materials and Methods

Study sites and sample collection

Water samples for eDNA analysis were collected from nine wetland sites in Macao (Fig. 1, the map was generated using ArcGIS[98]). These locations were selected to represent a comprehensive range of Macao’s urban aquatic habitats. They are comprised of 3 inland freshwater wetlands including Hac Sá Reservoir (HS), Ká Hó Reservoir Freshwater Wetland (KH) and Seac Pai Van Park Wetland (SPV), as well as 6 coastal sites, including estuarine habitats (Taipa Mangrove Wetland (TM), Coloane Village Wetland (CV), Cotai Ecological Protection Zones I (EI) and Cotai Ecological Protection Zones II (EII) and artificial urban lakes (Sai Van Lake (SVL) and Avenida da Praia Taipa Wetland (TW)). To capture temporal variation in community composition, sampling was performed across both summer (July, wet season) and winter (December, dry season) in 2024.

At each of the nine sites, six replicate water samples were collected during low tide. For each replicate, 2L of water were collected following the protocols of the Environmental DNA Sampling and Experiment Manual Version 2.1[99], using either pre-sterilized bottles or a decontaminated bucket and rope where direct access was limited. Samples were preserved with 1 ml of 10% benzalkonium chloride immediately after collection and transported to the laboratory in coolers within five hours to minimize DNA degradation. The six replicates from each site were filtered separately and then pooled in pairs, resulting in three combined samples per site for DNA extraction and sequencing. To monitor potential contamination during laboratory processing, 2L of Milli-Q water negative controls (3 per sampling season) were included. These controls were transferred into sampling bottles in the laboratory and were processed identically to the environmental samples, including filtration, DNA extraction, and PCR amplification.

Water sampling conducted within Cotai Ecological Protection Zone I (EI) and Cotai Ecological Protection Zone II (EII) was approved by the Environmental

Protection Bureau of Macao SAR (DSPA) (https://www.dspa.gov.mo/). Sampling at all remaining sites was approved by the Municipal Affairs Bureau of Macao SAR (http://www.iam.gov.mo).

DNA extraction, amplification and sequencing

Following the standardized protocols described in the Environmental DNA Sampling and Experiment Manual Version 2.1[100], total environmental DNA was extracted from the filter membrane using the DNeasy Blood & Tissue Kit (Qiagen). The purified DNA was eluted in 100 µL of Buffer AE, assessed for concentration and purity using a NanoDrop One spectrophotometer (Thermo Fisher Scientific) and finally stored at −20°C until shipment for sequencing.

Purified DNA samples were sent to BGI Genomics Co., Ltd. for downstream processing including PCR, library preparation and sequencing. Library size and concentration were verified using an Agilent 2100 Bioanalyzer, and qualified libraries were sequenced on BGI's DNBSEQ platform using paired-end 300 bp reads, with a targeted sequencing depth of approximately 100,000 reads per sample. Three gene regions were targeted: a ~170 bp fragment of the mitochondrial 12S rRNA gene (MiFish-U)[46], a ~ 313 bp fragment of the mitochondrial cytochrome c oxidase subunit (COI) gene (mlCOIintF / LoboR1)[101,102], and a ~400 bp fragment of the V4 region of 18S rRNA gene (TAReuk454FWD1 / TAReukREV3)[35]. All primer sequences and PCR conditions are listed in Table 1.

Bioinformatics analysis and taxonomic assignment

Demultiplexed raw sequence data were processed with the APSCALE pipeline, which incorporates tools such as Cutadapt and VSEARCH for automating essential metabarcoding preprocessing steps[103-105]. Firstly, primer sequences were removed from raw reads with Cutadapt[104], and the resulting paired-end reads were merged and quality-filtered with a maximum expected error (maxEE) of 1, and length threshold of ±50 bp of the provided amplicon sizes (e.g., 120–220 bp for 12S). Quality filtering was followed by dereplication and denoising under default APSCALE settings to produce exact sequence variant (ESV) tables.

Taxonomic assignments were conducted using local BLASTn searches against three reference databases: MIDORI2 for COI[106], SILVA for 18S[107], and MitoFish for 12S[108]. Species-level identifications were accepted if they met minimum similarity thresholds of 97% for COI, 99% for 18S, and 98% for 12S. For COI, taxonomic calls were further cross-validated with BOLDigger against the BOLD reference database[109]. Discrepancies at the species level, including conflicts between GenBank and BOLD or multiple BIN assignments in BOLD, were resolved based on geographic plausibility for chordates. Unresolved cases were conservatively reduced to the genus or higher level. Conversely, all similar discrepancies or unresolved cases were conservatively reduced to higher taxonomic rank for invertebrates, due to the lack of regional distribution data for invertebrates.

COI and 12S markers were used to target primarily fish, while COI and 18S were used to target invertebrate phyla. Nevertheless, given the broad taxonomic coverage of the COI marker, non-fish chordates was also retained when present to provide a more comprehensive overview of local biodiversity. Consequently, ESVs from Chordata and invertebrate phyla were retained for downstream analysis. Data filtering was conducted in multiple steps as follows: first, sequences detected in negative controls were excluded from all samples unless their read counts exceeded 20× those observed in the control. Next, for 12S, reads representing less than 0.1% of the highest read per ESV were removed due to the short target region and high read abundance, while for COI and 18S, reads with fewer than 10 counts were discarded.

To balance the removal of technical artifacts against the retention of ecologically meaningful rare taxa, a spatial-pattern filtering approach for singletons was applied. This logic is comparable to post-clustering curation algorithms like LULU[110], which use co-occurrence in multiple sample to identify errors. Specifically, singletons were removed if the corresponding

ESV was frequently detected elsewhere (i.e., present in ≥2 other samples), as such occurrences likely indicate index-hopping or 'tag-jumping'[111]. Conversely, unique ESVs that appeared exclusively as singletons across the entire dataset were retained as these ESVs lack a corresponding high abundance source elsewhere in the sample set that might generate technical artifacts, they are more likely to represent rare or spatially restricted ESVs[112].

Finally, knowing that removal of low frequency reads can significantly reduce sensitivity for rare species[113], any ESV identified to the species level with a confirmed local distribution was 'rescued' and retained regardless of the abundance thresholds. The final taxon presence was determined on a presence/absence basis (≥1 read after filtering).

Non-indigenous species identification

Non-indigenous species were identified using multiple sources, including global distribution databases such as Global Biodiversity Information Facility (GBIF), citizen science records (iNaturalist), regional field guides and literatures in the nearby region. The same approach was applied to both fish and invertebrates, however, due to limited regional taxonomic and distribution record for invertebrates, non-indigenous invertebrate species could not be confidently determined, and only those that are well documented were reported.

Comparison of eDNA results with traditional survey data

A reference species checklist was compiled from a technical report provided by the Macao Environmental Protection Bureau (DSPA)[38]. The report documents annual record of fish and invertebrate species observed within the Cotai Ecological Protection Zone using traditional survey approaches, including netting, visual surveys, and electrofishing[55]. After bioinformatics analysis and taxonomic assignment, the eDNA results were compared with this local checklist, which allow the findings from the eDNA analysis to be compared and verified. In the absence of regional DNA barcode reference library, this checklist also enabled validation of the ecological plausibility of detected taxa and enable direct comparison of the efficiency between conventional and molecular methods.

Statistical analysis

All statistical analyses were conducted primarily in R v4.5.0[114], with additional plotting performed in GraphPad Prism 9[115] and figure editing in Adobe Illustrator[116]. Data processing and organization were carried out using the dplyr (1.1.4) and tidyr (1.3.1) packages. Taxonomic composition across primer were visualized with chord diagrams generated using the circlize (0.4.16) package, while taxa overlap among primers was illustrated with Venn diagrams generated using eulerr (7.0.2) and exported with svglite (2.2.1).

Community composition analyses were conducted in phyloseq (1.52.0), including non-metric multidimensional scaling (NMDS). To ensure the assumptions of PERMANOVA were met, homogeneity of multivariate dispersions among groups was assessed using vegan (2.6.10)[117], followed by permutation tests (999 permutations) to evaluate whether group dispersions differed significantly. After confirming the non-significant of dispersions, community differences among sites were assessed using permutational multivariate analysis of variance (PERMANOVA). To investigate seasonal effects at individual sites, analysis of similarities (ANOSIM) were performed. These statistical analyses were chosen because they are appropriate for non-parametric comparisons of community composition data. Further comparisons of species composition, including distributions of non-indigenous species, were visualized using the UpSetR (1.4.0) package. Additional figures were generated using ggplot2 (3.5.2). Finally, species detected in the eDNA dataset were validated with a gap analysis using annual survey data DSPA[38] to assess the extent to which species recorded in these conventional surveys could be detected by eDNA analysis. This was performed by BLAST searches against the same reference databases used for taxonomic assignment of the water sample, including MIDORI 2 and BOLD reference database (COI), SILVA (18S), and MitoFish (12S).

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Author contributions

M.K. Leong[1] prepared the Introduction and Methods sections, formatted the manuscript, conducted sampling and experiments, and assisted in figure production. I.H. Lau[1] was responsible for data collection and analysis, prepared the Results and Discussion sections, performed sampling and experiments, and produced figures. F.O. Costa[2] provided advice on the metabarcoding approach and data analysis techniques and contributed to reviewing the manuscript. M.P. Cabezas[2] offered guidance on metabarcoding data analysis methodologies and participated in reviewing and refining the manuscript. K. Tagulao[1] conceptualized the project, designed the sampling/experiments, secured funding, coordinated research activities, and contributed to reviewing and finalizing the manuscript.

Competing interests

The authors declare no competing interests.

Data availability

Raw sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject **PRJNA1344804 (**https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1344804). Supplementary Information provides full lists of detected taxa, including taxonomic identification, primers, and the sites where each taxon was detected, as well as tables summarizing raw reads, merged reads, and reads retained after quality filtering for each library.

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Figures and figure legends

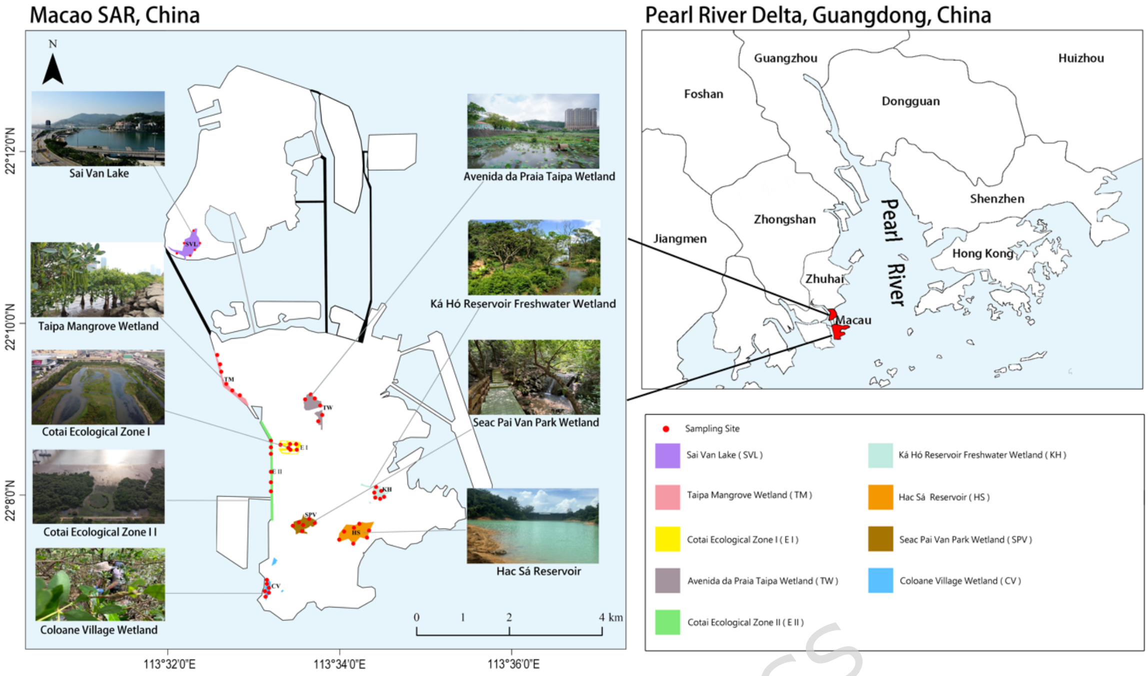

Figure 1.

Map of the study area showing the nine wetland sampling sites across Macao. The main panel displays the specific sampling locations (red dots) within the different wetland areas (colored polygons). The inset panel provides geographical context, showing the location of Macao within the Pearl River Delta Area, China. The site names and their corresponding abbreviated codes are as follows: SVL = Sai Van Lake; TM = Taipa Mangrove Wetland; TW = Avenida da Praia Taipa Wetland; EI = Cotai Ecological Protection Zone I; EII = Cotai Ecological Protection Zone II; KH = Ká Hó Reservoir Freshwater Wetland; HS = Hac Sá Reservoir; SPV = Seac Pai Van Park Wetland; and CV = Coloane Village Wetland.

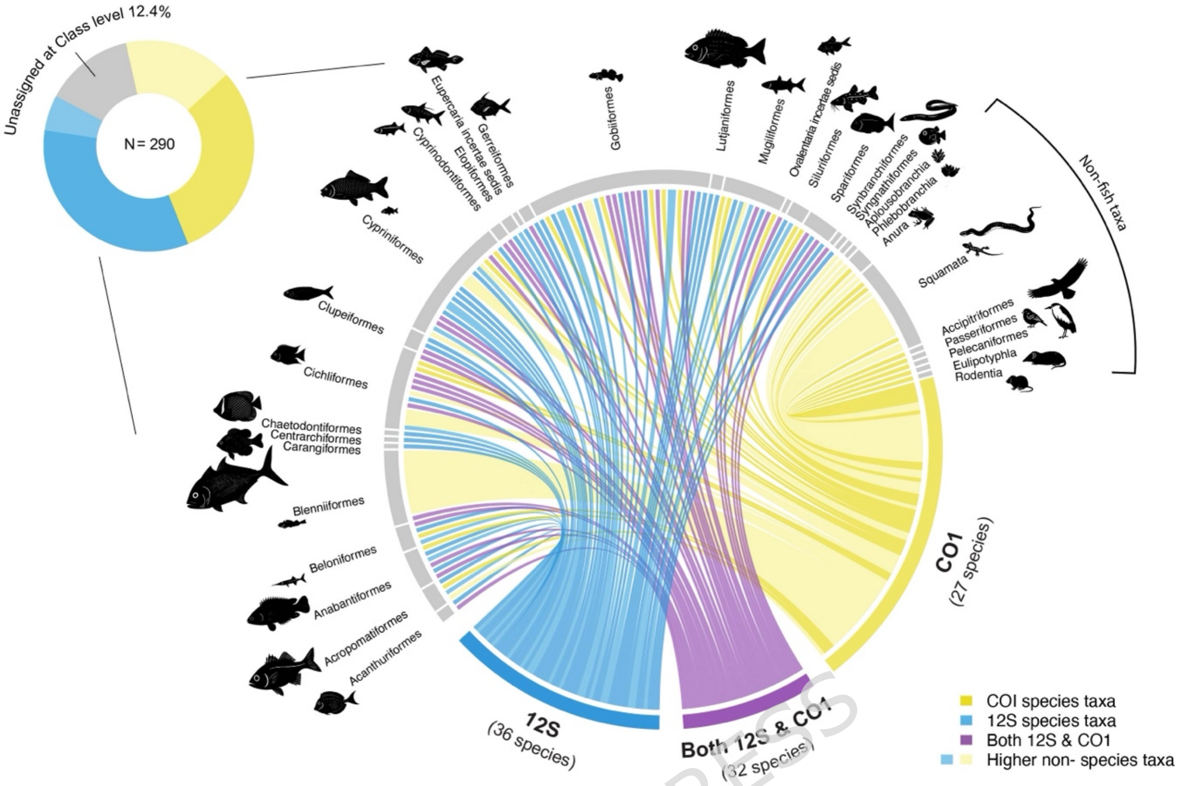

Figure 2.

Venn diagram showing the proportion of reads assigned only at the phylum level (grey), and a circle plot illustrating the order-level composition of detected chordate taxa across all samples, with ESVs assigned to the same species represented as a single taxon.

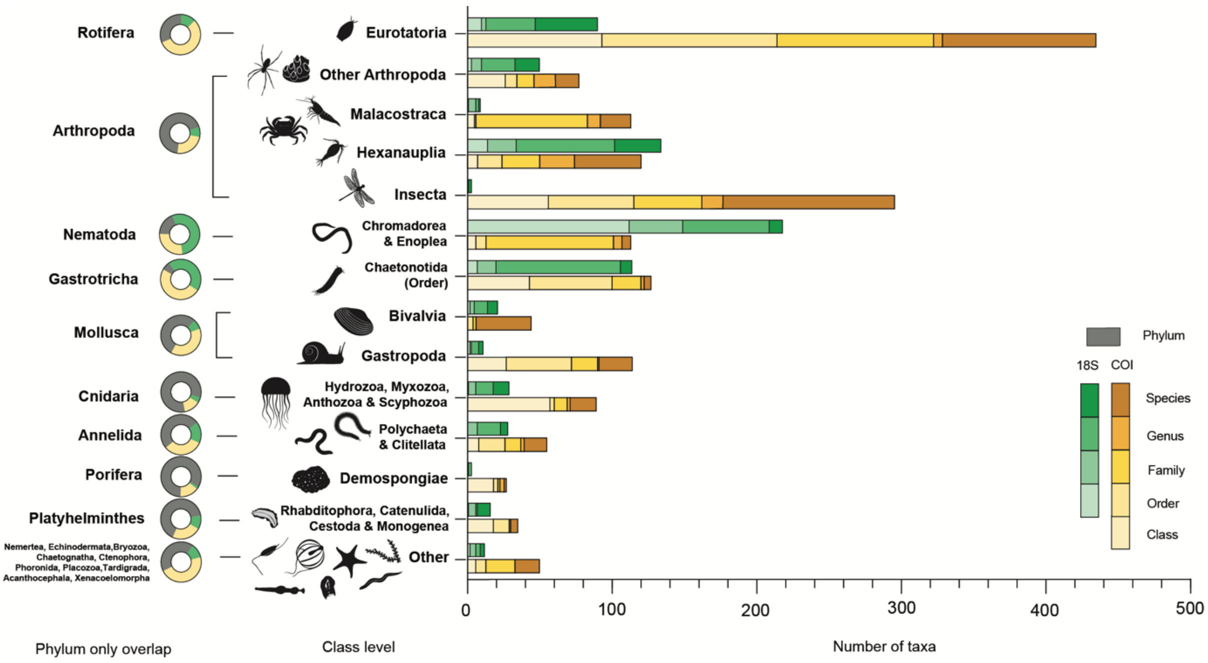

Figure 3.

Taxonomic resolution across phyla and orders based on eDNA metabarcoding. From left to right: phylum designation, Venn diagram illustrating the proportion of reads assigned only at the phylum level (shown in grey), order designation, and bar plot summarizing the number of taxa resolved at the Class level within each phylum. Taxa identified with 18S are shown in green and those identified with COI are shown in yellow.

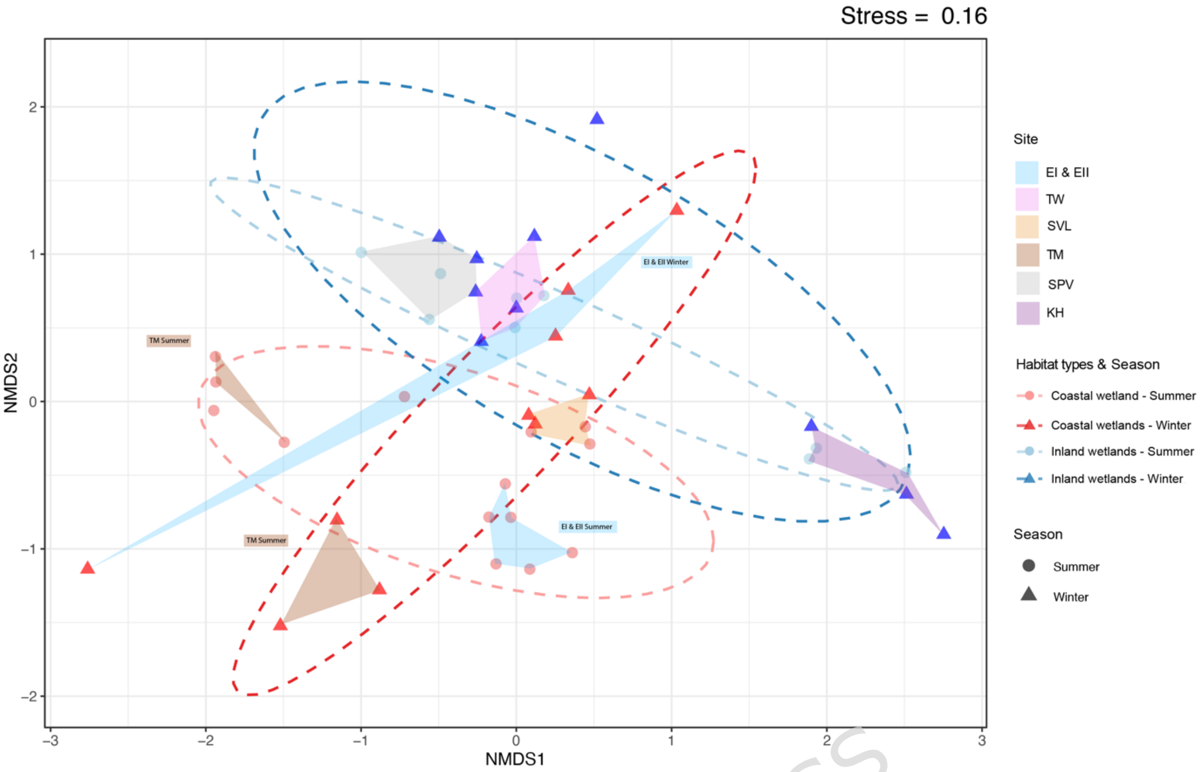

Figure 4.

Non-metric multidimensional scaling (NMDS) plots of fish community composition, with ellipses (80% confidence) illustrating grouping by habitat type and distinct site level differences. Ellipses were fitted after excluding outliers beyond 2 standard deviations. No overall seasonal effect was detected, but seasonal site groupings were supported by ANOSIM analysis at Cotai Ecological Protection Zones and Taipa Mangrove (EI & EII: R = 0.7; TM R = 0.9). Site codes are defined in the Methods and shown in Fig. 1.

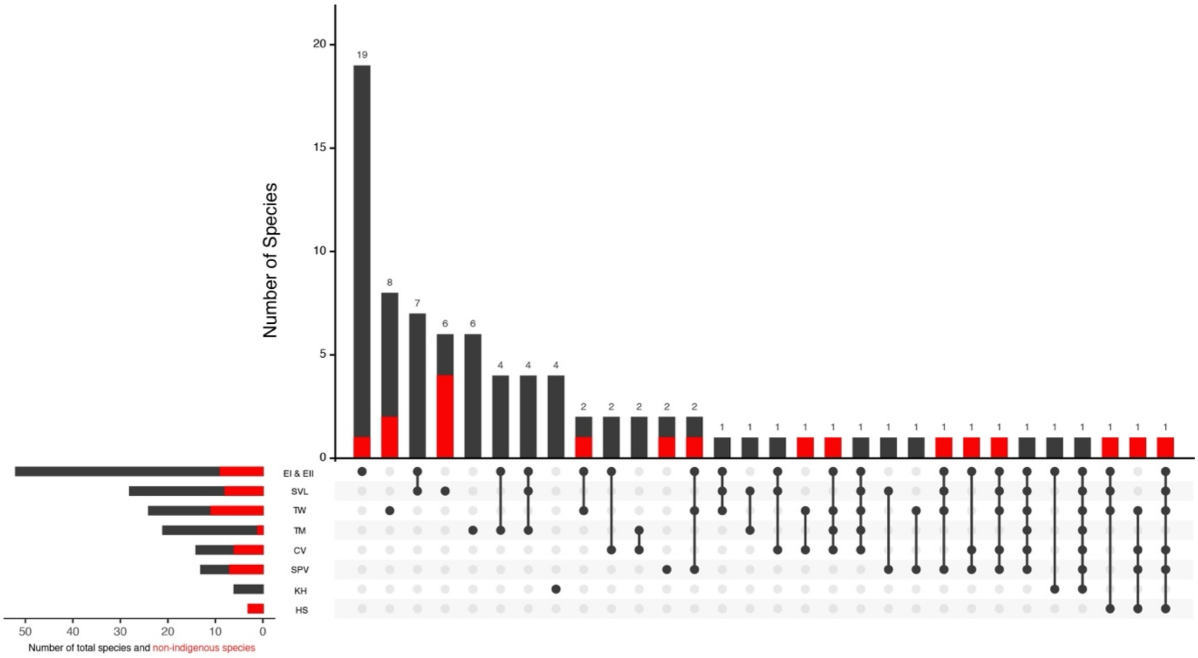

Figure 5.

UpSet plot showing shared fish species among sampling sites, total fish species richness per site, and the proportion of non-indigenous fish species. The vertical bars represent the number of species shared among different combinations of sites (intersections). The horizontal bars on the left indicate the total number of species detected at each site. Non-indigenous fish species are represented in red within the total species counts per site.

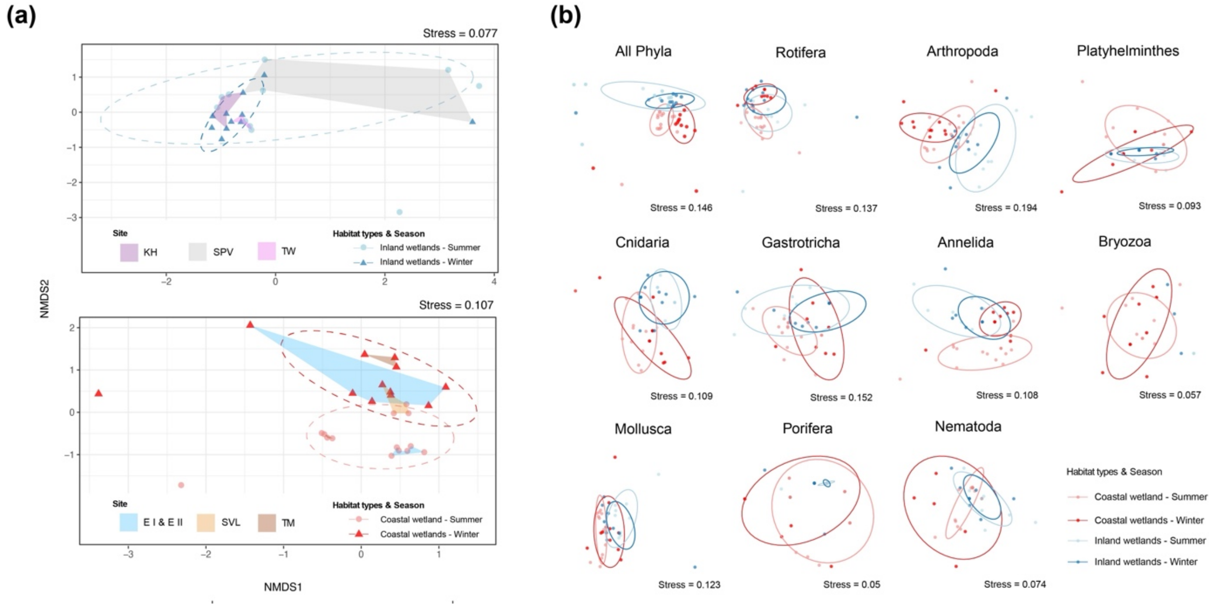

Figure 6.

(a) Non-metric multidimensional scaling (NMDS) plots of invertebrate community composition shown separately for Inland wetlands (top) and coastal wetlands (bottom). Individual data points are displayed with 80% confidence ellipses illustrating habitat and seasonal grouping, while shaded regions indicating distinct site communities. Ellipses were fitted after excluding outliers beyond 2 standard deviations. Seasonal differences were absent in inland freshwater wetlands (p = 0.1) but evident in coastal wetlands (p = 0.001). Site codes are defined in the Methods and shown in Fig. 1.

(b) NMDS ordination of invertebrate communities at the phylum level for different group across coastal and inland wetlands, Individual data points are shown with ellipses illustrated at 80% confidence. Only phyla with sufficient sample representation and interpretable stress valus are shown. Stress values for each ordination are displayed in the bottom left of the corresponding panel.

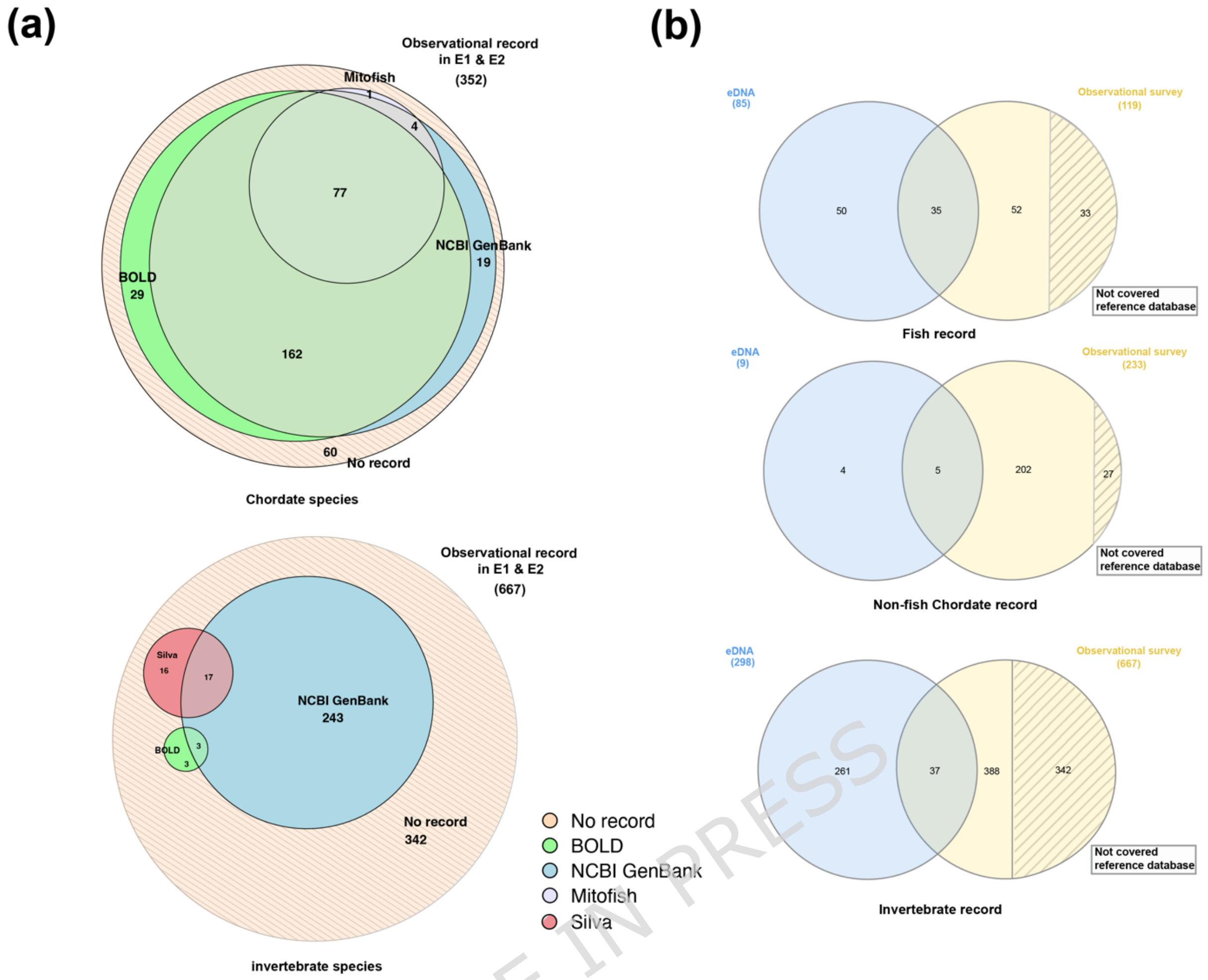

Figure 7.

(a) Reference database coverage for (top) chordate species (n=352) and (bottom) invertebrate species (n=667). Analysis for chordates assessed coverage of 12S and COI markers in BOLD, MIDORI 2 (GenBank) and Mitofish. Analysis for invertebrates assessed coverage of COI and 18S in BOLD, MIDORI 2 (GenBank), and SILVA database.

(b) Venn diagram comparing species detected by eDNA metabarcoding and species recorded data from observation surveys from Environmental Protection Bureau (DSPA) at Cotai Ecological Protection Zone I and Cotai Ecological Protection Zone II. The overlap represents species reported by both methods. Species recorded in traditional surveys but not available in the reference library are highlighted with line-pattern fill, indicating gaps in current sequence databases.

Tables

Table1: Primer sequences and PCR conditions

Primer	Sequence	PCR conditions			
		Denaturing	Annealing	Extension	Cycles
MiFish-U[46]	F: 5'GTCGGTAAAACTCGTGCCAGC-3' R:5'CATAGTGGGGTATCTAATCCCAGTTTG-3'	95°C 3 min (init); 98°C 20 s	65°C 15 s	72°C 15 s; 72°C 5 min (final)	35
mlCOIintF[101] / LoboR1[102]	F:5'-GGWACWGGWTGAACWGTWTAYCCYCC-3';	95°C 10 s (16×); 94°C 30 s (25×)	62°C 30 s, -1°C/cycle (16×); 46°C 30 s (25×)	72°C 60 s (all); 72°C 5 min (final)	41
	R: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	94°C 1 min (init); 94°C 30 s	45°C 1 min (1st); 54°C 1 min (30+15×)	72°C 1 min (5×); 72°C 45 s (15×); 72°C 5 min (final)	45
V4 18S TAReuk454FWD1 /	F: 5'-CCAGCASCY	95°C 5 min (init); 94°C 30 s (all)	57°C 45 s (10×); 45–49°C 45 s	72°C 1 min (all); 72°C	35

TAReukREV3[35]	GCGGTAATTCC-3'	(25×, sample-dependent)	2 min (final)