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THE eDNA MEKONG BIODIVERSITY STUDY IN THE MEKONG DOLPHIN MANAGEMENT AND PROTECTION ZONE, AND THE STUNG TRENG'S RAMSAR SITE

WWF-CAMBODIA

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Summary

This report describes the results of the Environmental DNA (eDNA) biodiversity surveys conducted in the Mekong River Dolphin Management and Protection Zone at the upper Mekong River in Cambodia. The surveys were conducted during both the dry and wet seasons in 2022.

Both the wet and dry season surveys were conducted for six days. The surveys documented the presence of fish, amphibian, reptile, bird and mammal species in the Mekong River based on Environmental DNA material found in water samples collected in *the Dolphin Management and Protection Zone* and *the Stung Treng's Ramsar site*. In total, 36 water samples were collected.

Analysis of the water samples, collected from the dry season survey, detected 147 potential taxa including 128 fish species, four amphibian species, five mammal species and 10 bird species. Eighty-six of 147 detected in the dry season sample and 52 of 86 species detected from the wet season sample analysis were identifiable within the GenBank Global Reference Database. I

Eight threatened species were detected from dry season samples, including the Critically Endangered Mekong Irrawaddy dolphin, goonch (*Bagarius yarrelli* -Vulnerable*)*, small scale mud carp (*Cirrhinus microlepis* - Vulnerable*)*, *Labeo Pierrei* (Vulnerable), elephant ear gourami (*Osphronemus exodon* -Vulnerable), *Scaphognathops bandanensis* (Vulnerable), Mekong herring (*Tenualosa thibaudeaui* - Vulnerable), and Wallago (*Wallago attu* - Vulnerable) while four threatened taxa were detected from the analysis of the wet season samples, including Mekong herring (*Tenualosa thibaudeaui* - Vulnerable), small scale mud carp *Cirrhinus microlepis* - Vulnerable*)*, *Scaphognathops bandanensis* (Vulnerable)

The DNA of the Notched mud carp (*Henicorhynchus lobatus*) was in the highest abundance encountered during the dry season, while Java barb (*Barbonymus gonionotus*) DNA was in the highest abundance detected in wet season sampling.

The Mekong River Commission has previously documented the presence of 41 invasive non-native fish species in the Mekong (MRC, 2021). Fishermen, in the study area, have reported catching two invasive non-native namely Pirapatinga, *Piaractus brachypomus*, and Nile tilapia, *Oreochromis niloticus.* However, in the current survey results did not confirmed any invasive non-native fish species.

The results of these surveys are important for the local communities occupying the areas around the deep water pools of the five sampling sites. The analysis of the data increases their awareness of the status of the fishery, the health ecosystem and aquatic resources available.

The information resulting from expanding the eDNA sampling sites to other parts of the study site, where many water deep pools are located, is important for local communities to strategically manage their aquatic resources.

Background

Rationale and aims of study

The 217-km section of the Mekong River from Kratie Province in the south up to the Lao border included the 180-km stretch of *the Mekong River Dolphin Management and Protection Zone* and the 37-km stretch of *the Stung Treng's Ramsar site,* covers approximately five percent of 4,350 km length of the Mekong River, which flows from Tibetan Plateau in China to the delta in Vietnam, where it enters the sea.

This section of river is unique and contains a diverse and rich mosaic of habitats including seasonally flooded riverine vegetation, sandbars, deep pools, rocky rapids, flooded islands, and river bank flooded forest.

These habitats are not only important for fish to shelter, feed, grow and breed, but they are also indispensable for a range of globally threatened species including the Irrawaddy river dolphin (*Orcaella brevirostris*), Cantor's giant softshell turtle *(Pelochelys cantorii),* the giant freshwater stingray (*Hemitrygon laosensis*), the Mekong giant catfish (*Pangasianodon gigas*), white shouldered ibis (*Pseudibis davisoni*), river tern (*Sterna aurantia*) and an Endangered subspecies of hog deer (*Axis porcinus annamiticus*), amongst other species.

This abundance of species and the diversity of habitats make this stretch of the Mekong River vitally important as a food source for the Cambodian people and the people living in the lower Mekong River Basin countries (Birdlife International, 2023; Mittermeier, 2019; Campbell T, 2020).

Anthropogenic activities threaten both the habitats and the species. These threats include climate change, conversion of the riparian forests, the removal river channel vegetation, urban and agricultural development, illegal fishing activities including gillnetting, electro-fishing, poisoning and overfishing, and hydropower dams on the main channel of the Mekong River and its tributaries that block fish migrations and alter the hydrology of the river locally and at scale.

Numerous studies of the biodiversity of the Mekong River have been conducted. However, the results obtained were never made available to local communities in a format that fisher people could utilize to strengthen the management and protection of the community fisheries. Creation of a Mekong biological inventory database would allow government agencies to track changes in species diversity. The baseline data can be utilized to design a water deep pool protection and monitoring plan, as current studies did not focus on data collection in a specific water deep pool but along the Mekong channel of the same study site through using different fishing gear types, market observation and interview with local communities.

Baseline data of the biodiversity, collected to date, is necessary for the provincial government agencies in Kratie and Stung Treng Provinces, local communities, as well as WWF to gain a better understanding of the current status of the biodiversity in the dolphin core zones and water deep pools, to determine the impact of illegal fishing practices on the freshwater fauna in this area.

Objectives

The objectives of this study were to:

1. Understand the presence and diversity of vertebrate species within the survey area;
2. Create a biodiversity database and establish a biodiversity baseline;
3. Understand distribution of biodiversity in the Mekong Dolphin Management and Protection Zone
4. Identification of non-native and invasive species, and species that maybe new records in Cambodia
5. Engage local communities in monitoring species of biodiversity using eDNA samples
6. Use evidence to inform and improve conservation decision making in the region.

Research Methodology

The Environmental DNA (eDNA) Mekong biodiversity study was conducted over two discrete survey periods, including a dry season survey in March 2022 and a wet season survey in July 2022. The survey team consisted of five researchers from WWF-Cambodia, the Fisheries Administration at the national level, and the Fisheries Administration Cantonments of Kratie and Stung Treng Provinces, as well as community representatives from villages adjacent to the five sampling sites. Two local motorized boats were used for the surveys. Each boat contained three researchers, GPS units, digital cameras and water sampling kits.

Study Sites and map

Water samples containing eDNA materials were collected at five sampling sites within the Mekong Dolphin Management and Protection Zone and the Stung Treng Ramsar Site, (Appendix 1 Figure 1 contains maps of the five sample sites).

Permissions and community engagement

The project was conducted in collaboration with the Fisheries Administration of Cambodia. The Provincial FiA Cantonments in Kratie and Stung Treng were implementing partners in these surveys. Representatives of five Community Fisheries (CFi) in villages adjacent to the sampling sites also participated in the eDNA surveys. Results of these surveys have allowed them to understand the aquatic diversity availability and engage in monitoring their trends based on the innovative tool.

Data Collection Methodology

The staff from the National FiA office, Provincial FiA Cantonments offices in Stung Treng and Kartie as well as WWF staff was virtually trained on the collection of water samples as well on the utilization of the NatureMetrics protocol. Five sampling sites were selected within a 217-km stretch of the Mekong River (Appendix 1, Figure 1). Samples were collected at the water surface. The surveying route was navigated from downstream to upriver and involved sampling in both the main and side channels of the river at each sampling site.

Water samples (see Table 1) were collected at the deep pool locations in the five sampling sites to obtain e-DNA material followed by NatureMetrics's Sampling Protocol Pump eDNA Kit. These samples were then shipped to the NatureMetrics Company based in the United Kingdom for laboratory analysis to identify species.

Table 1: The sampling sites, number of samples collected, volume of water filtered and date of samples collected

Sampling sites	Sampling seasons/Date	Number of Samples collected	Volume of water filtered
Kampi	Dry, March 2022	4	1,000-2,000ml
	Wet, July 2022	4	700ml
Koh Pdao	Dry, March 2022	4	2,000ml
	Wet, July 2022	4	700-800 ml
Tbong Kla	Dry, March 2022	4	2,700-3,500ml
	Wet, July 2022	4	700-900ml
Koh Santuk	Dry, March 2022	3	2,700-3,000ml
	Wet, July 2022	3	700ml
Ramsar	Dry, March 2022	3	3,000ml
	Wet, July 2022	3	700-900 ml

eDNA Sample Analysis Methodology

Swab barcoding

A total of 11 swab samples were collected from both surveys. The DNA from swabs taken from each of the fish samples was extracted using a commercial DNA extraction kit with a protocol modified to increase DNA yields. This DNA was amplified using a polymerase chain reaction (PCR) for a hypervariable region of the 12S rRNA gene. Determination of the success of the PCR was accomplished by running an agarose gel electrophoresis on the samples.

PCR reactions were successful for all 3 of the swab samples from dry season sampling (Appendix 2, Table 1) while PCR reactions were successful for all 8 of the swab samples from wet season sampling (Appendix 2, Table 2). The successful PCR products were purified and Sanger sequenced. Raw sequences were trimmed and cleaned. After cleaning, the sequences were compared to the NCBI *nt* reference database (GenBank) to identify which of the swabbed taxa represented novel additions to our database. All generated sequences were used to inform taxonomic assignment of the metabarcoding data.

Metabarcoding

eDNA from each filter of eDNA water sample was extracted using a commercial DNA extraction kit with a protocol modified to increase DNA yields. An extraction blank was also processed for the extraction batch. DNA was purified to remove PCR inhibitors using a commercial purification kit.

A total of 36 eDNA samples from both sampling surveys were collected. PCR reactions were successful for 17 of the 18-dry season samples (Appendix 2, Table 3) and all 18 samples of the wet season samples (Appendix 2, Table 4).

The dry season samples, 12 successful PCRs replicates were obtained for each of the 17 samples submitted for sequencing.

The wet season samples, 9-12 successful PCRs replicates were obtained for each of the 18 samples submitted for sequencing.

No bands were observed on electrophoresis gels for the extraction blank or negative controls.

All samples were successfully indexed electrophoresis bands were strong and of the expected size. No repeat reactions were necessary. Amplicons were purified and checked by gel electrophoresis, these were then quantified using a Qubit high sensitivity kit according to the manufacturer's protocol.

All purified index PCRs were pooled into a final library with equal concentrations. The final library was sequenced using an Illumina MiSeq V3 kit at 10.5 pM with a 20% PhiX spike in. Sequence data were processed using a custom bioinformatics pipeline for quality filtering, Operational Taxonomic Unit (OTU) clustering, and taxonomic assignment.

Both the negative and positive controls were as expected. Very few sequences were discarded prior to dereplication. This is indicative of high-quality data with minimal PCR and sequencing errors. A total of 1,286,024 high-quality sequences were included in the final dataset.

Consensus taxonomic assignments were made for each OTU using sequence similarity searches against the National Centre for Biotechnology Information (NCBI) (GenBank) reference database and a local reference database (Durand et al., 2022). Assignments were made to the lowest possible taxonomic level where there was consistency in the matches. Conflicts were flagged and resolved manually. Minimum similarity thresholds of 99%, 97%, and 95% were used for species-, genus- and higher-level assignments respectively.

In cases where there were equally good matches to multiple species, public records from Global Biodiversity Information Facility (GBIF) were used to assess which were the most likely to be present in Cambodia, Vietnam and Laos. Higher-level taxonomic identifications or multiple potential identifications were reported in cases that could not be resolved in this way.

The OTU table was then filtered to remove low abundance OTUs from each sample (<0.025% or <10 reads, whichever is the greater threshold for the sample). Unidentified, non-target, and common contaminant sequences such as human and livestock were then removed, as unidentified or misidentified taxa can result from incomplete or incorrect reference databases, and taxa may be missed due to low quality DNA, environmental contaminants, or the dominance of other species in the sample.

The abundance of taxa cannot be directly inferred from the proportion of total sequence reads. While the proportion of sequence reads is a consequence of abundance, it is also impacted by biomass, activity, surface area, condition, and distance from the physical sample, primer bias, and species-specific variation in the genome.

Results

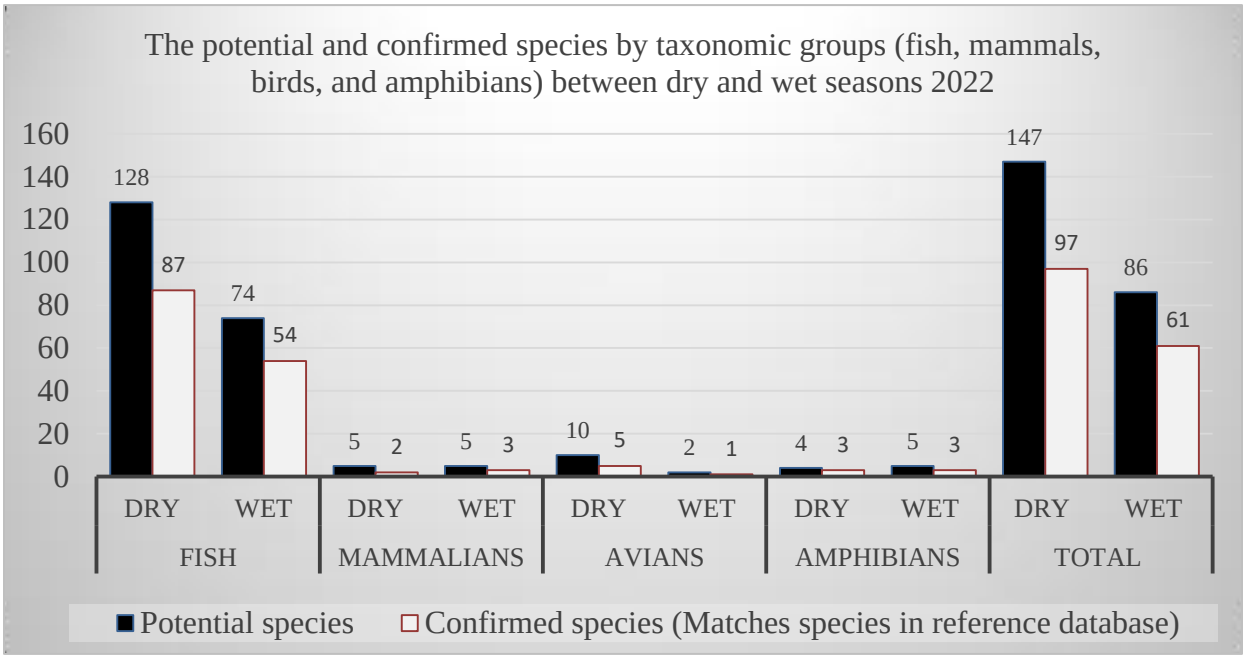
Overall species presence

The total of 147 potential species was detected from the analysis of the dry season water samples. This included 128 fish species, 10 avian species, five mammalian species and four amphibian species. Of the 147 potential species, 97 species were at least 99% similar to a species

in the global eDNA references databases. These 97 species included 87 fish species, two mammals, five birds, and three amphibians.

The wet season samples confirmed the presence of 86 potential taxa. This included 74 fish species, two bird species, five mammal species and five amphibian species. Of the 86 potential species, 61 species were at least 99% similar to a species in the global eDNA references databases. This included 54 fish species, three mammals, three amphibians and one bird species.

Figure 1: The potential and confirmed species by taxonomic groups of fish, mammalian, avian and amphibians between dry and wet season survey 2022 in the Mekong River



The average taxonomic richness varied between dry and wet seasons. The dry season samples resulted in taxonomic richness at 52.6 (ranging 29 to 70), significantly higher than taxonomic richness 14.89 (ranging 9 to 24) of wet season samples. The most abundant sequence found within the dry season was Notched mud carp (*Henicorhynchus lobatus*) while the most abundant sequence within the wet season was Java barb (*Barbonymus gonionotus*).

Mammalian Species

A total five potential mammal taxa were recorded by both sampling seasons, two species were confirmed included Irrawaddy dolphin *Orcaella brevirostris* (Critically Endangered), and Indochinese silvered langur *Trachypithecus germaini* (Endangered). Irrawaddy dolphin was confirmed at the two-sampling site (Kampi and Tboang Khlar), and Silvered langur was detected at Koh Santuk sampling site. The langur inhabits primarily lowland habitats, feeding on tree

leaves. Langur DNA likely entered the Mekong through runoff from upstream riparian forests along the Mekong River.

Otter species, such as the Smooth-coated otter (*Lutrogale perspicillata*-Vulnerable), likely still exist within the project site; however, sampling results did not detect DNA from an otter species.

Avian Species

A total of ten potential avian taxa were recorded from both sampling surveys, six species were confirmed.

During the wet season sampling survey, the Green backed heron (*Butorides striata)* was the only confirmed species. Five avian species were confirmed during the dry season survey including the white-breasted waterhen (*Amaurornis phoenicurus),* common Moorhen (*Gallinula chloropus*), Chinese pond-heron (*Ardeola bacchus*), brown hawk-owl (*Ninox scutulata*) and the Indian cormorant (*Phalacrocorax fuscicollis*). All species are listed as Least Concern on the IUCN's Red List.

The project area is located in the Key Biodiversity Area (KBA) from Kratie to Lao PDR and contains a significance of avian diversity. These species are strongly associated to the wetland that potentially their DNA materials would be collected during samples, for instance River tern (*Sterna aurantia,* VU), River Lapwing (*Vanellus duvaucelii*, NT), Small Pratincole (*Glareola lacteal*, LC), Little Cormorant (*Microcarbo niger*, LC), and Heron species (Jocqué, 2018) and (BirdLife International, 2023).

Reptilian Species

No reptilian species were recorded in either the wet or dry season eDNA surveys. In 2007 study, 40 species of reptiles were captured. A majority of these species do not live in the river but rather the adjacent terrestrial and wetland areas. The lack of reptiles in the e-DNA survey might be attributed to the sample site location and the lack of terrestrial sampling sites.

Amphibian Species

A total of seven amphibian taxa were accounted, four confirmed species of amphibians were recorded identified from both the wet and dry season surveys. All species are classified as Least Concern on the IUCN's Red list, including the Asian grass frog (*Fejervarya limnocharis*) and East Asian bullfrog (*Hoplobatrachus rugulosus*), Mortensen's frog **(***Sylvirana mortenseni*), Asian common toad **(***Duttaphrynus melanostictus*). The Asian grass frog and East Asian Bullfrog have a wide distribution and are common inhabitants of agricultural fields. These species are also a popular food source for local communities (Neang, T., 2010).

Historically, 72 amphibian species have been recorded in the Kingdom of Cambodia. The population status and ecological data for these species remain largely unknown (Poyarkov et al., 2021).

Fishes

A total of 128 potential fish taxa were recorded with 87 confirmed fish species from the dry season samples while 74 potential fish taxa with 54 confirmed fish species resulted from the wet season samples.

A total of nine fish species, listed as threatened by IUCN's Red List, were recorded during both eDNA surveys. These included one Endangered (EN), seven Vulnerable (VU), and one Near Threatened (NT) species. Seven fish species were documented as Data Deficient (DD) (see table below). One species was identified as invasive non-native species.

DNA from the genus *Schistura*, which contains numerous ASAP priority species, was detected, but could not be identified to species level.

Tetraodontidae *(*Pufferfish*)*

Categorised as Least Concern in the IUCN's Red List, *Pao abei* was one of the species in freshwater Pufferfish (Family Tetraodontiae) confirmed across five sampling sites in dry season and the Koh Santuk sampling site in wet season. It is distributed within the Meklong, Chao Phraya and Mekong basins.

Nemacheilidae (Stone loaches)

Several unique DNA detections of the family Nemacheilidae were made during the surveys. One was identified as belonging in genus *Nemacheilidae* and three to the genus *Schistura*. These records could potentially be very significant as many species in the genus *Schistura* are poorly known, and those that are known, are considered Critically Endangered by IUCN. Two detections were identified to species level - *Schistura fasciolata* and *Nemacheilus platiceps.* These species are listed as Data Deficient by the IUCN Red List. *Schistura fasciolata* was found during the dry season at Koh Pdao and the Stung Treng's Ramsar Site while *Nemacheilus platiceps* was found in the wet season in Koh Santuk.

Due to lack of data on Mekong fish species, population status and range, only the species *Nemacheilus platiceps* has documented distribution within Thai, Lao PDR, Cambodia and Vietnam (Juffe Bignoli, D. 2012).

Notopteridae (Featherback)

Three species of family Notopteridae (featherback) were confirmed from the Mekong River's samples. These species included the Indochinese featherback (*Chitala blanci*, NT) in wet season

survey, Ocellated Featherback (*Chitala ornate*, LC) from both survey seasons and the bronze featherback (*Notopterus notopterus*, LC) in dry season survey. The Indochinese featherback is endemic to the Mekong basin, and is found in Lao PDR, Thailand, Vietnam, and Cambodia (Vidthayanon, C. 2011).

Pangasiidae (Shark catfish)

Five potential taxa were recorded and four species of family Pangasiidae were confirmed as present in the study site. These included the Iridescent shark catfish (*Pangasianodon hypophthalmus* - Endangered), spot pangasius (*Pangasius larnaudii* – Least Concern), Trey chhwiet (*Pangasius macronema*, Least Concern), and *Pseudolais pleurotaeni* (**LC)**.

Even though *P. hypophthalmus* is listed as Endangered by the IUCN's Red list, its current distribution includes the Mekong River, Chao Phraya River, as well as large portions of the Mekong basins. Records of this species in the Mekong, could be attributed to its introduction into the river basins for aquaculture (Vidthayanon, C. & Hogan, Z. 2011).

Toxitidae (Archerfish)

Spotted archerfish *Toxotes chatareus* (Least Concern) was confirmed by the dry season survey at the Stung Treng's Ramsar Site. The species occurs mainly in brackish mangrove estuaries, and as well as freshwater rivers and small streams (Froese, R and Pauly, D. 2023).

Datnioididae

The Indonesian tiger perch (*Datnioides microlepis*) listed as Least Concern by the IUCN Red List. The population status of the Indonesian tiger perch is poorly known throughout its range. The species was recorded in the Koh Santuk sampling site during dry season (Ahmad, A.B. 2020).

Siamese tiger perch (*Datnioides pulcher*, Critically Endangered) has been reported significantly declined across its range within Indo-china and it was distributed in the Cambodia's Mekong River and potentially occurred within the sampling areas.

Goonch, *Bagarius yarelli*

The presence of goonch, *Bagarius yarelli*, was determined from the eDNA survey. *Bagarius yarelli* occurs in the Indian subcontinent, while three different Bagarius species are recognised in the Mekong. The Mekong species are not included in the eDNA reference database GenBank, and therefore we cannot be certain which of the Bagarius species our survey detected.

Invasive non-native species (INNS)

Cichlidae

Nile tilapia (*Oreochromis niloticus)* is one of the other *Oreochromis* known as an Invasive non-native species and it was confirmed by the wet season survey across the five sampling sites.

Table 2: The threatened species, ASAP species and Data Deficient species found within the samples.

Taxa	**Common Name**	**Scientific Species**	**Threatened/ DD**	**Confirmed**	
				Dry Season	**Wet Season**
Mammals	Irrawaddy dolphin	*Orcaella brevirostris*	CR	Yes	Yes
	Indochinese silvered langur	*Trachypithecus germaini*	EN	Yes	
Fish	Iridescent shark catfish	*Pangasianodon hypophthalmus*	EN		Yes
	Goonch	*Bagarius yarrelli*	VU	Yes*	
	Small scale mud carp	*Cirrhinus microlepis*	VU	Yes	Yes
		Labeo pierrei	VU	Yes	
	Elephant ear gourami	*Osphronemus exodon*	VU	Yes	
	Bandan sharp-mouth barb	*Scaphognathops bandanensis*	VU	Yes	Yes
	Mekong herring	*Tenualosa thibaudeaui*	VU	Yes	Yes
	Wallago	*Wallago attu*	VU	Yes	
	Royal knife	*Chitala blanci*	NT		Yes
		Dermogenys pusilla	DD	Yes	
	Giant Sharkminnow	*Osteochilus schlegelii*	DD	Yes	Yes
		Sikukia gudgeri	DD	Yes	Yes

		Schistura fasciolata	DD	Yes	
	Small scale Croaker	*Boesemania microlepis*	DD	Yes	
		Kryptopterus cheveyi	DD	Yes	
		Nemacheilus platiceps	DD		Yes

Discussion

Detection and monitoring of aquatic biodiversity has been a long-standing challenge for conservationists. Monitoring using eDNA is a relatively new tool that has proved valuable for monitoring aquatic and some terrestrial species. The two discrete eDNA Mekong biodiversity surveys during both the dry and wet seasons in 2022 resulted a total of 147 potential taxa in the dry season, of which 97 were identified to species level and 86 potential taxa in the wet season, of which 61 were identified to species level.

The dry season survey documented 128 fish species, four amphibian species, 10 avian species and five mammalian species while the wet season documented 74 fish species, 2 bird species, five mammalian and five amphibian species.

The eDNA survey results weren't confirmed any reptilian species and few species of the water bird and amphibian species. Within the study areas, this technique would not be viable to account these taxa. The direct observation technique for these taxa is most appropriate.

eDNA surveys have been conducted in the Mekong before. A similar study using eDNA was conducted across the Mekong River and Mekong Delta along 1,500km distance, from Vietnam to Thailand and Lao, with 15 sampling sites (Durand et al., 2022). A total of 287 OTU were recovered, of which 158 were identified to species level using reference sequences available in GenBank. Previously, WWF-Greater Mekong Programme partnered with France-based laboratory SPYGEN piloting eDNA monitoring in 2015 along the Mekong River from Tonle Sap Great Lake in central Cambodia, through Lao PDR to the Chiang Kong in northern Thailand (Grey. T.N.E, 2015). As a result, 175 species was detected with only 64 known fish species while the other 111 unidentified taxa not yet sampled by geneticists.

A large scale survey effort using conventional sampling, including fish capture and visiting to large urban markets, villages and fish traders for fishes, trapping methods for amphibian and reptile, and direct observation for birds within the Mekong River channel, principally in the

"central section" in 2006 and 2007. A study was conducted to inventory all taxa at the same stretch of the Mekong River.

The results of this survey documented 38 avian species, 8 mammalian species, 55 amphibian and reptilian species and 223 fish species (Bezuijen et al., 2008).

The species were classified based on conservation needs, including Very High Priority (VHP), High to Medium Priorities (HP &(MP).

White shouldered ibis, River tern, Woolly-necked stork, Lesser Adjutants are the focal conservation species, rated as VHP. Fish eagles, vultures, Owls, Ducks and Pigeons are ranked as high to medium priorities (HP) & (MP).

Among the eight mammalian species, silvered Indochinese langur, long-tailed macaque, Eld's deer, large flying-fox, two otter species, as well as sambar are HP & MP.

The 55 amphibian and reptile species recorded are all the common species, consisting of 16 frogs, 16 snakes, 17 lizards, six turtles.

Toxabramis species (a minnow) is a new national record of fish species in Cambodia.

Threatened, IUCN ASAP and Target Species

During the surveys, three threatened and, globally significant focal species were detected namely, Irrawaddy dolphin (CR), Indochinese silvered langur *(Trachypithecus germaini,* EN*),* Iridescent shark catfish *(Pangasianodon hypophthalmus,* EN*).* Seven vulnerable fish species were also confirmed, as well as the near threatened Royal knife *Chitala blanci.*

Neither of the eDNA studies detected focal species on the IUCN, ASAP or Cambodia's threatened species list which have been caught by local fishermen in the past. These threatened and endangered include the Siamese tiger perch (*Datnoides pulcher),* Bala shark minnow (*Balantiocheilos melanopterus),* Mekong giant catfish (*Pangasianodom gigas),* Giant barb (*Catlocarpio siamensis),* Isok barb (*Probarbus jullieni),* Thicklip barb (*Probarbus labeamajor),* Thinlip barb (*Probarbus labeaminor)* and Cantor's Asian giant soft-shell turtle (*Pelochelys cantorii).*

Invasive and Non-Native Species

The country list for Cambodia has recorded these fish as exotic species, including Bighead carp (*Hypophthalmichthys nobilis*), Silver carp (*Hypophthalmichthys molitrix*), North African catfish (*Clarias gariepinus*), Pangas catfish (*Pangasius pangasius*), Grass carp (*Ctenopharyngodon*

idella), Common carp (*Clarias gariepinus*), Roho labeo (*Labeo rohita*), and Mrigal carp (*Cirrhinus cirrhosus*). However, from five sampling sites within the 217-km stretch of the Mekong River were not able to collect the DNA materials from these species. To get results that accurately depict the faunistic biodiversity in this stretch of the Mekong River, additional sample sites for the random collection of water samples will be required to expand e-DNA sampling to other important portions of the study site.

The invasive and non-native species detected in the eDNA survey included only Nile tilapia (*Oreochromis niloticus)* in the wet season survey. However, another species in the genus *Oreochromis* - the Mozambique tilapia (*Oreochromis mossambicus),* as well as the Pirapatinga *(Piaractus brachypomus)* have been reportedly been caught by local fishers.

Conclusion and recommendations

Despite the current results of the eDNA biodiversity study within the Mekong River was limited, the approach is potential to monitor the aquatic diversity with ecological area with modifying the technique and improving the global database. The more eDNA surveys are being implemented, the expectation is better described the species and ecological niches of different species with improvement of the Mekong barcoding and more accurate species' assignment.

This study was also significant contribution to the first introduction of local based science approach, of which local fishermen were involved and learned the new technique and gained the knowledge and monitoring of the species diversity within their areas.

In addition, it has influenced the current management and conservation activities of the Irrawaddy dolphins. The Ministry of Agriculture, Forestry and Fisheries plans to demarcate the boundaries of both the dolphin core and buffer zones of *t*he Mekong Dolphin Management and Protection Zone, in which the water deep pools which contain the five sampling sites are located. Protection of those water deep pools means increased fish populations, increasing the amount of prey available for the Irrawaddy dolphins to have sufficient prey for food.

A long-term species monitoring program of the biodiversity in the Mekong River in the areas surrounding the deep water pools, is essential for tracking the population trends of threatened and endangered species as well as economically significant species, especially fish species.

The biodiversity the Mekong River is not limited to the species living in the river. The biodiversity of the Mekong contains a diversity of freshwater fish species, various species of water birds, as well as both freshwater and terrestrial mammals, amphibians and reptiles.

The results of this study recorded limited a number of species compared to the similar study covering the larger areas with greater sampling sites (Durand et al., 2022). This was attributed by

a limited sampling effort and likely limited a reference sequences in GenBank. An increase sampling effort is essential to cover various habitats and different water columns of the river (surface and bottom), of which different target taxonomic groups could be improving detection. In addition, improving the reference sequences in the GenBank is also greatly contributed to identify the species level and especially monitor the target, threatened and ASAP species.

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The sampling technique followed by NatureMetrics's protocol was pumped the river water and filtered eDNA materials. During dry season sampling was useful due to the low water turbidity obtaining high DNA material's index for PCR process. In contrast, during rainy season sampling, the river water turbidity was high causing difficulty of filter process and obtaining less volume of water with low DNA material's index. The future sampling techniques focus on the river flow sites such as the Mekong River, it is considered to take a filtration process to remove particles before filtering the eDNA materials into specimen tube.

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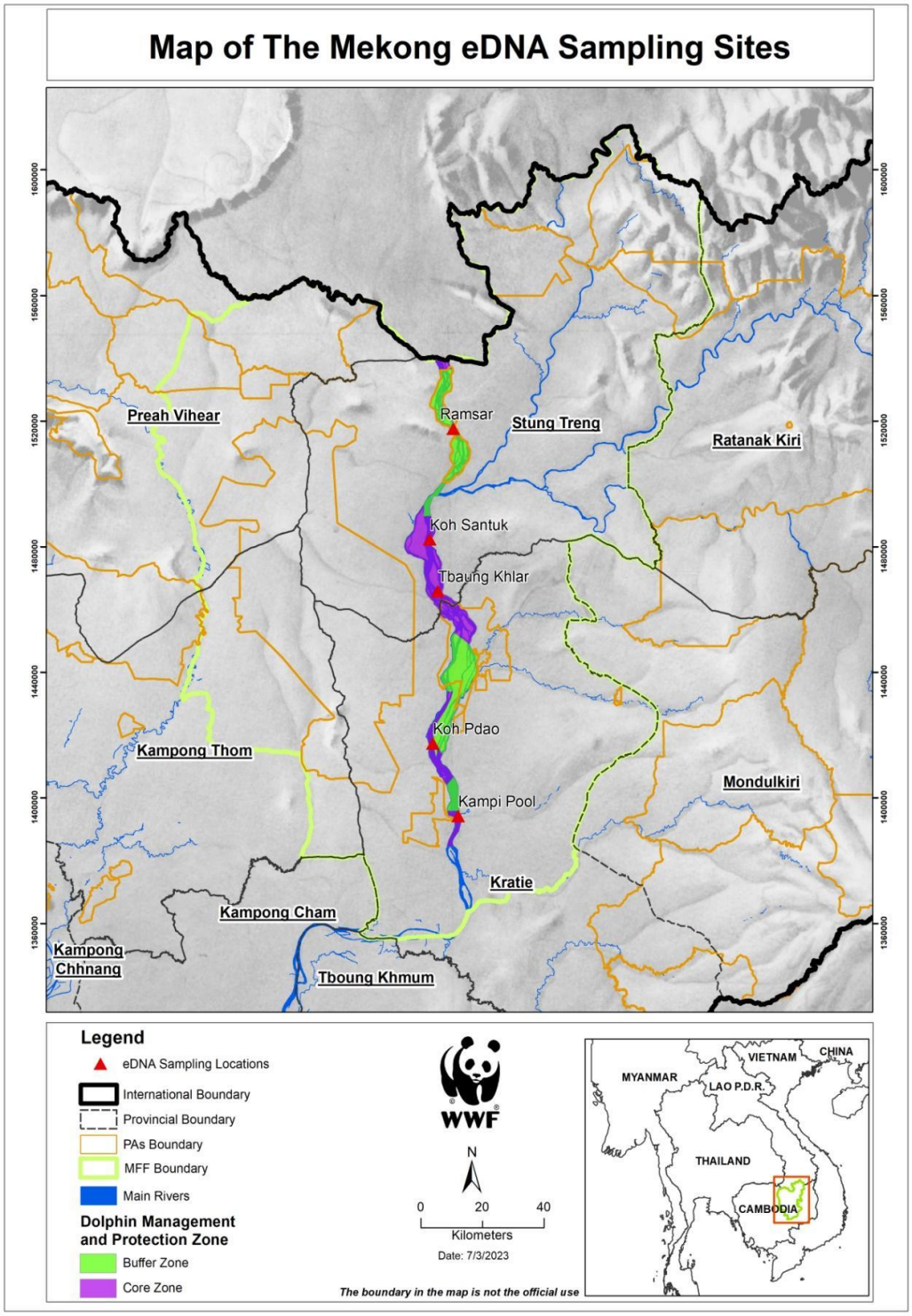
Appendixes

Figure 1. Map of the Mekong Dolphin Management and Protection Zone, the Stung Treng Ramsa Site and five sampling sites



Appendix 2:

Table 1: Summary of swab barcoding results from dry season survey in March 2022

No	Swab ID	Kit ID	Sequence	Taxon present in Genbank	Most similar taxon in Genbank	% Similarity	Sequence detected in Metabarcoding Data
1	*Labeo chrysophekadion*	Kit-7775110017000225777	ACTATGCTCAGCC…	YES	*Labeo chrysophekadion*	100	YES
2	*Kryptopterus geminus*	Kit-7775110017000226777	ACTATGCTCAGCC…	NO	*Heteropneustes fossilis*	99	YES
3	*Yasuhikotakia modesta*	Kit-7775110017000227777	ACTATGCTCAGCC…	YES	*Yasuhikotakia modesta*	100	YES

Table 2: Summary of swab barcoding results from wet season survey in July 2022

No	Swab ID	Kit ID	Sequence	Taxon present in Genbank	Most similar taxon in Genbank	% Similarity	Sequence detected in Metabarcoding Data
1	*Labeo chrysophekadion*	Kit-7775110017000225777	ACTATGCTCAGCC..	Yes	*Labeo chrysophekadion*	100	No
2	*Kryptopterus geminus*	Kit-7775110017000226777	ACTATGCTTAGCC…	No	*Heteropneustes fossilis*	99	No
3	*Yasuhikotakia modesta*	Kit-7775110017000227777	ACTATGCTCAGTCG…	Yes	*Yasuhikotakia modesta*	100	Yes
4	*Cynoglossum microlepis*	Kit-7775110017000229777	ACTATGCCTAGCTG…	No	*Cynoglossus trigrammus*	100	No
5	*Puntioplites falcifer*	Kit-7775110017000231777	ACTATGCTCAGCC…	No	*Semilabeo obscurus*	100	No
6	*Amblyrhynchichthys micracanthus*	Kit-7775110017000232777	ACTATGCTCAGCC…	No	*Amblyrhynchichthys truncatus*	100	Yes
7	*Mastacembelus armatus*	Kit-7775110017000228777	-	-	-	-	
8	*Hemiarius stormii*	Kit-7775110017000233777	-	-	-	-	

Table 3. The volume of water filtered and the resultant concentration of purified DNA and index PCRs from the samples collected during dry season survey in March 2022

No	Kit ID	Label	Volume filtered	Date received	DNA (ng/µl)	Index (ng/µl)
1	Kit-7771120017001856777	Kampi 01	1000ml	20-Apr-22	2.3	2.3
2	Kit-7771120017001843777	Kampi 02	2000ml	20-Apr-22	6.08	4.04
3	Kit-7771120017001825777	Kampi 03	2000ml	20-Apr-22	7.65	4
4	Kit-7771120017001852777	Kampi 04	2000ml	20-Apr-22	5.14	4.69
5	Kit-7771120017001839777	Koh Pdao 01	2000ml	20-Apr-22	21	3.48
6	Kit-7771120017001847777	Koh Pdao 02	2000ml	20-Apr-22	23.3	3.88
7	Kit-7771120017001850777	Koh Pdao 03	2000ml	20-Apr-22	9.35	2.97
8	Kit-7771120017001853777	Koh Pdao 04	2000ml	20-Apr-22	6.87	3.13
9	Kit-7771120017001845777	Koh San Tuk 01	3000ml	20-Apr-22	31.5	3.76
10	Kit-7771120017001846777	Koh San Tuk 02	2700ml	20-Apr-22	13.5	3.97
11	Kit-7771120017001835777	Koh San Tuk 03	3000ml	20-Apr-22	16.4	4.81
12	Kit-7771120017001844777	Ramsar 01	3000ml	20-Apr-22	2.6	5.56
13	Kit-7771120017001849777	Ramsar 02	3000ml	20-Apr-22	34.6	3.79
14	Kit-7771120017001841777	Ramsar 03	3000ml	20-Apr-22	7.65	3.09
15	Kit-7771120017001842777	Tbong Kla 01	3500ml	20-Apr-22	11.3	3.35
16	Kit-7771120017001837777	Tbong Kla 02	3500ml	20-Apr-22	22.4	3.68
17	Kit-7771120017001836777	Tbong Kla 03	2700ml	20-Apr-22	25.6	3.7
18	Kit-7771120017001828777	Tbong Kla 04	2700ml	20-Apr-22	19.9	-
19	Kit-7771120017001848777	Negative Control	2000ml	20-Apr-22	<0.01	3.05

Table 4: The volume of water filtered from the samples collected during wet season survey in July 2022

No	Kit ID	Sample ID	Volume filtered	Date received
1	Kit-7771120017001821777	Kampi 01	700ml	14-Sep-22
2	Kit-7771120017001851777	Kampi 02	700ml	14-Sep-22
3	Kit-7771120017001854777	Kampi 03	700ml	14-Sep-22
4	Kit-7771120017001855777	Kampi 04	700ml	14-Sep-22
5	Kit-7771120017001831777	Koh Pddao 01	700ml	14-Sep-22
6	Kit-7771120017001834777	Koh Pddao 02	700ml	14-Sep-22
7	Kit-7771120017001824777	Koh Pddao 03	700ml	14-Sep-22
8	Kit-7771120017001822777	Koh Pddao 04	800ml	14-Sep-22
9	Kit-7771120017001840777	Koh San Tuk 01	700ml	14-Sep-22
10	Kit-7771120017001819777	Koh San Tuk 02	700ml	14-Sep-22
11	Kit-7771120017001833777	Koh San Tuk 03	700ml	14-Sep-22
12	Kit-7771120017001832777	Ramsa 01	700ml	14-Sep-22
13	Kit-7771120017001826777	Ramsa 02	800ml	14-Sep-22
14	Kit-7771120017001823777	Ramsa 03	900ml	14-Sep-22
15	Kit-7771120017001820777	Tbong Kla 01	900ml	14-Sep-22
16	Kit-7771120017001838777	Tbong Kla 02	800ml	14-Sep-22
17	Kit-7771120017001830777	Tbong Kla 03	700ml	14-Sep-22
18	Kit-7771120017001827777	Tbong Kla 04	700ml	14-Sep-22
19	Kit-7771120017001829777	Negative Control	1000ml	14-Sep-22

Appendix 3:

2- Elephant ear gourami *Osphronemus exodon*, Vulnerable

Elephant ear gourami *Osphronemus exodon*, Vulnerable @ Pha Nem/WWF-Cambodia, confirmed by eDNA study

3- Small scale mud carp *Cirrhinus microlepis,* Vulnerable

A Small scale mud carp *Cirrhinus microlepis,* Vulnerable confirmed by the eDA study @Eam Sam Un/WWF-Cambodia

4- Isok barb *Probarbus jullieni*, Critically Endangered

Isok barb *Probarbus jullieni*, Critically Endangered did not confirmed by this study (photo was taken from local market) @ Eam Sam Un/WWF-Cambodia

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