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Environmental DNA exploration of fish diversity in the Balantak River, Central Sulawesi, Indonesia

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ABSTRACT

Introduction: Freshwater ecosystems play a crucial role in supporting human life, providing many ecosystem services. Species diversity is a valuable indicator for evaluating ecosystem health, gaining a broader perspective on ecological richness and ecosystem functions, but data are lacking for many rivers.

Objectives: To obtain baseline data on the upstream and downstream ichthyofauna of the Balantak River, Banggai Regency, using environmental DNA (eDNA) with a focus on diadromous species.

Methods: We collected, filtered and preserved water samples from upstream and downstream sites on the Balantak River followed by eDNA analysis (DNA extraction, PCR with MiFish 12S rRNA primers, Illumina sequencing, MitoFish bioinformatic analysis at 97 and 99 % identity thresholds) to produce taxonomically assigned Actinopteri amplicon sequence variants (ASVs).



Results: Our data are consonant with the expected dominance of diadromous and euryhaline taxa (75 %), high Gobiiformes biodiversity, and differences between upstream and downstream ichthyofaunal communities. Reads were assigned to Actinopteri ASVs from 12 orders, predominantly Gobiiformes, Anguilliformes, and Centrarchiformes. The 15 assigned families included Anguillidae, Eleotridae, Gobiidae, Rhyacichthyidae, Kuhlidae, and Terapontidae. Of the assigned genera (17 at 97 % and 25 at 99 %), *Anguilla* had the most reads (39.9 %), and *Sicyopterus* had the most ASVs (8).

Conclusions: These baseline data demonstrate the usefulness of eDNA metabarcoding for exploring ichthyofaunal biodiversity in small tropical watersheds while highlighting gaps in 12S rRNA sequence databases, reinforcing the need for integrative taxonomic research in Sulawesi watersheds and reliable reference databases. Most assigned taxa are vulnerable to exploitation for food and/or the ornamental fish trade. Four non-native taxa from Cichlidae, Cyprinidae and Clariidae are considered invasive. Implications for conservation include the need to address the unregulated and largely unreported freshwater ornamental trade and introduction of non-native invasive species.

Key words: amphidromy; Gobiiformes; metabarcoding; riverine biodiversity; Wallacea.

RESUMEN

Exploración del ADN ambiental de la diversidad de peces en el río Balantak, Sulawesi Central, Indonesia

Introducción: Los ecosistemas de agua dulce desempeñan un papel crucial en el mantenimiento de la vida humana, al proveer numerosos servicios ecosistémicos. La diversidad de especies constituye un indicador valioso para evaluar la salud de los ecosistemas, obtener una perspectiva más amplia y conservar la riqueza ecológica y las funciones ecosistémicas; sin embargo, faltan datos para muchos ríos.

Objetivos: Obtener datos de referencia sobre la ictiofauna de los tramos alto y bajo del río Balantak, en la regencia de Banggai, mediante ADN ambiental (eDNA) con énfasis en especies diádromas.

Métodos: Se recolectaron, filtraron y preservaron muestras de agua en sitios aguas arriba y aguas abajo del río Balantak, seguidas de análisis de eDNA (extracción de ADN, PCR con cebadores MiFish 12S rRNA, secuenciación Illumina, análisis bioinformático con MitoFish a umbrales de identidad de especie del 97 y 99 %) para generar variantes de secuencia de amplicones (ASVs) de Actinopteri con asignación taxonómica.

Resultados: Nuestros datos confirman el dominio esperado de taxones diádromos y eurihalinos (75 %), una alta biodiversidad de Gobiiformes y diferencias entre las comunidades ictiofaunales de los tramos alto y bajo del río. Las lecturas se asignaron a ASVs de Actinopteri pertenecientes a 12 órdenes, predominantemente Gobiiformes, Anguilliformes y Centrarchiformes. Las 15 familias asignadas incluyeron Anguillidae, Eleotridae, Gobiidae, Rhyacichthyidae, Kuhlidae y Terapontidae. Entre los géneros identificados (17 al 97 % y 25 al 99 %), *Anguilla* presentó el mayor número de lecturas (39.9 %), mientras que *Sicyopterus* tuvo el mayor número de ASVs (8).

Conclusiones: Se demuestra la utilidad del metabarcoding de eDNA para explorar la biodiversidad ictiofaunística en pequeñas cuencas tropicales, y resalta vacíos en las bases de datos de secuencias 12S rRNA, reforzando la necesidad de investigación taxonómica integrativa en las cuencas hidrográficas de Sulawesi y de bases de datos de referencia confiables. La mayoría de los taxones asignados son vulnerables a la explotación para consumo y/o como peces ornamentales, y cuatro taxones exóticos pertenecientes a Cichlidae, Cyprinidae y Clariidae se consideran invasores. Las implicaciones para la conservación incluyen la necesidad de abordar el comercio ornamental de agua dulce no regulado y en gran medida no reportado, así como la introducción de especies exóticas invasoras.

Palabras clave: anfidiromía; Gobiiformes; metabarcodificación; biodiversidad fluvial; Wallacea.

INTRODUCTION

Freshwater ecosystems cover a small fraction of the earth's surface, with estimates varying from around 0.5 % (Ottoni et al., 2023) to 2 % (Reid et al., 2019), but they provide habitat for around 40 % of fishes, with around 10 000-15 000 species described to date (Reid et al.,

2019; Tedesco et al., 2017; Wang et al., 2024). Freshwater ecosystems are among the most valuable in terms of ecosystem services of direct value to humans as well as intrinsic and option values (Albert et al., 2021; Dudgeon, 2020; Lynch et al., 2020; Reid et al., 2019). Freshwater fisheries are considered essential to meeting the Sustainable Development Goals (SDGs),

especially SDG 1 (No Poverty), SDG 2 (Zero Hunger), and SDG 15 (Life on Land) (Lynch et al., 2020). While freshwater ecosystems fall primarily under SDG 15, and the primary scope of SDG 14 comprises oceans, seas, and marine resources, SDG 14 and SDG 15 are both directly relevant for many aquatic taxa, in particular diadromous and euryhaline fishes (Hansen et al., 2023).

Despite growing awareness of their importance, many freshwater fishes and their habitats are imperiled, with threats growing in terms of their variety and severity (Albert et al., 2021). Key threats include exploitation as food and ornamental fish, the introduction of non-native species, direct and indirect impacts of habitat alteration (e.g. pollution, flow regulation, land-use change), local impacts of global climate change, and often synergistic interactions among multiple threats (Dudgeon, 2019; Nurjirana et al., 2024; Oikonomou, 2021; Reid et al., 2019; Sorensen, 2021). Extinction rates are considerably higher for freshwater taxa than for most other groups (Otoni et al., 2023; Williams-Subiza & Epele, 2021). Accurate and comprehensive biodiversity data are vital for understanding ecology, designing appropriate and effective conservation policies, assessing the impacts of threats and the effectiveness of actions taken to address the issues identified (Maasri et al., 2022; Oikonomou, 2021; Otoni et al., 2023). However, despite considerable progress in recent decades, much remains to be discovered in terms of species richness, functional diversity, evolutionary patterns, and biogeographical patterns across multiple spatial and temporal scales (Dudgeon, 2020; Maasri et al., 2022; Oikonomou, 2021).

Indonesia, the largest archipelagic nation, spans three major bioregions (Sundaland, Wallacea, and the Sahul Shelf) and is recognized as a mega-biodiversity country (Lohman et al., 2011). Sulawesi, the largest island in the Wallacea bioregion, is renowned for its diverse aquatic biota, including high levels of endemism (Ambo-Rappe & Moore, 2019; Lohman et al., 2011). Miesen et al. (2016) listed 226 fishes from 112 genera and 56 families that have been

reported from, or are likely to inhabit, Sulawesi freshwater habitats, including primary freshwater and diadromous species. The latter include catadromous species such as freshwater eels (genus *Anguilla*) and amphidromous species, including many Gobiiformes, which together contribute most of the riverine ichthyofaunal diversity (Wantania et al., 2024). Ongoing discoveries and new species records continue to enhance our understanding of the Sulawesi ichthyofauna (Gani, Nurjirana et al., 2021; Keith, Hadiaty et al., 2014; Ndobe et al., 2022; Nurjirana et al., 2024; Wantania et al., 2024). However, the Sulawesi primary freshwater and diadromous biota, including fish, are still understudied, raising the concern that aquatic populations, species and communities could be depleted or lost before they are even recorded (Ndobe et al., 2022; Ndobe et al., 2023; Nurjirana et al., 2024). Furthermore, invasive alien species have been recorded in several Sulawesi lakes and rivers (Herder et al., 2012; Herder et al., 2022; Paramata et al., 2025; Serdiati et al., 2020; Yanuarita et al., 2020). These studies highlight the need for further exploration in Sulawesi freshwater habitats, to fill the many gaps in our knowledge of the species present and their distributions. Such benchmarks are important not only to document the native species present but also to determine which species are truly non-native and to document their arrival and spread.

Addressing such gaps is important from scientific, conservation, fisheries, and environmental management viewpoints. Biodiversity is increasingly recognized as vital for resilience and sustainability, now and in the face of change (Bartholomew et al., 2024; Oliver et al., 2015). Data on fish species, including their distribution and status, are vital for resource management, in conservation, fisheries, and sustainable development, and baselines are vital for planning and monitoring progress (Albert et al., 2021; Bartholomew et al., 2024; Ojaveer et al., 2018). For example, the World Bank (under Standard ESS6) and other funding agencies increasingly mandate biodiversity assessments and action plans for all funded projects (Morley



et al., 2021; Nedopil et al., 2024). Furthermore, aquatic biodiversity monitoring can provide valuable indicators to assess ecosystem health (Robinson et al., 2022).

The exploration of biodiversity in freshwater and other ecosystems can be conducted using a variety of approaches, individually or in combination. Classic taxonomic surveys are now often combined with molecular methods such as DNA barcoding and environmental DNA (eDNA) metabarcoding (Chen et al., 2024; Iqram et al., 2024; Shu et al., 2021). Each of these approaches has strengths and limitations in terms of detection and identification (McElroy et al., 2020). Despite the advantages of classical surveys, such as enabling the collection of reference specimens, logistics, sampling gear type, seasonality and fish behavior are some of the many reasons why taxa can be missed when collecting specimens for classical and DNA-based identification (McElroy et al., 2020; Wantania et al., 2024). Although eDNA metabarcoding suffers from some of these limitations, in particular with respect to seasonality, one key advantage is that it can be considered a non-invasive method as it does not require direct observation or capture (Chen et al., 2024; Hidayani et al., 2024; Wang et al., 2024). The capacity to enable rapid identification, and even generate occurrence records, can be particularly advantageous in situations where collection could be dangerous, difficult, expensive, or threaten sensitive ecosystems and endangered taxa, including the detection and monitoring of endangered species or, conversely, non-native and potentially invasive species (Chen et al., 2024; Shu et al., 2021). It is therefore not surprising that eDNA methods are increasingly used for aquatic biodiversity surveys and monitoring, including both preliminary or baseline surveys and comprehensive biodiversity assessments across diverse ecosystems and taxonomic groups (Chen et al., 2024; McElroy et al., 2020; Porter & Hajibabaei, 2018; Ruppert et al., 2019). A recent review of the literature on aquatic eDNA (Takahashi et al., 2023) found the ten most used primer sets targeted four molecular markers: 12S rRNA (78.2 % of studies), with

COI, 16S rRNA, and Cytb collectively accounting for 20.2 %. The 12S rRNA molecular marker is commonly used for fish; the MiFish-U primer set, designed specifically for teleosts by Miya et al. (2015), accounts for 38 % of studies in Takahashi et al. (2023), providing superior detection and taxonomic resolution (Xu et al., 2024) and relative abundance data (Shu et al., 2021) in multi-primer tests).

Given these considerations, the purpose of this pilot study was to use eDNA metabarcoding targeting the 12S rRNA molecular marker to obtain baseline data on the biodiversity and composition of the fish species present in the upstream and downstream reaches of the Balantak River in Banggai Regency, Central Sulawesi, Indonesia, one of many understudied waterways in Sulawesi. Based on findings from other Sulawesi rivers, the Balantak River was expected to host predominantly diadromous taxa including amphidromous Gobiiformes, and to be at risk from non-native taxa. This study provides baseline data for the Balantak River that contributes to the body of knowledge on the ichthyofaunal biodiversity in Sulawesi, improving understanding of the native fish fauna and providing information on the spread of introduced species that are not native to Sulawesi or the Wallacea region. These data can inform conservation and fisheries management, provide a reference point for research and monitoring to understand the impact of human activities on aquatic ecosystems, and pave the way for improving and extending eDNA-based approaches in Sulawesi and beyond.

MATERIALS AND METHODS

The Balantak River in Banggai Regency, Central Sulawesi, Indonesia flows into the Gulf of Tolo on the east coast of Sulawesi Island, Indonesia and is typical of many such short rivers originating in the mountainous hinterland of the Banggai Regency. Two sampling sites were selected (Fig. 1): Site 1 in the downstream reaches (0°52'47" S & 123°22'37" E), and Site 2 in the upstream reaches of the Balantak River (0°52'59" S & 123°23'18" E). These sites were

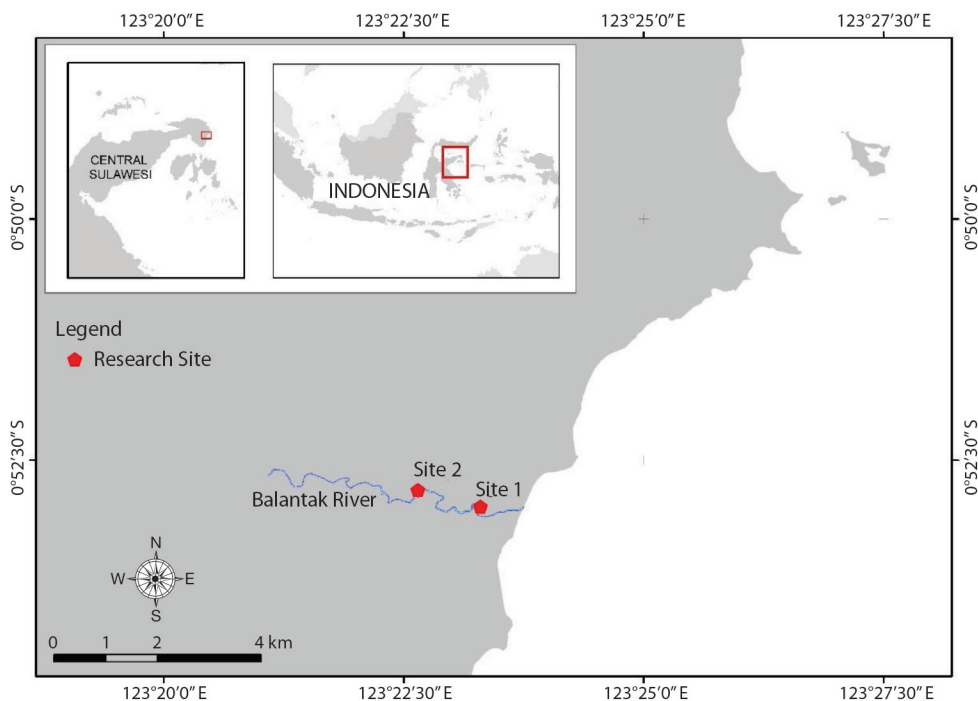


Fig. 1. Sampling sites on the Balantak River in Banggai Regency, Central Sulawesi, Indonesia.

chosen to sample the ichthyofauna in the lower reaches, flowing through the narrow coastal plain close to the estuary, including euryhaline taxa, as well as the ichthyofauna in the faster flowing upper reaches of the Balantak River, above the first rapids and waterfalls. Samples were collected on 21 July 2024. Sterile surgical gloves were worn during sample collection, handling, and all laboratory procedures. All equipment and work areas were sterilized (10 % bleach and/or 96 % ethanol). Tweezers and scissors were further sterilized using a Bunsen burner immediately before use. Water samples were collected in sterile plastic containers (approximately 5 L volume) and placed in coolboxes filled with ice for transport to the laboratory. All water samples were processed following the Oceanogen protocol (Madduppa & Sani, 2020). From each sample, 2 L of water was filtered through a 0.45 μm Pall membrane filter paper placed in a Nalgene rapid flow filter attached to a one-stage vacuum pump (Allie VE115K). The filters containing the DNA were

cut in half and each piece was placed in a 2 mL cryo-tube (Greiner Bio-One GmbH) filled with DNA/RNA Shield (Zymo Research). A negative control was also obtained by filtering 1 L of distilled water and treated in the same manner to monitor potential contamination. The tubes were labelled, sealed with parafilm and transported to Oceanogen in Bogor, Indonesia.

DNA extraction, amplification, and sequencing were conducted at the Oceanogen Laboratory in Bogor, Indonesia. DNA was extracted using Qiagen Blood and Tissue DNA Extraction Kits following the manufacturer's instructions. The first PCR stage amplified the target region of the 12S rRNA gene using the MiFish-U primer set (Miya et al., 2015). The 12S rRNA marker and the MiFish-U primer set were selected based on the considerations outlined above. The 12S rRNA marker has a comparatively high resolution for identifying teleost fishes, in particular tropical freshwater taxa and this relatively short DNA fragment is compatible with Next Generation metabarcoding



sequencing platforms. The MiFish-U primer is widely used (Takahashi et al., 2023) and has demonstrated comparatively high effectiveness in amplifying teleost DNA across a wide range of taxonomic groups (Xu et al., 2024; Zhu et al., 2023). Furthermore, this primer set is supported by a large and growing reference sequence database and on-line data analysis pipeline (Sato et al., 2018; Zhu et al., 2023). PCR was performed on a 96 Universal peqSTAR unit, with three replicates per sample. PCR product quality was visualized through electrophoresis. The three replicate PCR products from each sample were then pooled, bead-cleaned (AMPure XP) and indexed through a second PCR stage with IDT double indices and Illumina sequencing adapters for the Illumina Nextera DNA Unique Dual Index Set B. The indexed PCR product (amplicon library) was purified using AMPure XP beads, quantified using a Qubit fluorometer, and normalized to equal concentrations prior to DNA sequencing on a NovaSEQ6000.

Sequencing outputs (FASTQ files) were analyzed using the MitoNGS option in the MitoFish online portal (version 2025.06; Iwasaki et al., 2013; Sato et al., 2018; Zhu et al., 2023). Clean sequences were grouped into amplicon sequence variants (ASVs) assigned to the closest taxon in the GenBank 12S rRNA database and produce denoised lists of taxa identified. This process was repeated at two levels of identity: 97 % ID (highly likely to be correct at genus level) with minimum read abundance 0.01 and 99 % ID (likely to be accurate to species level) without a minimum read abundance. ASVs assigned to ray-finned fish (Actinopteri) taxa were tabulated, analyzed and visualized graphically in Microsoft Excel 365. The FASTQ read files were submitted to the NCBI GenBank Sequence Read Archive (SRA) under submission number SUB15073931, accession number PRJNA1223125.

Taxonomic validity of assigned taxa was verified through the World Register of Marine Species (WoRMS Editorial Board, 2025) and Eschmeyer's Catalog of Fishes (Fricke et al., 2025). Conservation status was obtained from

the (International Union for Conservation of Nature [IUCN], 2025) Red List. Geographical distribution, residency status (endemic, native, non-native) and human uses (food fisheries, ornamental fisheries, aquaculture) were obtained from FishBase (Froese & Pauly, 2025) and relevant literature. Indonesia spans three major biogeographical regions (Sundaland, Wallacea and the Sahul Shelf) with distinctive freshwater ichthyofauna (Kottelat et al., 1993). Therefore, the criterion for native residency status was based on known native distributions in Wallacea, and specifically in or near Sulawesi, rather than in Indonesia. Ichthyofaunal composition at upstream and downstream sites was described and compared graphically and with reference to relevant literature.

RESULTS

The Actinopteri ASVs assigned by the MitoFish pipeline in the "Species identified by MitoFish" table at 97 and 99 % percent identity thresholds were used for further analyses. ASVs in the "Higher taxa identified by MitoFish" table predominantly represented Gobiiformes without close matches in the sequence database and marine fish with low abundances. Non-fish vertebrate ASVs (≈ 5 % of length-filtered reads and 2-5 % of ASVs, excluded from further analyses) were assigned to mammalian taxa from four orders present in the study area and likely to shed DNA in the river: Artiodactyla (*Bos* sp., *Sus* sp.), Carnivora (*Canis lupus*), Primates (*Homo sapiens*), Rodentia (*Rattus* sp.). The number of reads and taxa assigned in the "Species identified by MitoFish" table varied considerably based on the percent identity threshold (Table 1). At the 97 % confidence level 307 753 reads were assigned to 32 Actinopteri taxa (putative species) from 12 orders, 15 families, and 25 genera. At the 99 % confidence level a total of 203 436 reads were assigned by the MiFish pipeline to 19 Actinopteri taxa (putative species) from 10 orders, 12 families, and 17 genera.

Assigned read abundance and distribution varied between the 12 orders assigned by the

Table 1

Summary of reads, ASVs and taxa assigned by the MiFish pipeline at 97 and 99 % sequence percentage identity thresholds for eDNA samples from two sites on the Balantak River in Banggai Regency, Central Sulawesi.

Parameter/ID level	Site 1		Site 2	
	Downstream 1	Downstream 2	Upstream 1	Upstream 2
Total reads-raw	507 834	556 680	625 460	536 414
Assembled reads after quality-filter, no N	359 529	401 556	415 409	341 175
Reads after length filter	147 752	154 683	209 391	194 456
Unique reads	13 973	14 157	15 587	14 051
DenoiSED ASVs	207	304	146	90
Actinopteri ASVs	202	300	142	86
Number of Actinopterygii Orders at species-level				
ID level 97 %	12	11	11	10
ID level 99 %	8	8	9	8
Number of Actinopteri Families				
ID level 97 %	14	14	14	13
ID level 99 %	10	10	11	9
Number of Actinopteri Genera				
ID level 97 %	19	23	24	18
ID level 99 %	12	14	16	10
Number of Actinopteri species level taxa/species identified without ambiguity				
ID level 97 %	20/15	25/19	30/22	22/17
ID level 99 %	11/10	17/13	18/13	11/8

MitoFish pipeline at the 97 % identity threshold (Fig. 2A). Gobiiformes (42.66 %) were the most abundant, closely followed by Anguilliformes (39.91 %) and Centrarchiformes (14.94 %). Cypriniformes read abundance was an order of magnitude lower (1.46 %) and the remaining seven orders (collectively 1.03 %) are not distinguishable in Fig. 2A. Syngnathiformes was only present downstream. Combined read abundance upstream was lower for Centrarchiformes and higher for Gobiiformes and Anguilliformes compared to downstream.

Relative read abundance by family differed between samples and sites, although similar numbers of families were detected. The 15 families assigned at the 97 % identity threshold (Fig. 2B) include three Gobiiformes families (Gobiidae 26.13 %, Eleotridae 12.14 % and Rhyacichthyidae 6.89 %) and two Centrarchiformes families (Kuhliidae and Terapontidae). The remaining orders were each represented by one family. Anguillidae had the highest overall read abundance (39.91 %). Gobiiformes

and Centrarchiformes communities differed between sites. Gobiidae reads were most abundant at the upstream site, followed by Rhyacichthyidae, and Terapontidae. Eleotridae were followed by Kuhliidae and Gobiidae at the downstream station.

Agreement between the 97 and 99 % ID lists at the genus level was high, with all taxa identified at the 99 % level also detected at the 97 % level. There were notable differences between upstream and downstream communities, especially with respect to Gobiiformes (Fig. 3). However, there were uncertainties regarding some taxa at the species level. Several ASVs were assigned to multiple species with ID > 99 %, in particular anguillid eels and Gobiiformes (genera *Sicyopterus* and *Glossogobius*), while some species-level assignments may represent closely related species lacking 12S rRNA reference sequences in the GenBank database.

The taxa assigned to the 32 ASVs in the “Species identified by MitoFish” table at the 97 % identity threshold, including alternative

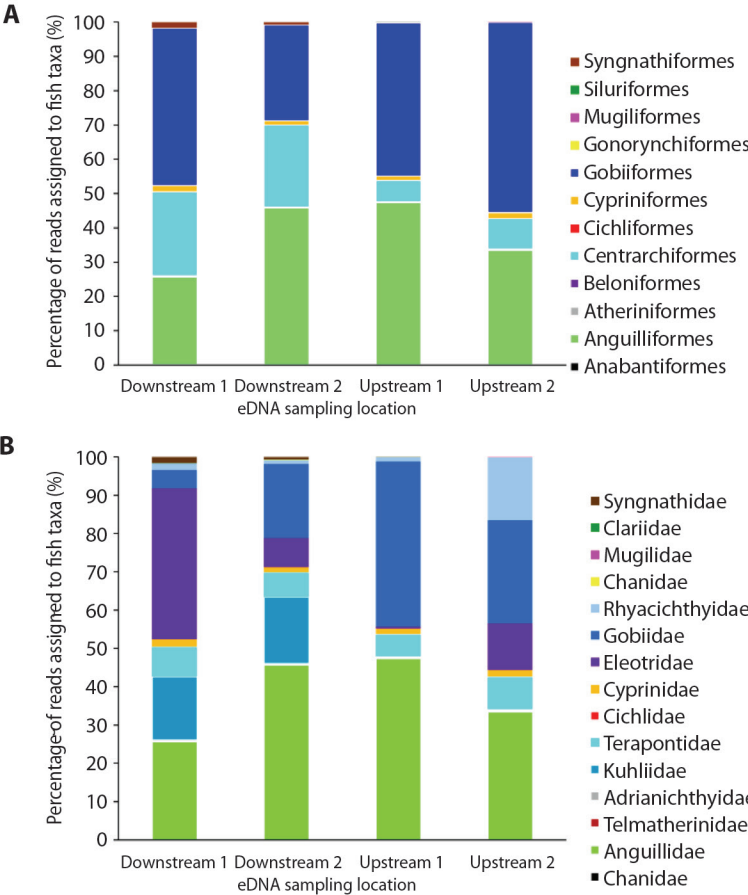


Fig. 2. Actinopteri orders (A) and families (B) detected through eDNA metabarcoding at downstream and upstream sampling sites on the Balantak River in Banggai Regency, Central Sulawesi, Indonesia (MitoFish, 97 % identity threshold).

identifications where given, included species assessed as at risk of extinction in the IUCN Red List (IUCN, 2025); endemic, native, and non-native taxa, and taxa of uncertain status; and fish caught or cultured as food and/or for ornamental use (Table 2). Among the identified taxa, *Sicyopterus* (Gobiidae) exhibited the greatest species diversity.

Table 2
Conservation status, origin, use, and previous reports from Sulawesi of taxa (ASVs) detected through eDNA barcoding at two stations in the Balantak River, Banggai Regency, Central Sulawesi, Indonesia (MitoFish 97 % identity threshold, 0.01 % read abundance threshold).

No	Order	Family	Genus/Species	Status ^a	Origin ^b	Use ^c	Report ^c
1	Anabantiformes	Channidae	<i>Channa striata</i>	LC	U	A,F	Yes
2	Anguilliformes	Anguillidae	<i>Anguilla interioris</i> <i>A. marmorata</i>	DD LC	N N	F F	Yes Yes
3	Atheriniformes	Telmatherinidae	<i>Telmatherina celebensis</i>	NT	E	F,O	Yes ^d
4	Beloniformes	Adrianichthyidae	<i>Oryzias celebensis</i>	LC	E	O	Yes ^e

No	Order	Family	Genus/Species	Status ^a	Origin ^b	Use ^c	Report ^c
5	Centrarchiformes	Kuhliidae	<i>Kuhlia marginata</i>	LC	N	F	Yes ^f
6	Centrarchiformes	Terapontidae	<i>Leiopotherapon plumbeus</i>	VU	A	F	No
			<i>L. unicolor</i>	LC	A	F	No
7	Cichliformes	Cichlidae	<i>Oreochromis niloticus</i>	LC	A	A,F	Yes ^g
8	Cichliformes	Cichlidae	<i>Cichlidae</i> sp.	-	A	O	Yes ^g
9	Cypriniformes	Cyprinidae	<i>Osteochilus</i> sp. CBM-ZF-12 420	-	A	F	Yes ⁱ
10	Gobiiformes	Eleotridae	<i>Belobranchius belobranchius</i>	LC	N	O,F ^j	Yes
11	Gobiiformes	Eleotridae	<i>Eleotris fusca</i>	LC	N	O,F ^j	Yes
12	Gobiiformes	Eleotridae	<i>Giuris tolsani</i>	NE	N	F	Yes ^h
13	Gobiiformes	Gobiidae	<i>Glossogobius illimis</i>	LC	N	-	Yes
14	Gobiiformes	Gobiidae	<i>G. aureus</i>	LC	N	F	Yes
			<i>G. flavipinnis</i>	NT	N		Yes ^d
15	Gobiiformes	Gobiidae	<i>Lentipes armatus</i>	NE	U	O ^k	No
16	Gobiiformes	Gobiidae	<i>L. palawanirufus</i>	NE	U	O ^k	No
17	Gobiiformes	Gobiidae	<i>Schismatogobius</i> sp. CBM:ZF:12 245	-	-	F ^j	-
18	Gobiiformes	Gobiidae	<i>Sicydium altum</i>	VU	A	-	No
19	Gobiiformes	Gobiidae	<i>Sicyopterus aiensis</i>	NT	A	F	No
			<i>S. cynocephalus</i>	LC	N	F,O	Yes
			<i>S. lengguru</i>	DD	U	-	No
20	Gobiiformes	Gobiidae	<i>S. lagocephalus</i>	LC	N	O ^k ,F ^j	Yes ^j
21	Gobiiformes	Gobiidae	<i>S. longifilis</i>	LC	N	O ^k ,F ^j	Yes ^j
22	Gobiiformes	Gobiidae	<i>S. marquesensis</i>	DD	U	F	No
23	Gobiiformes	Gobiidae	<i>S. squamosissimus</i>	NE	U	O ^k	No ^l
24	Gobiiformes	Gobiidae	<i>S. longifilis</i>		See above		
			<i>S. squamosissimus</i>				
25	Gobiiformes	Gobiidae	<i>Sicyopus zosterophorus</i>	LC	N	O ^k ,F ^j	Yes
26	Gobiiformes	Gobiidae	<i>Smilosicyopus leprurus</i>	LC	N	O ^k ,F ^j	Yes
27	Gobiiformes	Gobiidae	<i>Stiphodon semoni</i>	LC	N	O ^k ,F ^j	Yes
28	Gobiiformes	Rhyacichthyidae	<i>Rhyacichthys aspro</i>	DD	N	O ^k ,F	Yes
29	Gonorynchiformes	Chanidae	<i>Chanos chanos</i>	LC	N	A,F	Yes
30	Mugiliformes	Mugilidae	<i>Planiliza subviridis</i>	LC	N	F	Yes
			<i>Mugil cephalus</i>	LC	U	F	Yes ^m
31	Siluriformes	Clariidae	<i>Clarias batrachus</i>	LC	A	A,F,O	Yes ^g
32	Syngnathiformes	Syngnathidae	<i>Microphis</i> (formerly <i>Coelonotus</i>) <i>leiaspis</i>	LC	N	O ^k	Yes

Table caption: ^a IUCN Red List assessments (IUCN, 2025): LC = Least Concern; DD = Data Deficient; EN = Endangered; NT = Near Threatened; NE = not evaluated; - not assigned to species level. ^b IUCN Red List (IUCN, 2025) and FishBase (Froese & Pauly, 2025): A = alien, reported native distribution does not include Sulawesi; E = Sulawesi endemic; N = native; U = unclear; - no data. ^c IUCN Red List (IUCN, 2025) and FishBase (Froese & Pauly, 2025) unless indicated by further superscripts: A = aquaculture; F = food; O = ornamental; - = no data; Report = reported from Sulawesi. ^d Currently considered endemic to the Malili Lake complex (Lumbantobing, 2019b; Lumbantobing & Larson, 2019). ^e Reported from South and Southeast Sulawesi and a site in Timor Leste, but not from watersheds close to the study area. ^f (Haryono & Sauri, 2020; Miesen et al., 2016; Tweedley et al., 2013; Wantania et al., 2024). ^g Many reports of these and other Cichliformes in Sulawesi waters (see discussion). ^h (Astuti et al., 2023; Herder et al., 2022; Wantania et al., 2024). ⁱ Reports at genus level (Herder et al., 2022; Herjayanto et al., 2019; Ndobe et al., 2024; Serdiati et al., 2020; Serdiati et al., 2024; Wantania et al., 2024). ^j Amphidromous species fished for food as post-larvae, locally called nike or penja in Sulawesi waters (Nurjirana et al., 2019; Nurjirana et al., 2021; Nurjirana et al., 2022; Pangemanan et al., 2020; Sahami et al., 2020). ^k Traded on-line in the ornamental fish trade (advertisements/hobbyist posts present on 30 Nov 2024). ^l recently described from Java and Sumatra (Keith et al., 2015). ^m Currently considered a species complex with at least 15 species and likely confusion with several other genera in Mugilidae (Durand et al., 2017). ⁿ Reported from several sites in Sulawesi, including Luwuk (Keith et al., 2020).

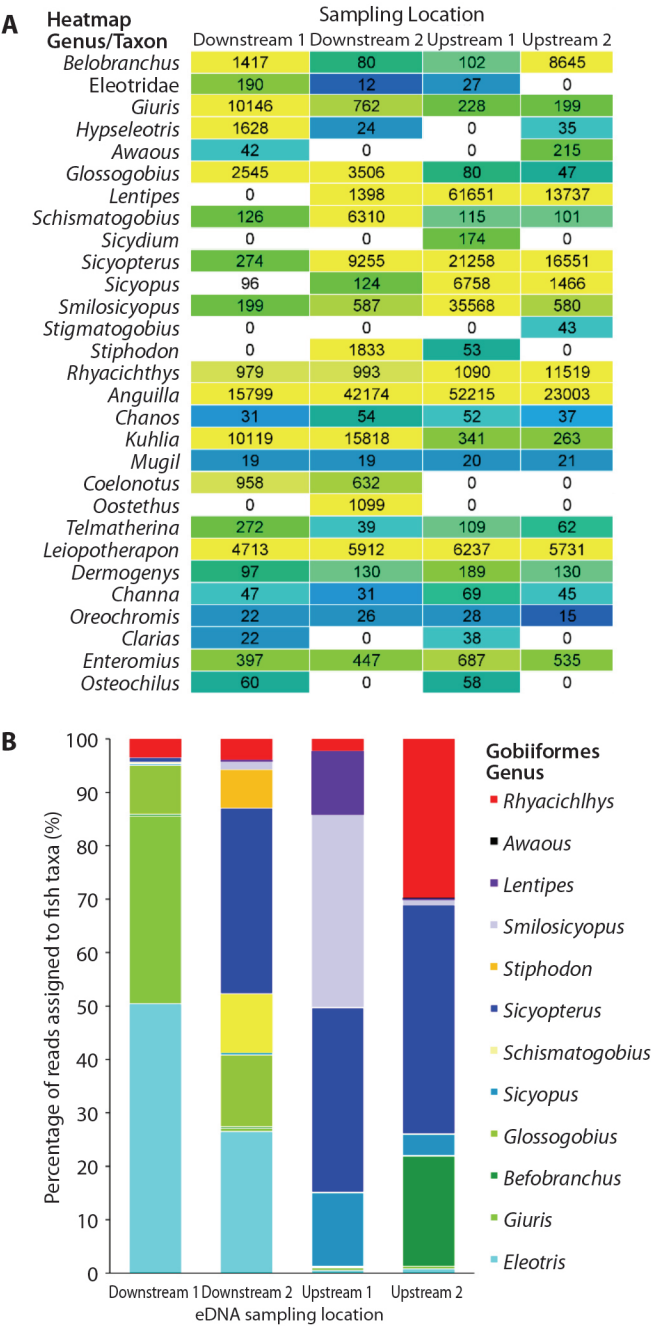


Fig. 3. Heatmap of Actinopteri genera (A), fill color from blue to yellow as assigned read volume increases; and relative (read) abundance of Gobiiformes genera (B) detected through eDNA metabarcoding at downstream and upstream sampling sites on the Balantak River in Banggai Regency, Central Sulawesi, Indonesia (MitoFish, 97 % identity thresholds).

DISCUSSION

The ability to detect species through eDNA not only enhances conservation efforts but also facilitates the management of aquatic resources by providing critical data on species distribution and abundance. At the study sites on the Balantak River in Banggai Regency, Central Sulawesi, eDNA metabarcoding identified Actinopteri (ray-finned fish) amplicon sequence variants (ASVs) representing at least 32 species-level taxa, of which 19 belong to the Gobiiformes. The observed conditions, including clear, fast-flowing waters and lush riparian vegetation, with little evidence of direct habitat degradation, provide habitat suitable for many amphidromous Gobiiformes (Keith & Lord, 2012; Keith, Lord et al., 2014), as evidenced by the high proportion (59 %) of gobies among the taxa identified. While these results do not imply that the Balantak River is free from threats to aquatic biodiversity, they indicate that this river supports a rich biodiversity worth conserving.

The ichthyofaunal community composition differed between the upstream and downstream stations, including endemic, native and alien species, as well as taxa of uncertain origin. The most abundant non-anguillid ASVs at the downstream station included *Eleotris fusca* Bloch and Schneider 1801, *Kuhlia marginata* Cuvier 1829, *Giuris tolsoni* Bleeker 1854, *Leiopotherapon* sp., *Glossogobius illimis* Hoese and Allen 2012, and *Sicyopterus longifilis* de Beaufort 1912. At the upstream station, the most abundant non-anguillid ASVs were all Gobiiformes, including *Smilosicyopus leprurus* Sakai and Nakamura 1979, *Rhyacichthys aspro* Valenciennes 1837, *Sicyopterus* sp. (likely *S. cynocephalus* Valenciennes 1837), *Belobranchus belobranchus* Valenciennes 1837, *Sicyopus zosterophorus* Bleeker 1856, *Lentipes armatus* Sakai & Nakamura 1979 and *L. palawanirufus* Maeda and Palla 2021.

The majority of ASVs (24, 75 %) were assigned to euryhaline or diadromous taxa that spend some stages of their life cycles in marine waters. The most numerous were the amphidromous Gobiiformes (20 ASVs), while

the sole Syngnathiformes taxon, the barhead pipefish *Microphis leiaspis* Bleeker, 1854 (formerly *Coelonotus leiaspis*), is also amphidromous (Jenkins et al., 2017). This pipefish was only identified at the downstream site, likely due to topography. This is consonant with a longitudinal survey of the ichthyofauna in the nearby Koyoan River (Ndobe et al., in press), also in Banggai Regency, where *M. leiaspis* was only observed in the lower reaches of the river, below the first waterfall. Together, these studies indicate that, despite the ability to swim past some rapids (Ishihara & Tachihara, 2008), this pipefish is likely unable to pass waterfalls to reach upstream montane habitat in these short, steep rivers.

The predominance of Gobiiformes taxa is not surprising, given that this order is the most speciose in Sulawesi and other Wallacean freshwaters (Kottelat et al., 1993; Kottelat & Whitten, 1996). The three catadromous taxa assigned are all widespread species likely present in the study area, namely anguillid eels (Pike et al., 2020); the greenback mullet *Planiliza subviridis* Valenciennes, 1836 (Bru, 2022); and the silver flagtail *Kuhlia marginata* (Hoese et al., 2019) reported from rivers in the Banggai Islands, close to the study site (Haryono & Sauri, 2020). The milkfish *Chanos chanos* Forsskal, 1775 is a widespread primarily marine euryhaline species known to enter estuaries and rivers and is popular in brackish-water aquaculture (Freyhof et al., 2019).

Multiple anguillid ASVs (one to five per station) had 99.5 %-100 % similarity to both the highlands long-finned eel *Anguilla interioris* Whitley 1938 and the giant mottled eel *Anguilla marmorata* Quoy & Gaimard 1824. Both *A. interioris* and *A. marmorata* have been reported from the surrounding region, and sympatric occurrence of anguillid eels is common in Sulawesi rivers and lakes (Aoyama et al., 2018; Arai, 2016; Hagihara et al., 2018; Serdiati et al., 2023; Sugeha, 2010). Both taxa may well be present, although the high similarities (> 99.5 %) with reference sequences for both species indicate that the 12S rRNA marker used cannot reliably distinguish between



these two congeners. This indication was reinforced by a phylogenetic reconstruction using ASV sequences from this study and all available 12S rRNA reference sequences for the genus *Anguilla* in the NCBI GenBank database (unpublished, available from the authors), where accessions of the two species had up to 100 % identity. The presence of anguillid eels is important from fisheries and conservation perspectives, as eels are valuable food fish and play important though still poorly understood ecological roles in tropical watersheds (Arai, 2016; Drouineau et al., 2018; Righton et al., 2021; Tembo et al., 2023). Tropical eels are heavily exploited from glass eel to silver eel stages globally (Jacoby et al., 2015) and in Sulawesi (Ambo-Rappe & Moore, 2019), often likely exceeding sustainable limits (Righton et al., 2021). Studies on anguillid eels cover many other regions of Sulawesi, including Poso Lake, Poso River and Tomini Bay; North Sulawesi and the Sulawesi Sea; the Makassar Strait including Palu Bay and the East coast of Sulawesi (Arai, 2016; Arai et al., 1999; Serdiati et al., 2023; Sugeha, 2010; Triyanto et al., 2021). However, although *A. marmorata* is reported from Peleng Island in the Banggai Archipelago, separated from the study area by a strait 30-50 km wide (Haryono & Sauri, 2020), data are lacking on anguillid eel populations and fisheries along the East coast of Sulawesi facing the Gulf of Tolo and Banda Sea. This study indicates that this lack of data does not reflect a lack of eels, highlighting the need for research on the genus *Anguilla* in this region.

The identification of terapontid Centrarchiformes ASVs as *Leiopotherapon plumbeus* Kner 1864 or *L. unicolor* Günther 1859 is likely a case of misidentification. *Leiopotherapon plumbeus* is considered endemic to the Philippines (Torres et al., 2021), while *L. unicolor* is considered restricted to Australia (Kennard et al., 2019). The only freshwater terapontid reported from Sulawesi is *Lagusia micracanthus* Bleeker, 1860, endemic to the Maros region of South Sulawesi (Hidayani et al., 2024; Vari & Hadiaty, 2012). Until recently, this species lacked COI DNA barcode sequences (Hidayani

et al., 2024) and still lacks 12S rRNA reference sequences. The comparatively low identity (97-98 % ID) indicates that this detection is more likely to represent a range extension for a species lacking 12S rRNA references sequences (e.g., *L. micracanthus*) or an undescribed terapontid rather than a range extension for *L. plumbeus* or *L. unicolor*.

Primary freshwater fish taxa detected included two Sulawesi endemic species: *Telmatherina celebensis* Boulenger, 1897 and *Oryzias celebensis* Weber, 1894. The sailfin silverside *T. celebensis* is considered endemic to the Malili ancient lake complex (Lumbantobing, 2019b) and is more likely a misidentification than a range extension of this species. Reasons for erroneous assignment include limitations of the molecular marker used in distinguishing between species in some taxa (Porter & Hajibabaei, 2018), such as Telmatherinidae (Hubert et al., 2015), and database gaps due to limited research on riverine Telmatherinidae (Stelbrink et al., 2014), especially in Eastern Sulawesi. The Celebes ricefish *Oryzias celebensis* (Lumbantobing, 2019a) is relatively widespread, and if confirmed this detection will extend its known range within Sulawesi. Likely introduced during prehistoric times, with a long history in culinary culture and considered native by local people, the origin of striped snakehead *Channa striata* Bloch, 1793 populations in Sulawesi remains uncertain (Ndobe et al., 2023).

Four alien invasive species detected in this study have been reported from Sulawesi in connection with deliberate introductions or releases from aquaculture (Herder et al., 2022; Herjayanto et al., 2019; Serdiati et al., 2020; Yanuarita et al., 2020). Barbs *Osteochilus* sp. and catfish *Clarias batrachus* Linnaeus 1758 are native to areas in Sundaland but not Sulawesi (Astuti et al., 2023; Chua & Lim, 2019; Ng & Low, 2019; Thinh et al., 2020). Barbs have been released in some Sulawesi freshwater bodies to increase fisheries yield (Yanuarita et al., 2020); alongside tilapia and other cichlids, they are among the many alien species potentially implicated in the decline of native fish in Sulawesi lakes (Herder et al., 2022; Serdiati et al., 2020). Tilapia in

Indonesia originated from Africa (Robin et al., 2023). In addition to *O. niloticus*, *Oreochromis* ASV's not identified to species level (< 97 % identity) may indicate the presence of hybrids or strains common in Indonesian aquaculture as reported from other Indonesian freshwater bodies (Herder et al., 2022; Ndobe et al., 2023; Yanuarita et al., 2020). The taxon assigned as Cichlidae sp. may be a hybrid. Cichlids released in Indonesia waters are primarily of South American or African origin (Robin et al., 2023) and at least one hybrid cichlid (dubbed flowerhorn cichlid) has been reported from other Sulawesi freshwaters, including the Malili lake complex (Hilgers et al., 2018).

The detection of alien species should be considered a wake-up call to evaluate and implement measures to prevent, eradicate, or manage the spread of invasive species that may negatively impact native biodiversity (Sorensen, 2021). On a recent map showing global hotspots of established alien species, most of Indonesia falls within the top 50 % category, and Sulawesi Island in the top 10 % category (Pyšek et al., 2020). Introduced species can cause harm to native species, populations, communities, and ecosystems through direct and indirect routes or processes, including predation, competition, hybridization, disease transmission, altering food-webs and energy flows (Bernery et al., 2022; Dudgeon, 2020; Sorensen, 2021). World-wide and across taxonomic groups, there is strong evidence that alien species invasions should be monitored and either eradicated or controlled, as interventions are more effective and less costly when detected and addressed at an early stage (Bernery et al., 2022; IPBES, 2023; Pyšek et al., 2020; Sorensen, 2021). Even when eradication of well-established invasive taxa is not feasible, impact mitigation remains an important strategy (Bernery et al., 2022; IPBES, 2023). Read abundances for invasive alien species detected in the Balantak River were comparatively low (1.55 %) but occurred at both stations. While these detections could represent the release of DNA from nearby fish-farms or waste from fish purchased and consumed by people living nearby (rather than

from wild populations of these species), this is unlikely at the upstream site, as aquaculture and human habitation are concentrated in the coastal plain. The presence of non-native fish DNA, especially at the upstream site, could signal early stages of alien species invasion processes. If the presence of potentially invasive fish species is confirmed, management measures for eradication or control might be feasible and appropriate (Dudgeon, 2020; IPBES, 2023; Pyšek et al., 2020; Sorensen, 2021).

Most native Gobiiformes taxa identified in this study have been reported from other rivers in Banggai Regency, in particular *B. belobran-chus*, *E. fusca*, *Sicyopterus lagocephalus* Pallas 1770, *Sicyopterus longifilis*, *Stiphodon semoni* Weber 1895, and *Sicyopus zosterophorus* (Gani et al., 2019; Gani, Adam et al., 2021). These studies also found specimens assigned to genus level but not identified to species level, especially in the genera *Lentipes*, *Schismatogobius* and *Sicyopterus*. A phylogenetic reconstruction (not included in this study) for the genus *Sicyopterus* indicated eight likely species-level taxa, with two unambiguously identified at the 99 % level (*S. lagocephalus*, *S. longifilis*). It is also worth noting that Gobiiformes taxonomy is under revision and could give rise to confusion; for example, the taxon *E. fusca* is now thought to comprise several cryptic species (Mennesson et al., 2018). Furthermore, a large proportion of Actinopteri reads were assigned to Gobiidae with a high degree of confidence but not assigned at lower taxonomic levels.

Assigned taxa from the Gobiidae, Tera-pontidae, and Mugilidae with reported distributions that do not include Sulawesi (Table 2) are unlikely to be introduced alien species. Some could be misidentifications due to data-base gaps or erroneous sequences. However, based on the distributions of other sicydiine gobies (Keith, Lord et al., 2014), new species discoveries and recent range extensions (e.g., an amphidromous goby previously considered restricted to the Philippines recently found in Sulawesi (Ndobe et al., 2023), some assignments may represent range extensions. *L. pala-wanirufus* was described from the Philippines



(Maeda et al., 2021) while *L. armatus* Sakai & Nakamura, 1979, previously considered restricted to Japan and Taiwan (Keith, Hadiaty et al., 2014) has since been reported from the Philippines (Tauli et al., 2022). One or both may well be present in Sulawesi, and if confirmed would represent similar range extensions to the recent records for nearby rivers of *Stiphodon annieae* Keith, Hadiaty et al. (2014), (Gani, Nurjirana et al., 2021) and *S. surrufus* Watson & Kottelat 1995 (Ndobe et al., 2022). Four *Sicyopterus* taxa given as alternative or sole identifications by the MitoFish pipeline are not recorded from areas close to the study site: *S. aiensis* Keith, Watson and Marquet (2004) is considered endemic to Vanuatu (Lord et al., 2010), *S. marquesensis* Fowler, 1932 to the Marquesas Islands in French Polynesia (Boseto, 2015); *S. lengguru* Keith, Lord et al. (2012) was described from Papua New Guinea (Keith et al., 2011); and *S. squamosissimus* from Java and Bali, Indonesia (Keith et al., 2015).

In addition to taxonomic uncertainties, the taxa identified in this study do not necessarily reflect the full fish diversity present in the Balantak River. The eDNA approach used relies on DNA traces shed by organisms present at or near the sampling stations and then collected in relatively small water samples, and there are many reasons why DNA from taxa present in the environment could be missed or could fail to be detected (Porter & Hajibabaei, 2018; Ruppert et al., 2019; Shu et al., 2021). As identification tools, molecular methods such as DNA barcoding and eDNA metabarcoding rely on the presence of homologous reference sequences; gaps or errors in the databases used can lead to misidentification or non-identification (McElroy et al., 2020; Moore et al., 2021; Moore et al., 2025; Porter & Hajibabaei, 2018). Other challenges in eDNA metabarcoding include the risk of contamination (false positives), so-called primer bias (likelihood of amplification across taxonomic groups), complex laboratory and bioinformatic analyses, technical (e.g. nucleotide read) errors, and interpretation of results (Porter & Hajibabaei, 2018; Shu et al., 2021; Wang et al., 2024). In this

study, negative samples were not collected in the field, but laboratory negative controls were negative. Discrepancies can arise due to DNA degradation before or after collection, as degradation rates are influenced by factors such as UV exposure, temperature, microbial activity, and pH levels (Perry et al., 2024; Ruppert et al., 2019). Reduced DNA quality from individuals presents some time ago may make detection less likely and increase the likelihood of erroneous read sequences (Porter & Hajibabaei, 2018). We could not find directly relevant data on DNA degradation for the methods, conditions and taxa in this study, but it is a possible source of unassigned ASVs and could have resulted in false negatives.

Even if DNA from all fish present was detected and correctly identified, the results are intrinsically limited as fish can move randomly or following patterns at various spatial and temporal scales (Perry et al., 2024), and riverine fish communities can change over time, for example between seasons (Wantania et al., 2024). Furthermore, some fish (e.g., large-bodied species or adult individuals) may shed more eDNA than others (e.g., smaller fish or juvenile stages) (Maruyama et al., 2014), and some taxa are more easily amplified and detected than others during the laboratory processes (Miya et al., 2020). Despite the possibility of false positives due to fish DNA from food waste at the downstream site, the taxa identified likely underestimate the ichthyofaunal diversity of the Balantak River. This is indicated by the high proportion of Actinopteri reads assigned to family level (predominantly Gobiidae) with a high degree of confidence but not assigned at lower taxonomic levels. This study was conducted during the South or East monsoon. Further sampling to account for seasonal variation would provide a better picture of ichthyofaunal composition. Furthermore, given the large proportion of diadromous species, sampling over a greater spatial as well as temporal span (e.g., across watersheds in the region) would enable a better assessment of species status as well as fish biodiversity in the many short rivers along the East coast of the Banggai Regency.

This study contributes baseline data for natural resources in Central Sulawesi, at the heart of the Wallacea bioregion. The results call for further research, in particular integrative taxonomic studies to validate or correct some of the taxonomic assignments, and fill gaps in public sequence databases (Haÿ et al., 2023; Riley et al., 2023). Once the presence of a species is confirmed, its local conservation status can be evaluated, and if necessary, management strategies (*in-situ* and, if necessary *ex-situ*) can be developed and implemented (Maasri et al., 2022). All native genera identified are known to be exploited for human use (Table 1), and the gaps in IUCN Red List coverage highlight the ongoing need for assessments. The majority of taxa detected were diadromous or euryhaline, reinforcing the importance of maintaining connectivity between coastal and riverine ecosystems and throughout watersheds (Shao et al., 2019; Lagarde et al., 2021; Wantania et al., 2024). This study demonstrates that many ecologically and economically important species are using both marine and freshwater environments in this and nearby watersheds during their life-cycles. Therefore, it is vital to protect both environments, and the land-sea interface where they meet, crossing institutional and administrative boundaries in terms of governance and stakeholder groups with an interdisciplinary and collaborative approach. Furthermore, the detection and control of non-native species and the prevention of further introductions should be taken seriously in the Balantak River, and across all Sulawesi freshwaters.

While long-term conservation of freshwater ecosystems, their biodiversity, and ecosystem services require addressing major global challenges, especially related to climate change, local action is vital (Dudgeon, 2019; Oikonomou, 2021). Management strategies for Sulawesi freshwaters, such as the Balantak River watershed, should focus on conserving the rich native ichthyofauna, in particular diadromous species, while eliminating or controlling alien invasive species. Options include but are not limited to habitat protection or management; fisheries management including data collection

and regulations, especially for ornamental fisheries and trade; conservation breeding and sustainable aquaculture practices that provide both conservation and economic benefits; and developing a comprehensive and reliable 12S rRNA database for Sulawesi freshwater fishes supported by voucher specimens.

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