First DNA Barcoding Records of Fish Species from Totok and Makalo Estuaries, North Sulawesi, Indonesia

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Abstract

The Totok and Makalo River estuaries in North Sulawesi, Indonesia, serve as vital habitats teeming with fish biodiversity, supporting coastal ecosystems and local fisheries. This study aimed to identify fish species and elucidate their genetic relationships through DNA barcoding of the Cytochrome Oxidase I (COI) gene. Fish samples were collected from both estuaries using nets and traps, followed by molecular analysis involving polymerase chain reaction (PCR) amplification and Sanger sequencing. The results confirmed the presence of 11 fish species, including Osteomugil engeli, Polydactylus plebeius, and Gazza minuta, with high identification accuracy (over 98% sequence similarity to reference databases). Notably, Paracentropogon rubripinnis and Nematalosa come were recorded for the first time in North Sulawesi waters, expanding the known distribution of these species. Significant genetic variation was detected in P. rubripinnis, potentially indicating cryptic speciation or local adaptation to environmental gradients. Phylogenetic analysis revealed close genetic affinities between certain species and populations from Taiwan and Bangladesh, suggesting historical migration patterns or shared ancestry, possibly facilitated by ocean currents. These findings underscore the rich genetic diversity in these estuaries, which are influenced by factors like salinity, temperature, and anthropogenic pressures. The study emphasizes the urgency of conservation strategies, including habitat protection and sustainable fishing practices, to preserve biodiversity and maintain ecosystem services. By providing baseline data on species composition and genetic connectivity, this research contributes to broader efforts in marine biodiversity monitoring and informs policy for coastal management in Indonesia's biodiversity hotspots.

Keywords: *DNA barcoding, COI gene, first record, Paracentropogon rubripinnis, Nematalosa come, estuarine fish*

Introduction

Indonesia, the largest archipelagic country in the world, is characterized by an impressive diversity of marine species (Hutomo and Moosa, 2005; Nugraha *et al.,* 2023); however, information on genetic resources in Indonesian aquatic waters, such as fish, is still scarce (Dahruddin *et al*., 2017). This lack of data is even more pronounced in estuarine ecosystems - key transitional zones between freshwater and marine environments that are known habitats for various fish species, including spawning, feeding, and nursery areas (Cardoso, 2021). Despite their ecological and economic importance, extensive molecular surveys on fish diversity in Indonesian estuaries, although they remain ecologically and economically understudied.

The Totok and Makalo estuaries, located in the southeast of Minahasa Regency, North Sulawesi, are rich in marine resources with significant fisheries potential. However, these estuarine ecosystems are threatened by environmental degradation due to human activities, especially traditional small-scale gold mining that takes place locally, called "dompeng." These activities disturb important habitats (through sediment disturbance), affect water quality, and pose a threat to fish biodiversity due to habitat degradation and potential heavy metal contamination (Meutia *et al*., 2023). Studies have shown that the nearby Buyat Bay has high accumulation of heavy metals (Manengkey, 2010; Rumampuk and Warouw, 2015), which may also affect these estuaries.

While recent surveys of fish communities in estuaries of North Sulawesi have employed morphological methods (Bataragoa *et al*., 2023; Dauhan *et al*., 2024), molecular systematic surveys have never been conducted at the Totok and Makalo estuaries. This lack of information prevents accurate species identification, understanding of the population connectivity, and efficient biodiversity monitoring. DNA barcoding with the mitochondrial Cytochrome Oxidase I (COI) gene has been particularly successful in identifying fish species, providing higher taxonomic resolution that differentiates between morphologically challenging taxa (Hebert *et al*., 2003; Ward *et al*., 2005). This molecular approach is particularly useful when there

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is a lack of expertise in traditional morphology is lacking or when characters are not sufficiently diagnostic (Bemis *et al*., 2023).

The objectives of this study were to document fish species found in the Totok and Makalo estuaries using COI barcoding, to add new molecular records for North Sulawesi and Indonesia, to record environmental variables as measures of habitat status, to compare the phylogenetic relationships among these taxa with those sampled from other geographic regions globally, and to provide a baseline database for monitoring biodiversity changes and establishing conservation priorities in mining-affected estuarine ecosystems. This study represents the first combined molecular and environmental survey of fish diversity within these estuaries, thereby providing critical baseline data for understanding the coastal ichthyofauna of North Sulawesi under anthropogenic influence.

Materials and Methods

Sample collection

Fish specimens were collected from the Totok and Makalo River estuaries, Southeast Minahasa Regency, North Sulawesi, Indonesia (Figure 1), during June 2024. Three sampling replicates were performed at each station (Totok and Makalo) to capture spatial variation. Eleven specimens representing morphologically distinct taxa were obtained using beach seines (Rozas and Minello, 1997). Each specimen was photographed for morphological documentation, assigned a unique laboratory code, immediately preserved in 95% ethanol, and stored at room temperature until DNA extraction. Ethanol preservation effectively removes residual saltwater, dehydrates cellular tissue, and reduces DNA degradation, thereby improving the quality of extracted DNA for molecular analysis (Ward *et al*., 2005; Nagy, 2010).

Environmental parameter measurements

Water quality parameters were measured directly at each sampling station using a multi-parameter water quality meter (Horiba U-52G). The parameters measured included temperature (°C), pH, dissolved oxygen (mgL^{-1}), and salinity (ppt). Three replicate measurements were made at each station during fish sampling, and average values were calculated. These data describe environmental conditions and explore potential relationships between water quality and fish species distribution. Measured parameters were evaluated against Indonesian seawater quality standards for marine biota (Republic of Indonesia, 2021).

Laboratory analysis

Tissue samples (~10 mg or small muscle pieces) were collected from fish muscle, and DNA was extracted using the Chelex 10% protocol (Walsh *et al*., 2013). The extracted DNA was then PCR amplified using the universal fish primers FISH F1 (5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3') and FISH R1 (5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3') (Ward *et al*., 2005), following the protocol of the BIONESIA laboratory (Bali, Indonesia).

The total PCR reaction volume was 26 µL, consisting of 2 µL of DNA template, 1.25 µL of each primer (10 µM), 9 µL of ddH_2O, and 12.5 µL of Ready-mix. Amplification was performed using an Applied Biosystems™ 2720 Thermal Cycler with the following profile: initial denaturation at 94°C for 3 min, followed by 38 cycles of denaturation at 94°C for 30 s, annealing at 50-59°C for 30 s, and extension at 72°C for 60 s, with a final extension at 72°C for 2 min. PCR products were visualized on a 1% agarose gel stained with GelRed®. Positive samples were sequenced using the Sanger dideoxy method at PT. Genetika Science Jakarta (Sanger *et al*., 1977).

Data analysis

Chromatogram sequences were edited in MEGA 11 (Tamura *et al*., 2021) by trimming the forward and reverse primer sequences to obtain the core region. This sequence alignment strategy was chosen to ensure accurate representation of genetic variation and minimize errors in phylogenetic tree construction (Zou *et al*., 2024). Species identification was performed using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI).

Evolutionary distances between sequences were calculated using the Tamura-Nei method with base substitution rates per site. Rate heterogeneity was modelled with a gamma distribution (shape parameter = 1). Thirty-two nucleotide sequences were analyzed (ambiguous positions were removed by pairwise deletion; there were a total of 607 positions in the final dataset). A phylogenetic tree was generated using the neighbor-joining method (Saitou and Nei, 1987). Clade confidence was evaluated using bootstrap support (1,000 iterations). All evolutionary analyses were conducted using MEGA 11.

Descriptive water quality analyses were conducted to characterize environmental conditions and spatial differences that may influence fish distribution patterns and genetic diversity.

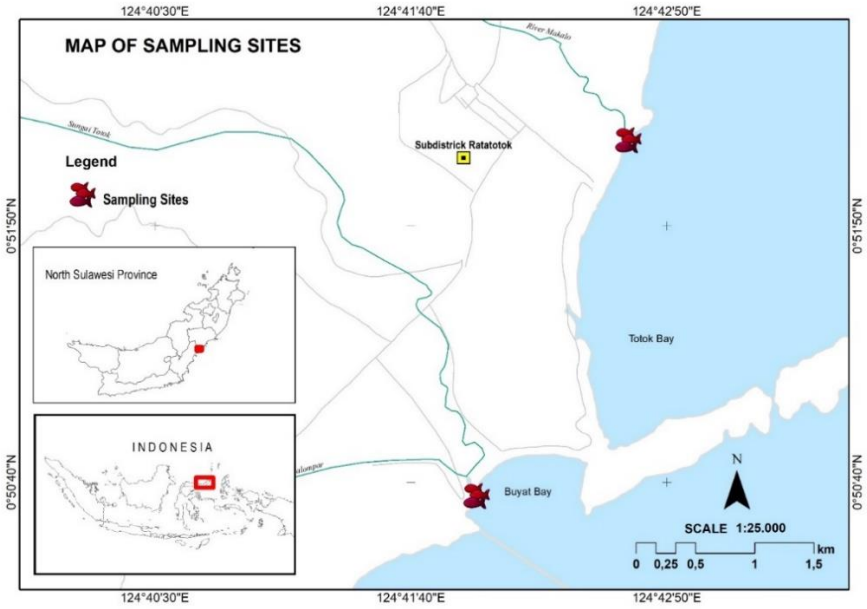

Figure 1. Map showing sampling locations in the Totok and Makalo River estuaries, Southeast Minahasa Regency, North Sulawesi, Indonesia

Results and Discussion

Water quality measurements revealed distinct environmental profiles between the Totok and Makalo River estuaries (Table 1). Temperatures ranged from 25.41°C to 28.27°C, with consistently alkaline pH values (7.53–8.44), indicating conditions typical of a marine-influenced tropical estuary. These values comply with Indonesian seawater quality standards for marine biota (temperature: 28–30°C natural; pH: 7–8.5) as specified in Appendix VIII of Government Regulation No. 22/2021 (Republic of Indonesia, 2021), and are within acceptable ranges for estuarine fish communities (Cardoso, 2021).

The most striking difference was observed in salinity patterns. The Totok River estuary exhibited significantly higher salinity (mean: 5.93 ppt, range: 1.05–14.56 ppt), with Station 1 showing strong marine influence (14.56 ppt), while the Makalo River estuary consistently exhibited lower salinity (mean: 2.23 ppt, range: 0.38–3.43 ppt), indicating stronger freshwater dominance. These differences reflect varying levels of saltwater intrusion, tidal dynamics, and freshwater discharge between the two systems, which directly impact species distribution and osmoregulatory demands on fish communities (Abdulmalik-Labe *et al*., 2023).

Dissolved oxygen levels were generally adequate for fish survival in both estuaries (5.9–6.76 mgL^{-1}), meeting the minimum threshold of >5 mgL^{-1} specified in Indonesian seawater quality standards (Republic of Indonesia, 2021). However, slightly lower concentrations in Makalo (mean: 6.18 mgL^{-1} vs. 6.61 mgL^{-1} in Totok) likely reflect anthropogenic disturbance. Direct field observations during sampling in June 2024 documented intensive artisanal gold mining operations ("dompeng") in the Makalo River Estuary, characterized by mechanical disturbance of the benthic substrate, visible sediment plumes in the estuarine waters, and changes in riverbank morphology. These mining operations resulted in a significant decrease in water clarity compared to the Totok Estuary. Previous studies in nearby Buyat Bay have documented heavy metal contamination associated with similar mining activities (Manengkey, 2010; Rumampuk and Warouw, 2015), raising concerns about potential impacts on fish biodiversity in this system.

Molecular species identification and COI barcoding effectiveness

BLAST analysis showed that 11 sequences were identified at the species level with query coverage between 90% and 100%, and sequence identities ranging from 87.28% to 100% (Table 2). After quality trimming, the COI fragments varied from 654 to 715 bp in length.

Seven species from six families were found in the Totok River estuary, including *Osteomugil engeli* (Mugilidae), *Nematalosa come* (Clupeidae), *Polydactylus plebeius* (Polynemidae), *Upeneus sulphureus* (Mullidae), *Plectorhinchus gibbosus* (Haemulidae), and the Carangidae family with two species, *Caranx sexfasciatus* and *Gazza minuta*. In

the Makalo River estuary, four species from four different families were recorded: one belonging to the Ophichthidae family (*Eleutherochir opercularis*), one belonging to the Tetrarogidae family (*Paracentropogon rubripinnis*), one belonging to the Engraulidae family (*Stolephorus waitei*), and one belonging to the Gobiidae family (*Psammogobius biocellatus*).

The high identification success confirms COI barcoding as a reliable tool for estuarine fish identification in Indonesian waters, with the COI marker providing substantial interspecific divergence while maintaining intraspecific conservation (Hebert *et al*., 2003; Ward *et al*., 2005; Dahruddin *et al*., 2017; Bemis *et al*., 2023). The lower sequence similarity for *P. rubripinnis* (87.28%) may indicate intraspecific genetic variation between Indonesian and Korean populations or limited database representation, underscoring the need to expand barcode reference libraries for underrepresented regions like the Coral Triangle. These 11 new COI sequences submitted to GenBank provide valuable reference data for future molecular identification, eDNA metabarcoding, and identification of early life stages that often lack diagnostic morphological characters (Kumar *et al*., 2020).'

First molecular records and biogeographic significance

This study reports two major molecularly documented range expansions. The molecular record of *Paracentropogon rubripinnis* (Family: Tetrarogidae) is the first report for Indonesian waters. Although widely distributed in the temperate and subtropical western Pacific, including neighbouring countries to the north (Republic of Korea, Japan, and Taiwan), no previous scientific record has been reported from Indonesia based on morphology or molecular systematics. The Makalo River Estuary specimen had 87.28% identity to the Korean reference sequence (GenBank: NC_062460.1) with 99% query coverage, confirming this identification.

Nematalosa come represents the first recorded occurrence in the waters of North Sulawesi. The specimen from the Totok River Estuary had 98.93% similarity to the reference sequence (GenBank: GU673751) and 93% query coverage. Although this species has been reported previously in the waters of southern Bali, northern Bali, and southern Java (Andriyono *et al*., 2020), this discovery extends its distribution to the north, including the waters of Sulawesi, where it was previously unrecorded, thus expanding knowledge about its biogeographic range throughout the Indonesian archipelago.

These finding are noteworthy in light of recent extensive morphological surveys in various estuary systems in North Sulawesi, including those from the Tondano River (Bataragoa *et al*., 2023, 2024; Dei *et al*., 2024), Sario River (Dauhan *et al*., 2024), and Bahu River (Sampe *et al*., 2024). Both *P. rubripinnis* and *N. come* were not present in these morphological inventories, which demonstrates the complementary value of molecular approaches to species identification. Such applications are especially useful in the case of cryptic species, juveniles, and damaged specimens (Bemis *et al*., 2023), as barcodes yield definitive taxonomic assignments independent of life stage or observer bias.

The occurrence of *P. rubripinnis* in tropical North Sulawesi waters presents interesting biogeographic questions. This discovery may represent (1) an extension in the range facilitated by oceanographic conditions or a shift associated with climate-affected distribution; (2) historical oversights in surveys due to the cryptic morphology and small size of the species; or (3) a previously undetected rare natural population. The relatively low sequence identity (87.28%) in comparison with Korean samples suggests potential genetic divergence, which needs to be addressed by expanded sampling and using multi-locus molecular markers.

Table 1. Water quality parameters measured at sampling stations in Totok and Makalo River estuaries

Location	Replicate ID	Temperature (°C)	pH	Dissolved Oxygen (mgL^{-1})	Salinity (ppt)
Totok River Estuary	1	28.27	8.26	6.46	14.56
Totok River Estuary	2	25.41	7.53	6.62	1.05
Totok River Estuary	3	26.39	7.65	6.76	2.17
Mean ± SD		26.69 ± 1.46	7.81 ±0.39	6.61 ± 0.15	5.93 ± 7.78
Makalo River Estuary	1	26.42	8.44	5.94	0.38
Makalo River Estuary	2	26.64	7.96	5.90	2.87
Makalo River Estuary	3	27.50	8.09	6.70	3.43
Mean ± SD		26.85 ± 0.56	8.16 ± 0.24	6.18 ± 0.44	2.23 ± 1.58

Table 2. Molecular identification of fish species from Totok and Makalo River estuaries using COI barcoding

Lab ID	Location	bp	Species; Family; Common Name	Accession Number	Query Cover (%)	Identity (%)
BIOSUB288.026	Totok River Estuary	715	*Osteomugil engeli;* Mugilidae; Engle's Mullet	OQ386328.1	99	100.00
BIOSUB288.027	Totok River Estuary	700	*Nematalosa come;* Clupeidae; Perth Herring or Gizzard Shad	GU673751.1	93	98.93
BIOSUB288.002	Totok River Estuary	689	*Polydactylus plebeius;* Polynemidae Striped Threadfin	KJ202188.1	99	99.71
BIOSUB288.007	Totok River Estuary	654	*Upeneus sulphureus;* Mullidae; Yellowstripe Goatfish	MK348216.1	100	99.39
BIOSUB288.032	Totok River Estuary	700	*Plectorhinchus gibbosus;* Haemulidae; Brown Sweetlips or Harry Hotlips	KJ202186.1	97	99.71
BIOSUB288.033	Totok River Estuary	684	*Caranx sexfasciatus;* Carangidae; Bigeye Trevally	MN795449.1	100	99.85
BIOSUB288.035	Totok River Estuary	680	*Gazza minuta;* Leiognathidae; Toothed Ponyfish	NC_026232.1	100	99.41
BIOSUB288.028	Makalo River Estuary	698	*Eleutherochir opercularis;* Ophichthidae; Finny Snake Eel	KF265061.1	90	99.37
BIOSUB288.029	Makalo River Estuary	689	*Paracentropogon rubripinnis;* Tetrarogidae; Red-finned Velvetfish	NC_062460.1	99	87.28
BIOSUB288.030	Makalo River Estuary	712	*Stolephorus waitei;* Engraulidae; Waite's Anchovy	MH380735.1	91	98.61
BIOSUB288.031	Makalo River Estuary	693	*Psammogobius biocellatus;* Gobiidae Twin-spot Goby	HQ909449.1	98	98.83

Note: bp = sequence length (base pairs) after contig assembly and trimming. Query coverage and sequence identity indicate match quality with GenBank reference sequences.

Table 3. Average pairwise genetic distances among fish species from Totok and Makalo River estuaries based on COI sequences using the Tamura-Nei model

Description	Genetic Distance (d)	Percentage (%)	Standard Deviation (SD)
All samples (n=32)	0.2957	29.57	0.074
Totok River Estuary (n=7 species)	0.3014	30.14	0.027
Makalo River Estuary (n=4 species)	0.3229	32.29	0.026
Outgroup (*Mobula birostris*)	0.3843	38.43	0.024

Note: Genetic distances represent the number of base substitutions per site calculated using the Tamura-Nei model. Rate variation among sites was modelled using a gamma distribution (shape parameter = 1). Analysis performed using MEGA11 with 1000 bootstrap replications. All ambiguous positions were removed using pairwise deletion option. Higher values indicate greater genetic differentiation between samples.

Genetic diversity, environmental quality, and mining impacts

The Tamura-Nei genetic distance analysis revealed moderate interspecific divergence across the dataset (Table 3). The average genetic distance of all samples was 0.2957 (29.57%), consistent with the interspecific divergence patterns observed in other fish DNA barcoding studies (Dahruddin *et al*., 2017; Bemis *et al*., 2023), indicating that COI can be used effectively to separate species.

The average genetic distance among samples from the Makalo River Estuary was higher than that from the Totok River 0.3229 or 32.29% vs 0.3014 or 30.14%. This difference is largely due to the taxonomic representation of the Makalo samples, which include distantly related taxa (*e.g., P. rubripinnis* — Tetrarogidae and *E. opercularis* — Ophichthidae). However, environmental factors may also contribute to these patterns.

Consistent with this interpretation, environmental measurements showed that the Makalo Estuary had lower DO (mean: 6.18 mgL^{-1} vs. Totok mean= 6.61 mgL^{-1}) and distinct salinity profiles (mean: 2.23 ppt vs. 5.93 ppt). These observations coincided with active "dompeng" mining operations observed during fieldwork. These mining activities cause extensive disturbance of the benthic substrate due to sediment mobilization and significantly alter water chemistry through the influx of heavy metals and increased turbidity, potentially reducing primary productivity (Meutia *et al*., 2023). Previous research in nearby Buyat Bay has shown significant bioaccumulation of mercury, arsenic, chromium, cadmium, and lead in organisms and sediments (Manengkey, 2010; Rumampuk and Warouw, 2015) that could pose similar contamination risks to the Makalo system.

Anthropogenic disturbances can strongly impact fish communities through multiple pathways. Species that thrive in disturbed habitats may increase in abundance, while sensitive species may decline or disappear (Weiskopf *et al*., 2020; Hajji and Lucas, 2024). This selective pressure not only alters community composition but can also affect genetic diversity at the assemblage level. Furthermore, pollution-induced population declines can reduce effective population size, leading to genetic drift and local differentiation (Mastretta-Yanes *et al*., 2024).

Similar cases in other estuarine systems demonstrate the potentially profound impacts of sediment disturbance or hydrological changes on fish reproduction and biodiversity. For example, dam construction in the Yangtze River Estuary destroyed important spawning grounds for *E. sinensis*, resulting in decreased fertility and reproductive success due to excessive salinity or turbidity caused by suspended sediment (Chen *et al*., 2023; Zhang *et al*., 2023). Experimental restoration of coastal ecosystems also shows that lower habitat heterogeneity is negatively associated with fish diversity and density (Schulz *et al*., 2020).

Some of the taxa in this study have ecological traits that make them more vulnerable to mining disturbance. Benthic species, such as *P. biocellatus*, require undisturbed sediment for shelter or breeding and are therefore highly sensitive to habitat loss. Planktivorous organisms, such as *S. waitei*, may be affected by decreased primary production resulting from higher turbidity. The consequences of these changes in food supply can also extend to higher trophic levels. Euryhaline species such as *O. engeli* can also accumulate heavy metals from consuming organic matter and sediments contaminated with heavy metals (Abdulmalik-Labe *et al*., 2023).

However, it should be noted that our sampling design (few samples per species) provides limited power for comprehensive analyses of intraspecific genetic diversity or population structure. The genetic distances calculated in this study primarily reflect interspecific, rather than intraspecific, variation. Further field research at the population level involving both disturbed (mined) and undisturbed sites is needed to provide robust evidence regarding mining-related impacts on within-population genetic diversity, gene flow, and genetic structure (Abdulmalik-Labe *et al*., 2023; Mastretta-Yanes *et al*., 2024). Furthermore, comprehensive environmental monitoring, including water quality, sediment quality, and heavy metal analysis, is needed to assess the correlation between mining activities and biodiversity patterns before developing evidence-based conservation management.

Phylogenetic relationships and regional connectivity

Phylogenetic analysis using the neighbor-joining method revealed distinct evolutionary relationships among species and their geographic populations (Figure 2). Bootstrap support values varied between 65 and 100, with high support value (>95) confirming well-supported phylogenetic signals in the dataset.

The Makalo River Estuary species exhibited interesting biogeographic patterns. *Stolephorus waitei* and Taiwanese specimens clustered together with high bootstrap support (100), indicating a close evolutionary relationship, and suggesting possible historical connections between these populations. This pattern likely reflects larval dispersal, enabled by ocean currents connecting the Western Pacific, given the high fecundity and planktonic larval stages of *Stolephorus* spp. (Mulya *et al*., 2021; Gao *et al*., 2023). *Paracentropogon rubripinnis* clustered with species from the Republic of Korea, albeit with low bootstrap support (65), suggesting a complex evolutionary history, such as multiple dispersal events and/or long periods of isolation. *Eleutherochir opercularis* from Makalo also showed a close genetic relationship with the Taiwanese population (bootstrap support: 100), consistent with regional connectivity patterns.

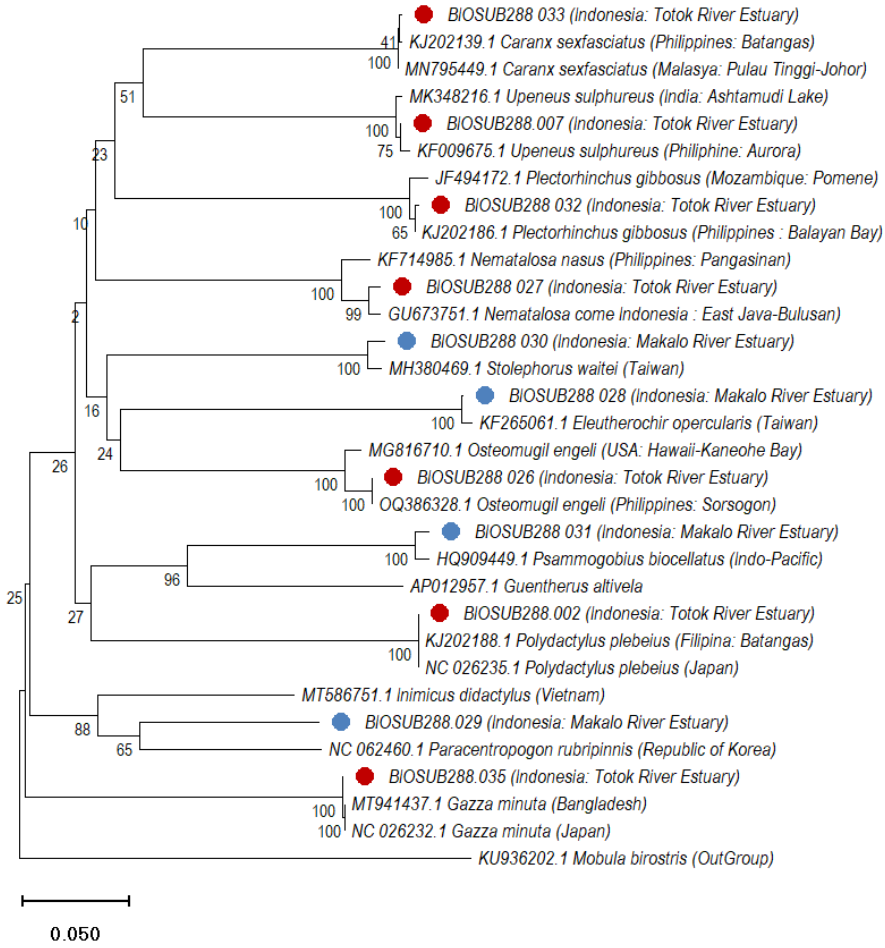

Figure 2. Neighbor-joining phylogenetic tree based on COI sequences showing evolutionary relationships among fish species from Totok and Makalo estuaries and reference sequences from GenBank.

Several species from the Totok River Estuary showed broad biogeographic distributions and genetic conservation across large geographic scales. *Gazza minuta* clustered with haplotypes from Bangladesh (bootstrap support: 100), suggesting widespread distribution of *G. minuta* in tropical Indo-Pacific waters (Habib and Islam, 2021). *Plectorhinchus gibbosus* had high bootstrap support (100) when clustered with the Mozambican population, indicating genetic conservation identity across a broad geographic area (the Indian and Pacific Oceans). Other species showed striking Indo-Pacific connectivity: *Caranx sexfasciatus* formed a cluster with individuals collected in the Philippines and Malaysia (bootstrap support: 100), and *Osteomugil engeli* grouped with samples from Hawaii and the Philippines (bootstrap support: 100), indicating broad dispersal capacity for these widespread species.

These phylogenetic patterns reveal that the Totok and Makalo estuaries harbour species with diverse biogeographic histories, ranging from locally adapted populations to populations that maintain genetic connectivity with distant regions through recent or historical gene flow. The complex oceanography of the Indo-Pacific, including major hydrodynamic systems such as the Indonesian Throughflow, the North Equatorial Current, and monsoon-facilitated ocean currents, is thought to have driven marine population dispersal and genetic connectivity (Alfaro *et al*., 2018). Species exhibiting diverse biogeographic affinities also contribute to the complex community structure of the Coral Triangle biodiversity region (Nikijuluw, 2019), including a temperate migratory species (*P. rubripinnis*), widespread Indo-Pacific generalists (*G. minuta, P. gibbosus*), and regional endemics (*N. come*).

Study limitations and future research directions

The current work is a first molecular and environmental survey with several limitations. The small sample size likely underestimates the actual

species richness, since fish communities in estuaries are highly diverse and temporally variable because of migration events, reproductive periods, and environmental changes (Cardoso, 2021). The single sampling event in June 2024 is only a snapshot in time, and species that are present at other times of the year or day may have been overlooked. Moreover, the use of beach seines alone may introduce taxonomic and size-biases that might underestimate small or cryptic species or species in deeper waters.

Although they provide valuable reference information, water quality measurements are only snapshots and fail to account for diurnal or seasonal variation in parameters relevant to fish ecology or mining impacts. The inferences made regarding relationships among environmental variables, species ranges, and genetic patterns are suggestive rather than definitive. Causal relationships cannot be firmly established without replicated sampling, controlled comparisons between impacted and reference site, before after designs, or other experimental approaches that control for confounding factors.

Future work should focus on expanded temporal and spatial coverage across seasons to fully document species richness and compare mining-impacted areas with reference sites. Multidisciplinary environmental surveys encompassing water quality, sediment quality, and heavy metal analyses should be conducted to quantify mining impacts and assess relationships between environmental conditions and diversity. Population-level sampling of key indicator taxa using multiple molecular markers is needed to evaluate genetic diversity, structure, connectivity patterns, and anthropogenic impacts on genetic variation. Careful integration of morphospecies data with molecular data would help develop robust reference collections for both traditional taxonomic identification approaches as well as modern molecular methods. The use of eDNA metabarcoding should complement traditional survey methods to detect rare or elusive species.

Despite these limitations, this study has provided important baseline molecular and environmental information for poorly studied estuaries and demonstrated the utility of a combined methodology to inventory fish diversity under anthropogenic pressure. The new molecular records, in combination with environmental context and conservation considerations, form a basis for monitoring and evidence-based management of coastal environments in North Sulawesi.

Conclusion

This COI DNA barcoding survey identified 11 fish species from the Totok and Makalo estuaries, providing the first molecular evidence of ichthyofaunal diversity in these North Sulawesi systems. Notable first records include *Paracentropogon rubripinnis* in Indonesian waters and *Nematalosa come* in North Sulawesi. Environmental characterization revealed contrasting salinity regimes, with reduced dissolved oxygen in Makalo associated with artisanal gold mining. Phylogenetic analyses demonstrated significant interspecific divergence and connectivity with Indo-Pacific populations. Eleven new COI sequences were deposited in GenBank. These findings underscore the need for ecosystem-based management, including mining regulations and habitat protection, to conserve estuarine biodiversity in North Sulawesi.

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