Short Communication

What's in the water: using environmental DNA metabarcoding to detect fish biodiversity in the Cambodian Mekong

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Globally, freshwater biodiversity faces increasing threats (Dudgeon *et al*., 2006) and the fish community of the Lower Mekong Basin (LMB) exemplifies this trend. Climate change, hydroelectric development, and an increasing human population are major stressors in the basin (Lauri *et al*., 2012; Pokhrel *et al*., 2018; Yoshida *et al*., 2020), which covers approximately 571,000 km^2 across Cambodia, Thailand, Laos and Vietnam. In Cambodia, the Mekong enters the country at its border with Laos and flows 480 km south to the border with Vietnam. As it flows southward it is fed by large tributaries, including the Sekong, Sesan and Sre Pok (3S) rivers that drain southern Laos, central Vietnam and northeastern Cambodia. In addition, Southeast Asia's largest lake— the Tonle Sap—lies in western Cambodia. As a tropical watershed with a recurring wet season and associated flooding of expansive wetlands, the lake supports a productive and diverse community of over 1,000 fish species (Hortle, 2009a; Rainboth *et al*., 2012). The seasonal flood pulse drives fish abundance in the basin, as species are adapted to utilize seasonally available, highly productive habitats (Poulsen *et al*., 2002). This ecosystem supports an annual fish harvest in excess of two million tons and consisting of hundreds of species, providing food security for over 70 million people (Hortle, 2009b; FAO, 2020). This means that monitoring the fish community is essential, both as an early warning system for biodiversity losses and for evaluating impacts of conservation measures. However, Cambodia exemplifies challenges common to biodiversity monitoring in large, tropical river systems, including difficulties with accessing remote areas, extreme seasonal conditions, resource-limited management agencies, and a need for numerous gears and significant expertise to capture and identify hundreds of species. Given the growing stressors in the region, developing approaches for effective biodiversity monitoring is particularly critical.

Collection of genetic material from the environment (eDNA) is a non-invasive and increasingly applied approach to characterize biodiversity in freshwater systems and monitor for endangered species (Deiner *et al*., 2016; Evans & Lamberti, 2018; Doi *et al*., 2021; Laporte *et al*., 2021; Yao *et al*., 2022). Notably, eDNA metabarcoding is highly sensitive and has the potential to detect greater numbers of species compared to traditional capture methods (McColl-Gausden *et al*., 2021). Although applications of metabarcoding in tropical rivers have been challenged by a lack of reference sequences (Jerde *et al*., 2021), the technology is beginning to be applied in Southeast Asia. For example, metabarcoding has been used in the Chao Phraya Basin of Thailand to detect patterns in biodiversity to inform conservation efforts (Blackman *et al*., 2021), and it has been used in the LMB to distinguish patterns in fish diversity across ecological gradients and evaluate hypothesized barriers to fish dispersal (Durand *et al*., 2022).

We performed a pilot evaluation of the feasibility of metabarcoding for quantifying fish diversity in the Cambodian Mekong through the collection of water-

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borne eDNA. Sampling occurred between February and April 2022 throughout the Mekong River; the 3S rivers; and the Tonle Sap (Fig. 1, Table 1). Collection of eDNA samples was permitted by the Cambodian Fisheries Administration and conducted with support from the Inland Fisheries Research and Development Institute.

Samples were collected with single-use aquatic eDNA kits (Jonah Ventures, Boulder, Colorado, USA), which included nitrile gloves, a 60 ml syringe, a 5 μm filter cartridge, and a 1 ml syringe of Longmire's solution to stabilize DNA for storage and transport. These kits were selected because no special equipment is required

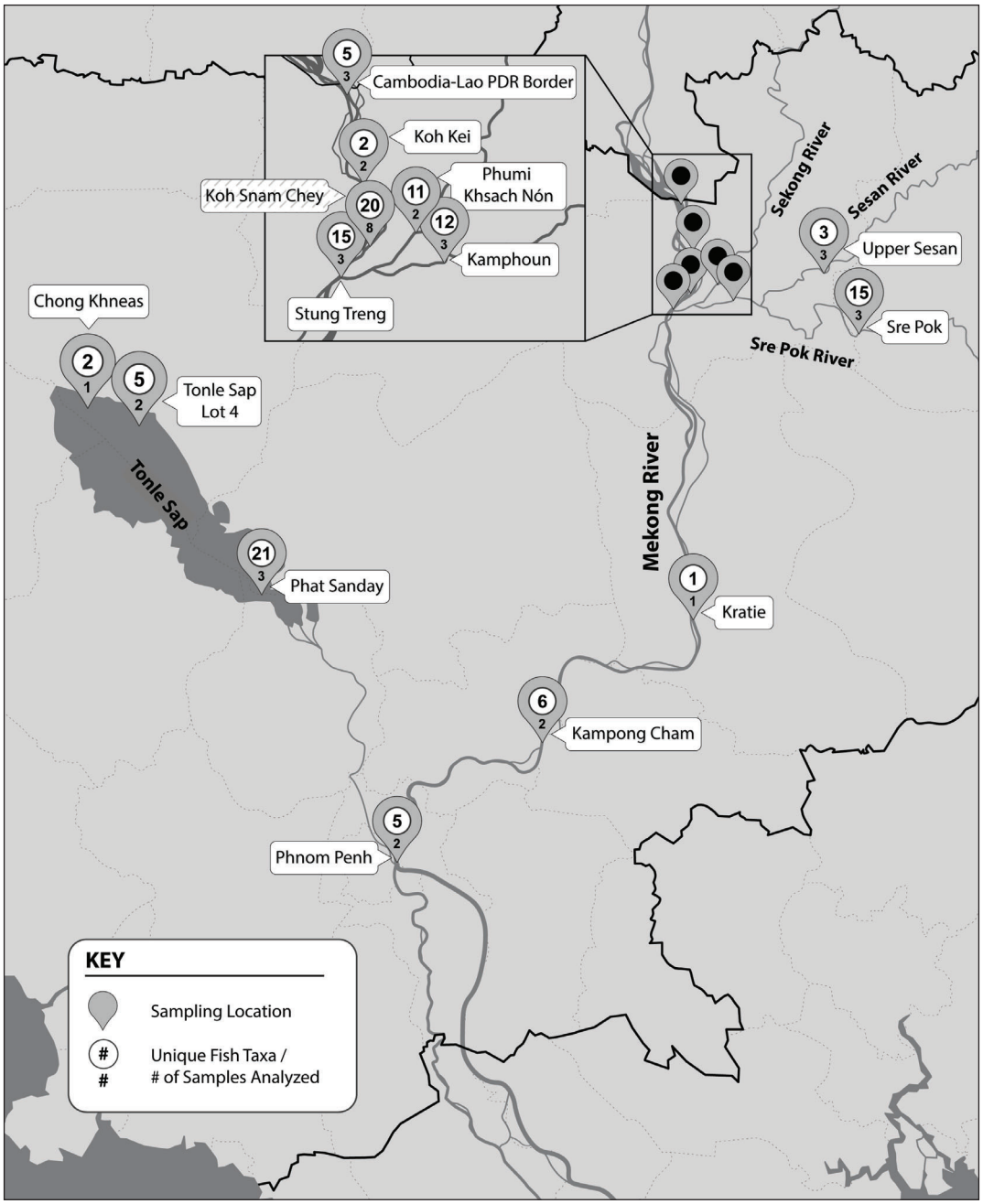

Fig. 1 The Lower Mekong Basin, Cambodia and locations where eDNA samples were collected. Each location is labelled with the total number of taxa detected in metabarcoding analysis, as well as the total number of samples from that location which yielded results.

Table 1 Locations, filter volumes and biodiversity detected in samples. Samples in which no DNA sequences were detected or for which no sequences could be assigned to fish taxa are excluded from the summaries of volume and taxa detections.

Site	# Samples Yielding Results / # Samples Collected	River	Mean Volume Filtered per Sample Kit (ml)	Total Volume Filtered at Site (ml)	Unique Fish Taxa	Families	Genera	Species
Border	3/9	Mekong	180	540	5	2	5	5
Koh Kei	2/3	Mekong	175	350	2	2	2	2
Koh Snam Chey	8/9	Mekong	150	1200	20	7	17	19[1]
Stung Treng [2]	0/3	Mekong	-	-	-	-	-	-
Kratie	1/3	Mekong	180	180	1	1	1	1
Kampong Cham	2/3	Mekong	120	240	6	4	5	5[1]
Phnom Penh	2/3	Mekong	120	240	5	5	5	5[1]
Stung Treng	3/3	3S (Sekong)	120	360	15	8	13	14
Phumi Khsach Nón	2/3	3S (Sekong)	120	240	11	9	10	11[1]
Kamphoun	3/3	3S (Sesan)	100	300	12	6	11	11[1]
Upper Sesan	3/3	3S (Sesan)	125	375	3	2	3	3
Sre Pok	3/3	3S (Sre Pok)	120	360	15	7	12	15[1]
Chong Khneas	1/2	Tonle Sap	30	30	2	2	2	2
Tonle Sap Lot 4	2/3	Tonle Sap	38	75	5	3	3	4
Phat Sanday	3/3	Tonle Sap	20	60	21	11	17	21[1]
Totals	38/56			4.55 l	123	69	106	31

[1] Exact sequence variants that could only be assigned to genus level were detected in these sites. Each of these is included in the species count as a single species for the site where it was detected. Fish taxa at the family and order level are not included in the genera or species counts. See Table 2 for details.
[2] Samples collected from the mainstem Mekong River in Stung Treng city failed to generate any sequence reads. This site is not included in Fig. 1, and the only Stung Treng site shown is that on the Sekong River above the confluence in Stung Treng city.

and samples stabilized with Longmire's solution do not require refrigeration in the field. Further, the filters are enclosed in a cartridge that reduces potential for contamination, and the single-use nature of the kits eliminates the need for decontamination of equipment. As an additional precaution to reduce contamination, field staff were instructed to collect upstream of where they were wading or of the boat used to reach the sample location. At most locations, a sample was collected from the right bank, middle of the channel, and the left bank, for a total of three samples per location. Samples were collected approximately five centimetres beneath the water's surface in all locations except for the site on the Mekong River near Koh Snam Chey (shaded label in Fig. 1 inset), where three samples apiece were collected at depths of 1, 35 and 60 m (Table 1).

DNA metabarcoding employed MiFish primers (Miya *et al*., 2015), which target the 12S region of the mitochondrial genome and are known to provide genetic resolution to the species level. PCR amplification was performed in replicates of six, none of which were pooled. Each round of PCR included a non-template control to identify any laboratory cross-contamination. Metabarcoding produced hundreds of thousands of sequences, which were processed using a custom bioinformatics pipeline that summarized the number of unique exact sequence variants (ESV) amplified in each water sample. ESV assignments were based on percent similarity to reference sequences from GenBank (release 248), plus five unpublished sequences from specimens sequenced by Jonah Ventures (*Clupeoides borneensis*, *Henicorhynchus entmema*, *Puntioplites falcifer*, *Trichopodus trichopterus* and *Ompok siluroides*). A full description of laboratory methodology can be found in the supplemental materials of Campbell *et al*. (2022). A recursive matching algorithm assigned ESVs to known species according to sequence similarity, and if below a similarity threshold ESVs were assigned to higher taxonomic levels. In most cases, ESVs were designated to species, but genus, family, and order level assignments did occur. We then used R statistical

Table 2 Fish species detected in 38 eDNA samples collected throughout the Lower Mekong Basin. Two exact sequence variants (ESVs) were only assigned to Siluriformes, six were only assigned to Pangasiidae, and four were only assigned to Cyprinidae and are not explicitly included in this table, although these orders and families are represented at each site by other genus and species level assignments. For taxa identified only to the genus level, the number of unique ESVs assigned to that genus is provided in the species column. The number of ESVs assigned to each species are given in parentheses after the species name. VU=Vulnerable, EN=Endangered, CR=Critically Endangered, per IUCN (2022). Scientific names are based on NNEF (2021).

	Mainstem Mekong						3S Basin					Tonle Sap		
Species (# of ESVs)	**Border**	**Koh Kei**	**Koh Snam Chey**	**Kratie**	**Kampong Cham**	**Phnom Penh**	**Stung Treng**	**Phumi Ksach Nón**	**Kamphoun**	**Upper Sesan**	**Sre Pok**	**Chong Khneas**	**Tonle Sap Lot 4**	**Phat Sanday**
Osteoglossiformes														
Notopteridae														
Chitala ornata (2)					X									
Notopterus notopterus (3)	X								X					X
Clupeiformes														
Clupeidae														
Clupeichthys aesarnensis (5)									X	X				
Clupeoides borneensis (1)												X	X	
Sardinops sp. (1)										X				
Engraulidae														
Lycothrissa crocodilus (2)						X								
Cypriniformes														
Cyprinidae														
Amblyrhynchichthys micracanthus[1] (2)											X			
Barbonymus altus (7)			X				X		X		X			
Barbonymus gonionotus (10)	X		X					X			X			X
Catlocarpio siamensis[2] CR (2)													X	
Cirrhinus microlepis VU (5)	X		X						X					
Cosmochilus harmandi (1)			X											
Cyclocheilichthys apogon (1)														X
Cycloheilos enoplos (5)		X							X					
Epalzeorhynchos sp. (2)											X			
Hampala dispar (2)			X											
Henicorhynchus entmema (2)							X							
Henicorhynchus siamensis (3)					X		X							X
Hypsibarbus malcolmi (2)	X													
Labeo chrysophekadion (3)											X			
Labiobarbus leptocheilus (1)				X			X							
Labiobarbus sp. (2)														X
Mystacoleucus marginatus (3)			X					X	X					
Onychostoma meridionale (4)			X						X					
Osteochilus vittatus (4)														X

Table 2 Cont'd.

Species (# of ESVs)	Mainstem Mekong						3S Basin					Tonle Sap		
	Border	Koh Kei	Koh Snam Chey	Kratie	Kampong Cham	Phnom Penh	Stung Treng	Phumi Ksach Nón	Kamphoun	Upper Sesan	Sre Pok	Chong Khneas	Tonle Sap Lot 4	Phat Sanday
Osteochilus melanopleurus (1)														X
Osteochilus microcephalus (1)					X									
Puntioplites falcifer (4)			X				X		X		X			
Puntioplites proctozystron (1)														X
Puntioplites sp. (1)											X			
Scaphognathops bandanensis VU (3)	X		X				X							
Sikukia gudgeri (11)			X				X				X			
Danionidae														
Raiamas guttatus (3)			X						X					
Rasbora dusonensis (1)			X					X			X			
Rasbora sp. (1)							X							
Xenocyprinidae														
Paralaubuca typus (2)						X			X					
Botiidae														
Yasuhikotakia eos (5)			X					X			X			
Yasuhikotakia lecontei (3)			X								X			
Cobitidae														
Acantopsis dinema (4)							X	X	X					
Nemacheilidae														
Nemacheilus platiceps (1)			X											
Schistura sp. (1)			X											
Siluriformes														
Ailiidae														
Laides longibarbis (1)			X					X						
Bagridae														
Hemibagrus sp. (3)					X			X						
Hemibagrus spilopterus (1)								X						X
Mystus atrifasciatus (1)														X
Mystus sp. (3)						X								X
Clariidae														
Clarias macrocephalus (1)						X								
Pangasiidae														
Pangasianodon gigas[2] CR (5)													X	
Pangasianodon hypophthalmus[2] EN (1)													X	
Pangasius macronema (4)			X		X	X					X			X
Pseudolais pleurotaenia (2)							X	X						

Table 2 Cont'd.

Species (# of ESVs)	Mainstem Mekong						3S Basin					Tonle Sap		
	Border	Koh Kei	Koh Snam Chey	Kratie	Kampong Cham	Phnom Penh	Stung Treng	Phumi Ksach Nón	Kamphoun	Upper Sesan	Sre Pok	Chong Khneas	Tonle Sap Lot 4	Phat Sanday
Siluridae														
Ompok siluroides (1)														X
Beloniformes														
Belonidae														
Xenentodon cancila (2)											X			X
Zenarchopteridae														
Dermogenys siamensis (1)														X
Dermogenys sp. (1)														X
Perciformes														
Ambassidae														
Parambassis sp.[3] (1)							X							
Anabantiformes														
Anabatidae														
Anabas testudineus (1)														X
Channidae														
Channa micropeltes (1)														X
Channa striata (5)							X	X				X		X
Nanidae														
Pristolepis fasciata[4] (4)							X	X		X	X			X
Osphronemidae														
Trichopodus trichopterus (2)														X
Gobiiformes														
Gobiidae														
Gobiopterus sp.[3] (1)											X			
Papuligobius ocellatus (2)		X	X				X							

[1] ESVs were assigned to *A. truncatus*, however this formerly monotypic genus has been split into two species: *A. truncatus* in the Sundaland region and *A. micracanthus* in the northern Indochinese region (Ng & Kottelat, 2004). We therefore presume that the species detected was in fact *A. micracanthus*.

[2] These species were detected at Tonle Sap Lot 4, where they were released prior to eDNA sample collection as part of a mark-recapture study.

[3] Sequences were assigned to species in these genera, but because these species are not known to occur in Cambodia, they are presumed to be incorrect assignments based on poor resolution of the MiFish primers for species within these genera, incomplete genetic reference libraries for the region, or both. As such, the sequence assignments were retained at the genus level, as they were presumed to represent actual diversity at the sites where they were detected.

[4] There is ongoing uncertainty regarding the taxonomic placement of this species. It was assigned to Anabantiformes and Pristolepidae by Kottelat *et al*. (1993), but placed in Nandidae within Perciformes by Nelson (2006). Though the genetic reference library assigned the species to Pristolepidae, we have applied its most latest assignment to Nanidae in Anabantiformes by NNEF (2021).

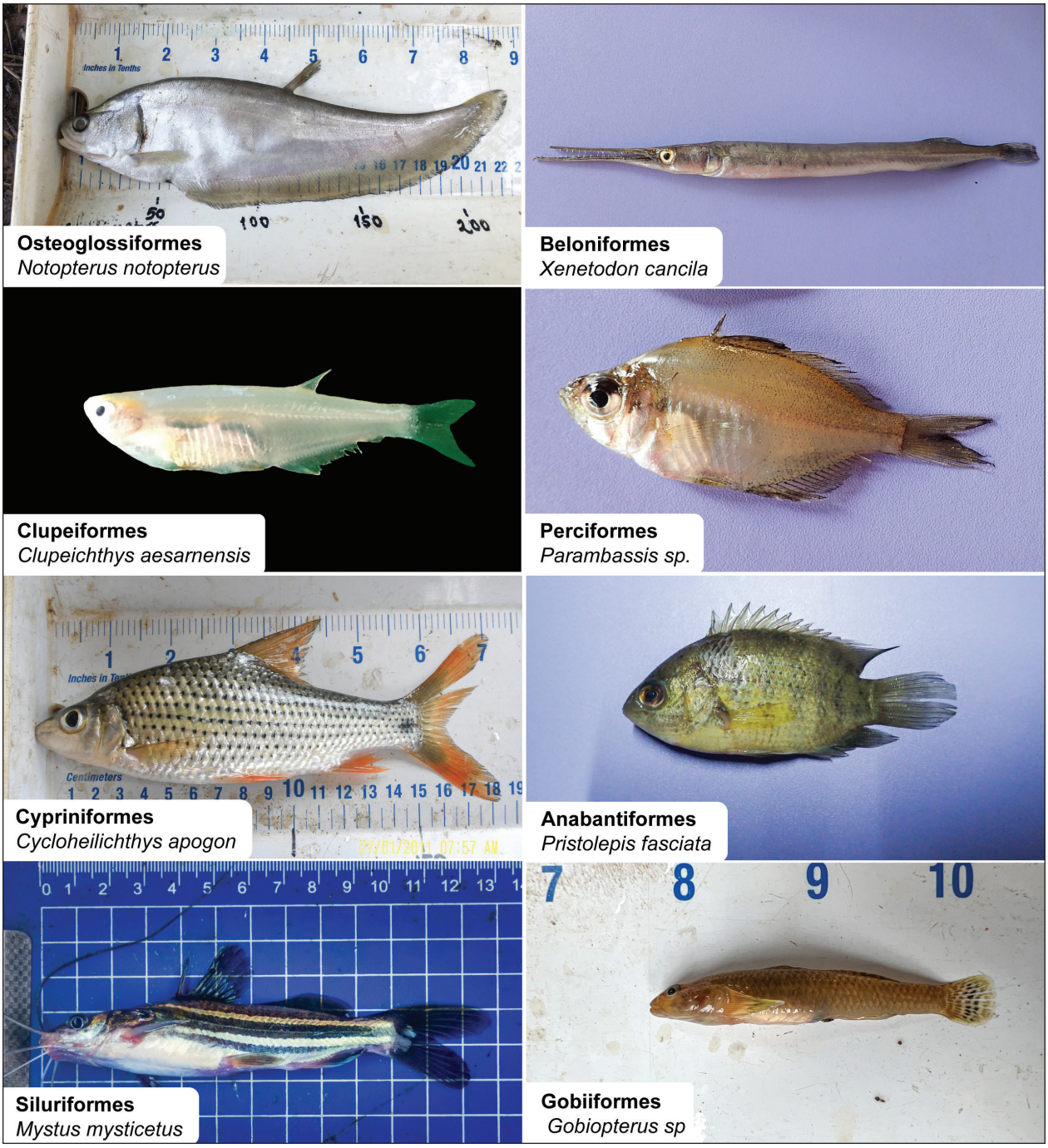

Fig. 2 Fish species among those detected in eight orders in the metabarcoding samples.

software (R Core Team, 2022) for subsequent filtering and analysis.

In total, 56 eDNA samples were collected. Of these, we excluded 11 for PCR failure, three for only having unidentifiable ESVs, and four that only contained mammalian DNA (e.g., *Bos* spp., *Sus scrofa* and *Homo sapiens*). This left 38 samples for analysis. Sequence and sampling data have been uploaded to the NCBI SRA database (BioProject ID: PRJNA1003506). Across these samples, a total of 161 fish ESVs were assigned to 63 fish taxa that represented eight orders, at least 23 families, at least 49 genera, and at least 55 species (Table 2). Two

ESVs could only be assigned to order (both Siluriformes) and ten were only assigned to family (four to Cyprinidae and six to Pangasiidae). A number of ESVs were assigned to genera, including three ESVs each assigned to *Mystus* and *Hemibagrus*, two ESVs each to *Labiobarbus* and *Epalzeorhynchos*, and one ESV each to *Dermogenys*, *Puntioplites*, *Rasbora*, *Sardinops* and *Schistura* (Table 2). Each of these genera assignments were also represented by species level assignments of other ESVs, except for *Epalzeorhynchos*, *Sardinops* and *Schistura*.

We compared all taxa identified in the samples to a list of species known to occur in the Mekong (Jerde *et al*., 2021). Two species from the Western Hemisphere were excluded from our final dataset due to possible laboratory cross-contamination. Another two taxa—*Parambassis ranga* and *Gobiopterus lacustris*—are not known to occur in Cambodia, but other species within these genera do. As *P. ranga* and *G. lacustris* were the sole representatives of these genera in the reference library, this indicates high sequence similarity with Cambodian representatives of these genera, or else the matching algorithm would have only assigned them to genus. We retained these two sequences as genus level taxonomic representatives at the sampled locations. This result, along with other assignments to genus or higher levels, highlights the need for mitochondrial DNA vouchers from all Cambodian fish species.

The detected taxa represented a wide variety of species (Fig. 2), including the Critically Endangered *Catlocarpio siamensis* and *Pangasianodon gigas*, and the Endangered *P. hypophthalmus* (IUCN, 2022). Detection of these iconic species was expected given their release at the project site in the Tonle Sap (Campbell *et al*., 2022), but these results confirm that metabarcoding is able to detect them in natural settings. Sequences belonging to *Cirrhinus microlepis* and *Scaphognathops bandanensis*—both listed as Vulnerable (IUCN, 2022)—were also detected. Further, we detected a sequence belonging to genus *Schistura*, which suggests that cryptic species like loaches may be effectively detected with eDNA, although more work is needed to improve genetic reference libraries for such diverse genera (Jerde *et al*., 2021). The *Schistura* detection occurred only in the sample collected at a depth of 35 m at Koh Snam Chey, suggesting that some species may not be detected from surface sampling alone.

An average of 8.78 fish taxa were detected at each site, and varied from 21 at Phat Sanday to one at Kratie (Fig. 1, Table 1). We used taxa accumulation curves to evaluate whether the number or volume of samples adequately captured taxonomic richness at a regional scale, as well

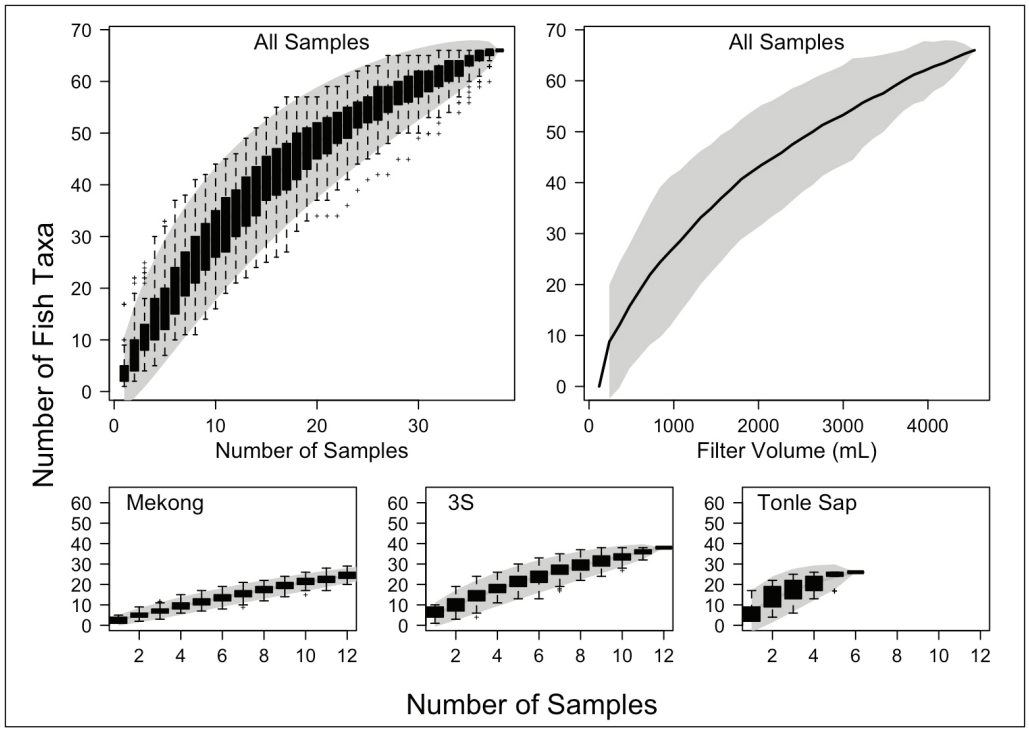

Fig. 3 Fish taxa accumulation curves based on the number of samples from all sites in the Lower Mekong Basin in Cambodia (top left), the Mekong mainstem, 3S basin and Tonle Sap sites. The top right panel is based on total filter volume for all samples.

as basin-wide using all samples. Accumulation curves increase with increasing number of samples and should reach an asymptote, at which point additional samples will not detect more species. The curves were constructed using the '*specaccum*' function available in the *vegan* package for R (Oksanen *et al*., 2022). For the number of samples collected in each region and across the entire basin, the number of species increased but did not reach an asymptote (Fig. 3). The same pattern applied to sample filter volume. The curves indicate that our limited sampling did not provide adequate taxonomic coverage in any region, therefore we cannot evaluate differences in regional levels of diversity.

Our pilot study illustrates some tradeoffs to consider when implementing eDNA metabarcoding studies in the LMB. High turbidity at sample locations precluded the ability to filter large volumes with the sample kits. Larger pore size filters may allow for filtration of greater volumes (e.g., Durand *et al*., 2022), but this carries greater risk of sample contamination with PCR inhibitors (Herder *et al*., 2014), which are abundant in turbid systems (Kumar *et al*., 2021). When filtering smaller volumes (e.g., < 500 ml), finer pore sizes capture significantly more DNA than filters with larger pore sizes (Jeunen *et al*., 2019). Therefore, the best option for the application of single-use kits is to increase the number of samples. Indeed, sample volumes as low as 100 ml collected with kits similar to those we used can effectively detect biodiversity in turbid, tropical systems if suitable replication is achieved (Blackman *et al*., 2021). Clearly, studies seeking to use eDNA to quantify biodiversity will require greater numbers of samples, and consequently greater volumes of water filtered to maximize the number of species detected. In addition, collecting samples from multiple depths may increase detection of benthic species. In the laboratory, increasing the number of PCR replicates may increase detection species with low DNA concentrations. However, this runs the risk of increased chances for cross-contamination. Finally, using eDNA in the most efficient and meaningful way possible in the LMB will require comparisons between the data from traditional sampling and metabarcoding data to better understand the strengths and limitations of a molecular approach. Studies seeking to do so are currently in progress.

Global loss of aquatic biodiversity not only threatens vital fisheries, but also imperils numerous other benefits that freshwater biodiversity provides to humanity (Lynch *et al*., 2023). In the LMB, eDNA is a valuable tool for monitoring biodiversity and informing conservation approaches. Significant benefits provided by eDNA metabarcoding compared to traditional sampling methodologies are its scalability and relatively lower cost and effort. Standardized eDNA sampling may be useful for detecting species diversity in areas that are not represented in current LMB monitoring programs (Halls *et al*. 2013). Pending refinement of best practices in sampling design and buildout of genetic reference libraries, eDNA may also be useful in rapid biodiversity inventory applications such as environmental impact assessments associated with dam development. Each of these potential benefits, when considered in the context of the urgent need for improved fish biodiversity monitoring throughout the LMB, suggest that applications of eDNA studies in the basin may provide important information for fisheries management, conservation efforts, and policy decisions in the region.

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