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Trails of river monsters: Detecting critically endangered Mekong giant catfish *Pangasianodon gigas* using environmental DNA

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HIGHLIGHTS

- Species specific assays were developed for IUCN CR Mekong giant catfish.
- eDNA potential to detect threatened biodiversity in complex tropical environments.
- Accounting for false-absences critical for eDNA survey of poorly known species.

ARTICLE INFO

ABSTRACT

Pressures on freshwater biodiversity in Southeast Asia are accelerating, yet the status and conservation needs of many of the region's iconic fish species are poorly known. The Mekong is highly species diverse and supports four of the six largest freshwater fish globally, three of which, including Mekong giant catfish (*Pangasianodon gigas*), are Critically Endangered. Emerging environmental DNA (eDNA) techniques have potential for monitoring threatened freshwater biodiversity, yet have not been applied in complex and biodiverse tropical ecosystems such as the Mekong. We developed species-specific primers for amplifying Mekong giant catfish DNA. *In situ* validation demonstrated that the DNA amplification was successful for all samples taken in reservoirs with known presence of Mekong giant catfish independent of fish density. We collected water samples from six deep pools on the Mekong, identified through Local Ecological Knowledge, in Cambodia, Lao PDR, and Thailand. DNA was extracted and amplified from these samples using the designed primers and probes. Mekong giant catfish DNA was detected from one sample from the species' presumed spawning grounds on the Mekong mainstream, near the border between northern Thailand and Lao PDR. eDNA sampling using species-specific primers has potential for surveying and monitoring poorly known species from complex tropical

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aquatic environments. However accounting for false absences is likely to be required for the method to function with precision when applied to extremely rare species that are highly dispersed within a large river system. We recommend that such approach be utilised more widely by freshwater conservation practitioners for specific applications. The method is best suited for baseline biodiversity assessments or to identify and prioritise locations for more rigorous sampling. Our methods are particularly relevant for systems or species with limited baseline data or with physical characteristics that logistically limit the application of conventional methods. Such attributes are typical of large tropical rivers such as the Mekong, Congo, or Amazon.

1. Introduction

Southeast Asia is facing a biodiversity crisis driven by unprecedented rates of growth in populations, economies, and per-capita consumption (Sodhi et al., 2010). This has led to a higher concentration of threatened terrestrial reptile, bird and mammal species than any other region globally (Sodhi et al., 2010). Patterns of freshwater biodiversity and endangerment in tropical Asia are less well known, but freshwater ecosystems often support a higher proportion of threatened species than marine or terrestrial equivalents (Loh et al., 2005; Revenga et al., 2005; Dudgeon et al., 2006; Strayer and Dudgeon, 2010). Given that trends in water use, energy production and consumption, and associated environmental degradation are projected to continue rising across Southeast Asia, understanding and monitoring the status of freshwater biodiversity is critical.

The accelerating pressures on freshwater ecosystems in tropical Asia are encapsulated by the Mekong; the largest river in Asia and ranked amongst the top three rivers globally in terms of fish diversity (Campbell, 2009). Large stretches of the lower Mekong Basin (comprising Myanmar, Thailand, Lao PDR, Cambodia, and Vietnam) remain relatively pristine, but could be irreversibly altered by planned infrastructure developments. Many Mekong fish species are also currently impacted by heavy exploitation, particularly large-bodied and migratory species (Allan et al., 2005). The Mekong supports a unique assemblage of freshwater megafauna (species > 90-kg and >180-cm long) including four of the six largest freshwater species globally (Hogan, 2011). Three of these species: Mekong giant catfish (*Pangasianodon gigas*), giant carp (*Catlocarpio siamensis*), and dog-eating catfish (*Pangasius sanitwongsei*), are assessed as Critically Endangered by the IUCN Red List, with declines driven primarily by heavy fishing pressure and habitat loss or degradation (Jenkins et al., 2009; Hogan, 2013a,b). The Mekong giant catfish is described as a contemporary example of overharvest (Allan et al., 2005).

Despite their notable role in regional culture, economies, and food security, knowledge of the current status and distribution of many charismatic flagships of Mekong freshwater biodiversity, including the Mekong giant catfish, is limited. This paucity of knowledge largely results from of a lack of effective survey and monitoring techniques. Because of population declines and associated fishing bans, the majority of recent (post-2005) records of Mekong giant catfish occurrence are from incidental by-catch by fishermen in Cambodia, Lao PDR, and Vietnam (e.g. Hogan et al., 2004). There is thus an urgent need for effective survey methods for the species in order to both monitor distribution trends and to identify priority sites for conservation interventions.

Advances in molecular biology have provided means to monitor single species, or to assess community biodiversity, from aquatic or terrestrial environments using environmental DNA (eDNA), i.e. DNA left behind by the organism in the environment. This approach has demonstrated potential to detect rare and cryptic species, with examples in the literature rapidly growing, and has been validated on different taxonomic groups (including vertebrates and invertebrates) and different ecosystems (Dejean et al., 2012; Taberlet et al., 2012; Machler et al., 2014; Biggs et al., 2015; Thomsen and Willerslev, 2015). eDNA sampling of rivers in North America has been shown to enable cost-effective detection of fishes occurring at low densities, such as along invasion fronts (Jerde et al., 2011; Goldberg et al., 2015). These methods are opening new perspectives for improving environmental monitoring and informing management and policy (Kelly et al., 2014).

The majority of published applications of eDNA sampling have occurred in controlled conditions and/or in temperate environments with low species diversity (Thomsen and Willerslev, 2015). The applicability of eDNA sampling for effective conservation management in the biodiverse tropics is less clear. To test the efficacy of eDNA sampling for detecting threatened tropical species in a challenging environment, we developed, and independently verified, specific assays (primers and probes) for Mekong giant catfish. These assays were then applied to water samples collected in reservoirs with confirmed presence of Mekong giant catfish and from the Mekong river across the known range of the species.

2. Materials and methods

2.1. Design, in silico and in vitro validation of an assay for Mekong giant catfish

We designed species-specific primers and a probe for amplification of Mekong giant catfish DNA. All available cytochrome b sequences for the Mekong giant catfish and other species from the Pangasiidae family 16 out of the 28 species from the

Table 1

Number of samples with positive detection of Mekong giant catfish from aquaculture ponds in Thailand during In situ validation of species-specific assay. Densities of catfish in each reservoir defined as follows: **High**: ± 12 MGC – wild individuals captured in the Mekong at least 20 years ago ("the parents") (40–50 kg weight), in a pond of 3200 m^2 with a 2–3 m depth; **Medium**: ± 40 large MGC (40–60 kg) – some up to 20 years old + about 100 smaller MGC of less than 10 kg (F1, second generation bred from wild parents), in a pond of 24, 000 m^2 with a 4 m depth; **Low**: ± 10 MGC – 25 cm length (1–2 kg) in a pond of 6400 m^2 with a 2–3 m depth.

Sample name	Density of Mekong giant catfish in the reservoir	Number of positive replicates
Res1 n°1	Medium	12/12
Res1 n°2	Medium	12/12
Res1 n°3	Medium	12/12
Res2 n°4	Low	12/12
Res2 n°5	Low	12/12
Res2 n°6	Low	12/12
Res3 n°7	High	12/12
Res3 n°8	High	12/12
Negative	Species absent	0/12

genera *Heicophagus, Pangasianodon, Pangasius and Pseudolais* (FishBase, 2016), were retrieved from GenBank® and then aligned using Geneious® software (version R6, http://www.geneious.com, Kearse et al., 2012). The PrimerQuest® program (IDT, Coralville, USA. Retrieved 12 December, 2012. http://www.idtdna.com/Scitools) was used to design primers and probes. The designed primers were first validated *in silico* by assessing their specificity using the ecoPCR program (Bellemain et al., 2010; Ficetola et al., 2010) on the EMBL-Bank release 114 (released in December 2012). An *in vitro* validation was then undertaken using real time PCR upon 10 Mekong giant catfish DNA samples, extracted from captive fish in Thailand. The limit of detection (LOD, i.e. the minimum amount of target DNA sequence that can be detected in the sample) was calculated by running a dilution series of a known amount of DNA, ranging from 10^{-1} ng/μL to 10^{-10} ng/μL. The real time amplification (qPCR) was performed in 12 replicates in a final volume of 25 μL, using 3 μL of template DNA, 12.5 μL of TaqMan Environmental Master Mix 2.0 (Life Technologies®), 6.5 μL of ddH2O, 1 μL of Forward primer (Pgigas_cytb_F: CTAACCTGGATTGGTGGCAT, 10 μM), 1 μL of Reverse primer (Pgigas_cytb_R: AAGAAGAGGAAGTACAAGATGGAG, 10 μM) and 1 μL of probe (Pgigas_cytb_Pr: CCAATAATGATGAATGGATGTTCGACTGGC, 2.5 μM) under thermal cycling 50 °C for 5 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 30 s. and 58.8 °C for 1 min. qPCR negative controls (with 12 replicates as well) were performed in parallel to detect potential contamination. Samples were run on a BIO-RAD® CFX96 Touch real time PCR detection system in a room dedicated to amplified DNA analysis with negative air pressure and physically separated from the DNA extraction room.

2.2. In situ validation of Mekong giant catfish assay

In situ validation of Mekong giant catfish specific assay was undertaken using eight water filtration samples collected from ponds of the Srichiangmai Fisheries Research Centre in Nong Khai Province, Thailand, which contained captive Mekong giant catfish at different densities, from "low" to "high" (Table 1).

Sampling was performed from the shore using, for each sample, a filtration capsule (Envirochek HV® 1 μm, Pall Corporation, Ann Arbor, MI, USA), sterile tubing and joints and a portable peristaltic pump (Vampire Sampler, Bürkle GmbH®, Germany) with a flow of 1.67 L/min. For each sampling, 10 litres were filtered near the surface (maximum depth 40 cm). After filtration, 125 mL of ethanol was added to each capsule, and the capsules were kept refrigerated at 4 °C for a few days until sent to the laboratory. The transport to the laboratory took 4 days. DNA extraction was conducted, following a modified protocol from Valentini et al. (2016), in a room dedicated to processing water samples equipped with positive air pressure, UV treatment and frequent air renewal. Laboratory personnel wore full protective clothing (disposable coveralls, hood, mask, laboratory-specific shoes, overshoes and two pairs of gloves) that was put on in an airlock foyer before entering the processing room. Filtration capsules were emptied of the ethanol and filled with 125 mL conservation buffer (Tris–HCl 0.1 M, EDTA 0.1 M, NaCl 0.01 M and N-lauroyl sarcosine 1% with pH 7.5–8). They were then left at 56 °C for 2 h, agitated manually for 5 min and emptied into three 50 mL tubes. In total, approximately 120 mL were retrieved in three tubes that were centrifuged for 15 min at 15,000 g. The supernatant was removed with a sterile pipette, leaving 15 mL of liquid at the bottom of the tube. Subsequently, 33 mL of ethanol and 1.5 mL of 3 M sodium acetate were added to each 50 mL tube. After a quick manual shaking, samples were stored for 24 h at −20 °C. The three subsamples per site were then centrifuged for 30 min at 15,000 g and 6 °C and the supernatant was discarded. After this step, 360 μL of ATL Buffer of the DNeasy Blood & Tissue Extraction Kit (Qiagen) were added in the first tube, the tube was vortexed and the supernatant was transferred to the second tube. This operation was repeated for the third tube. The supernatant in the 3rd tube was transferred in a 2 mL tube and the DNA extraction was performed following the manufacturer's instructions. Two mock extractions were performed in parallel to monitor possible contaminations. Real time DNA amplifications (including mock samples) were carried out in 12 replicates using the specific primers designed in this study for Mekong giant catfish, following the protocol described above. qPCR negative controls (12 replicates) were performed in parallel to detect potential contamination. A dilution series of Mekong giant catfish DNA, ranging from 10-1 ng/μL to 10-4 ng/μL, was used as a qPCR standard. Those standards were

added in the qPCR plate (with the tubes containing the eDNA samples sealed) in a separate room from the one where the eDNA extractions were performed.

2.3. Selection of sampling sites in the Mekong

Mekong giant catfish is endemic to the lower and middle Mekong basin and was historically distributed throughout the Mekong from the coast of Vietnam to northern Lao PDR (Hogan, 2013a). Whilst understanding of the species' migration patterns is incomplete, it is believed to migrate along the Mekong and Tonle Sap River, between spawning sites in northern Thailand and Lao PDR, to nursery grounds in the Tonle Sap Lake, Cambodia. Water sampling was undertaken at six locations in the Mekong between the confluence of the Tonle Sap and Mekong, close to Phnom Penh in Cambodia, to northern Thailand (Fig. 1).

Broad sampling locations were selected based on historic catch records indicating presence of Mekong giant catfish, Local Ecological Knowledge (LEK) on the species' distribution, as well as logistics and accessibility constraints. LEK data was sourced during previous projects for Mekong giant catfish conservation, as well as informal interviews with experienced fishermen. Considering that Mekong giant catfish are primarily a benthopelagic species relying on deep pools as refugia, especially in the dry season when river levels are low (Poulsen et al., 2002), we targeted sampling at deep pools using hydrographic maps produced by the Mekong River Commission combined with informal interviews with senior fishermen who identified specific pools where the species had been captured in the past. (MRC, 1996; Halls et al., 2013). Sampling was performed in April and May 2014, at the end of the dry season, with relatively low river flow compared to the rest of the year.

2.4. eDNA sampling in the Mekong

At each site ($n = 6$, Fig. 1), samples were collected from a boat in vertical series at two points within the pool: first at the downstream lip of the deep pool, and second, at the deepest point in the pool. At each sampling point, an immersion cylinder (Diameter 75 mm, capacity 1L; Bürkle GmbH®, Germany) was used to collect the water at different depths: (1) near-bottom, (2) water column, and (3) surface (Table 2).

The maximum depth at the sampling point was measured with a depth sounder. The near-bottom samples were collected one metre above the substrate. For the mixed water column samples we subtracted two metres from the total depth (so that the sampling covered the column starting 1 m above the substrate up to 1 m below the surface) then divided this depth by 10. One litre of water was collected at each of the 10 depth points along the water column. Before each sampling round, immersion cylinders and buckets were sterilised with 10% chlorine bleach and a new sterile tube was used for each water filtration. At each depth, 10 litres were collected and emptied into a sterile bucket. Immediately after water collection, filtration was performed from the buckets, using the same filtration equipment as described for aquaculture ponds in Thailand. Capsules were then filled with conservation buffer (Tris–HCl 0.1 M, EDTA 0.1 M, NaCl 0.01 M and N-lauroyl sarcosine 1% with pH 7.5–8) and kept on ice in a cooler and then refrigerated at 4 °C until sent to the laboratory. Personnel wore gloves at all times during the sampling process. DNA extraction and amplification were performed following the protocol described in "*In situ* validation of Mekong giant catfish assay" section. In total, 36 water samples were collected, filtered and analysed (30 litres filtered for each sampling point, and 60 litres filtered for each deep pool).

3. Results

3.1. In silico, in vitro and in situ validation of the assay for Mekong giant catfish

The amplified fragment length was 83 bases, which is appropriate for amplification of environmental DNA that is usually degraded (Deagle et al., 2006). The results of the *in silico* test showed that, using this assay, no other species present in the Mekong was amplified. However; four species of *Micropterus* (*M. cataractae, M. notius, M. punctulatus* and *M. treculii*) and one species of *Rineloricaria* (*R. uracantha*), all occurring in the continental United States, could potentially be amplified with 2 or 3 mismatches on each primer. When using the probe in combination with the primers, only Mekong giant catfish is amplified. The *in vitro* tests showed that limit of detection of primers and probe was 5.10^{-8} ng of DNA/µL. The *in situ* tests (from aquaculture ponds containing Mekong giant catfish in Thailand) confirmed the reliability of the assay. For all samples collected in those ponds, the DNA amplification was successful, with all PCR replicates positive independent of fish density in the reservoirs (Table 1). The field, extraction and PCR negative controls did not show any amplification, validating the primer pair and the probe, confirming the purity of the consumables used and the absence of cross contaminations.

3.2. Field survey results

Among the 36 samples analysed over the 6 sites, one sample (site A in Fig. 1; sample A5 in Table 2), corresponding to a water-column sample, showed a positive amplification of Mekong giant catfish, in one replicate out of 12. Extraction and PCR negative controls did not show any amplification.
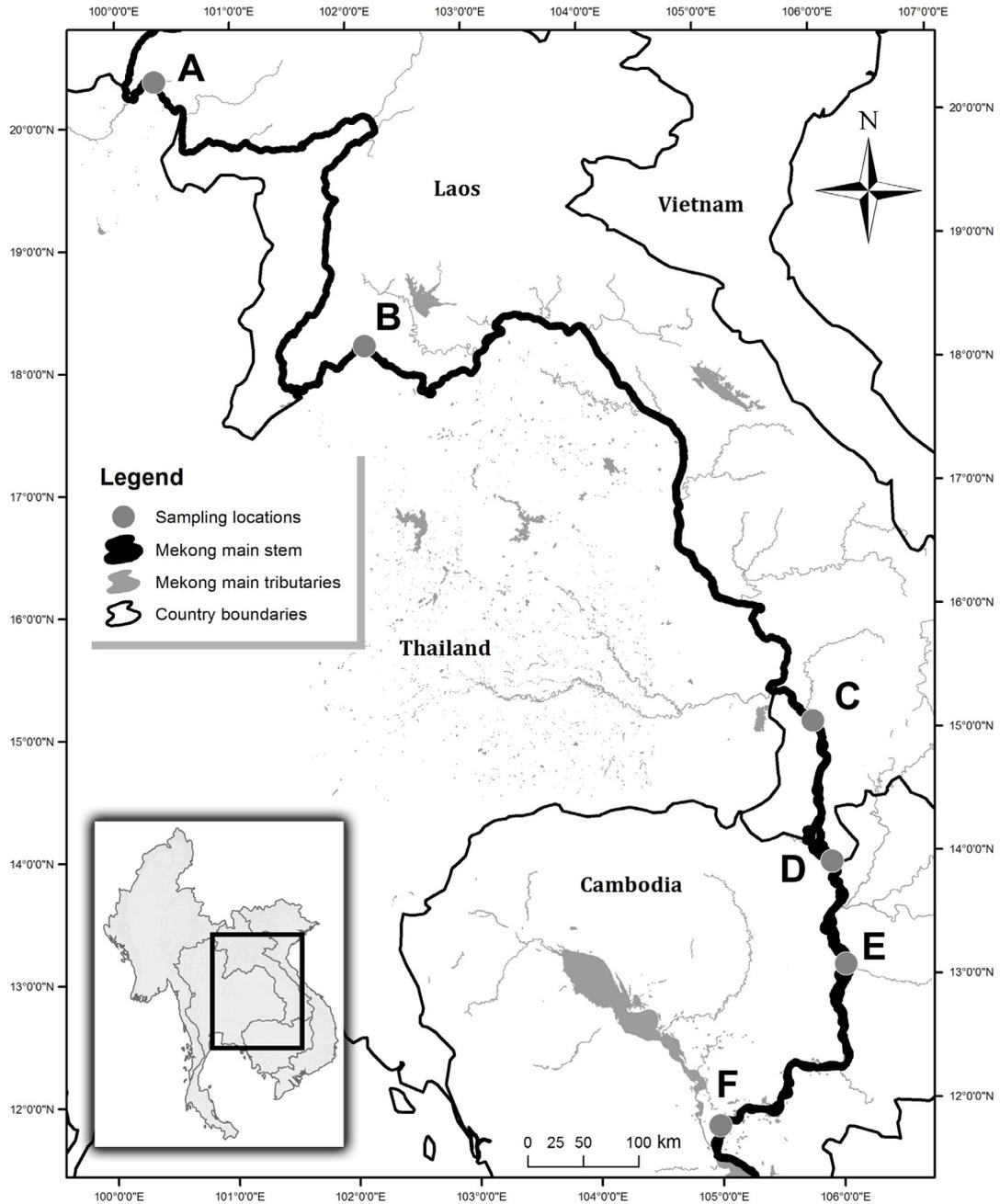
Fig. 1. Sampling sites for Mekong giant catfish along the Mekong River in Cambodia, Lao PDR, and Thailand. More details of each sampling site are given in Table 2.

4. Discussion

Detection and monitoring of elusive and rare species has been a long-standing challenge for conservationists. This challenge is particularly acute in environments such as large tropical rivers, which typically host highly threatened species in low densities and are not amenable to conventional survey methods. Environmental DNA (eDNA) sampling offers an innovative approach to overcome the challenges associated with detecting rare species in freshwater ecosystems (Goldberg et al., 2011). However, to our knowledge, there are no published studies using eDNA sampling in biodiverse tropical freshwater environments to target poorly understood threatened species. While eDNA methods are rapidly gaining popularity, with many publications bolstering the conception of the approach as a "silver bullet" for rare species surveys

Table 2
Details of sampling sites surveyed during field surveys collecting water samples for Mekong giant catfish eDNA analysis on the Mekong river.

Site	Sample number	Site	Location	Date (2014)	GPS N	GPS E	Depth (m)	Type
A	A1	Upstream of Ban Pla Tang		13/05	20′21.892	100′21.434	0	Surface
	A2	Upstream of Ban Pla Tang	Deep pool	13/05	20′21.892	100′21.434	26.65–0.91	Water column
	A3	Upstream of Ban Pla Tang		13/05	20′21.892	100′21.434	29.57	Bottom
	A4	Upstream of Ban Pla Tang		13/05	20′23.111	100′20.652	0	Surface
	A5	Upstream of Ban Pla Tang	Up-stream	13/05	20′23.111	100′20.652	24.08–0.91	Water column
	A6	Upstream of Ban Pla Tang		13/05	20′23.111	100′20.652	24.99	Bottom
B	B1	Nong Pla Beuk		22/04	18′12.716	102′07.625	6.10	Bottom
	B2	Nong Pla Beuk	Up-stream	22/04	18′12.716	102′07.625	5.1–1	Water column
	B3	Nong Pla Beuk		22/04	18′12.716	102′07.625	0	Surface
	B4	Nong Pla Beuk		22/04	18′12.501	102′07.562	28.65	Bottom
	B5	Nong Pla Beuk	Deep pool	22/04	18′12.501	102′07.562	27.65–1	Water column
	B6	Nong Pla Beuk		22/04	18′12.501	102′07.562	0	Surface
C	C1	Pakse		05/05	15′04.485	105′49.851	0	Surface
	C2	Pakse	Up-stream	05/05	15′04.485	105′49.851	17.29–1	Water column
	C3	Pakse		05/05	15′04.485	105′49.851	18.29	Bottom
	C4	Pakse		05/05	15′05.586	105′49.692	0	Surface
	C5	Pakse	Deep pool	05/05	15′05.586	105′49.692	26.43–1	Water column
	C6	Pakse		05/05	15′05.586	105′49.692	27.43	Bottom
D	D1	Dolphin pool		02/05	13′55.939	105′57.524	0	Surface
	D2	Dolphin pool	Up-stream	02/05	13′55.939	105′57.524	21.86–1	Water column
	D3	Dolphin pool		02/05	13′55.939	105′57.524	22.86	Bottom
	D4	Dolphin pool		02/05	13′56.033	105′56.936	0	Surface
	D5	Dolphin pool	Deep pool	02/05	13′56.033	105′56.936	35.58–1	Water column
	D6	Dolphin pool		02/05	13′56.033	105′56.936	36.58	Bottom
E	E1	Koh Khnea		01/05	13′05.675	106′02.912	0	Surface
	E2	Koh Khnea	Up-stream	01/05	13′05.675	106′02.912	12.72–1	Water column
	E3	Koh Khnea		01/05	13′05.675	106′02.912	13.72	Bottom
	E4	Koh Khnea		01/05	13′07.746	106′03.506	0	Surface
	E5	Koh Khnea	Deep pool	01/05	13′07.746	106′03.506	14.24–1	Water column
	E6	Koh Khnea		01/05	13′07.746	106′03.506	15.24	Bottom
F	F1	Phat Sanday		29/04	11′48.207	104′58.773	0	Surface
	F2	Phat Sanday	Up-stream	29/04	11′48.207	104′58.773	17.9–1	Water column
	F3	Phat Sanday		29/04	11′48.207	104′58.773	18.9	Bottom
	F4	Phat Sanday		29/04	11′50.679	104′59.703	0	Surface
	F5	Phat Sanday	Deep pool	29/04	11′50.679	104′59.703	19.73–1	Water column
	F6	Phat Sanday		29/04	11′50.679	104′59.703	20.73	Bottom

(Thomsen and Willerslev, 2015; Valentini et al., 2016), our study serves to demonstrate both the potential and the limitations of these methods.

Here we demonstrate that by using eDNA barcoding with a specifically designed genetic assay, it is possible to detect the critically endangered Mekong giant catfish from reservoirs where the species is known to be present. Our detection in the natural habitat of this species was limited to a single positive detection, from the only known spawning grounds on the Mekong mainstream at the border between northern Thailand and Lao PDR. When we consider the scale of the river relative to the extremely low density of the species, it still represents a promising outcome. Our limited results from the natural habitat of the giant catfish do not reflect a problem with the molecular methods underlying eDNA research, rather they highlight the rarity of Mekong giant catfish eDNA in the sampled water. In addition, it shows the importance of obtaining prior knowledge of detection probabilities, rigorous statistical design of sampling strategies, and accounting for 'false absences' through analytical approaches such as occupancy modelling (MacKenzie et al., 2002).

Despite supporting high species richness, including more species of very large-bodied fish and more total fish biomass than any other river system (Baran et al., 2012), and providing critical ecosystem services, there is little systematic monitoring of freshwater biodiversity in the Mekong. Thus, the status of many iconic threatened species is poorly known. Currently available information is primarily based on interviews with fishermen, self-reporting of catches by fishers, or sampling of fish captured through commercial or subsistence harvests (Fily and d'Aubenton, 1965; Bao et al., 2001; Halls et al., 2013). These conventional methods have limited potential to detect rare species, and the information they provide can be inaccurate or incomplete, and time-consuming or costly to collect.

Conventional aquatic species survey methods that are commonly used in North America and Europe, such as electrofishing or seine netting, are rarely applied at an adequate scale for research in the Mekong because of the large geographic scale and transboundary nature of the catchment, large number of long-distance migratory fish species, financial constraints, and logistically challenging physical characteristics of the river. Deep pools in the Mekong can reach depths

up to 70-m, which makes the application of conventional methods impossible in many sites. One alternative option for monitoring species in deep pools is sonar sampling (Viravong et al., 2006). However, this method typically only provides information on biomass and does not enable species identification. Given these knowledge and methodological constraints eDNA sampling may be the most effective way to detect presence of threatened species in many sites, particularly if combined with preliminary site selection based on Local Ecological Knowledge (LEK).

4.1. Factors influencing detection probability of the giant catfish and recommendations for future eDNA sampling

Despite sampling throughout the known historic range of Mekong giant catfish across three countries, we obtained the species' DNA from only one of 12 replicates in the water column sample at the Ban Plan Tung sampling site (site A, Fig. 1). Intrinsically, the detection probability of rare species will be low (Yoccoz et al., 2001; Kéry and Schmidt, 2008). The probability of collecting eDNA in any given river water sample is influenced by a cascade of events:

First, the species is or was recently present at the sampling location or some distance upstream. In this study, we used LEK to target sampling sites for eDNA collection in order to increase the chances of detecting our focal species. The literature contains highly variable results regarding the downstream transport distance of eDNA in rivers, and the distance downstream at which an organism's eDNA is detectable seems to differ amongst species (Deiner and Altermatt, 2014; Pilliod et al., 2014; Civade et al., 2016). There are currently no published studies available which examine this process in a river as large as the Mekong. Having demonstrated proof-of-concept of detecting rare species using species-specific primers, we recommend that future studies design sampling using a site-occupancy framework (*sensu* Schmidt et al., 2013), with multiple samples collected within short periods of time from sampling sites, to account, and model, for non-detection. Such an occupancy sampling approach could form a framework for long-term monitoring of Mekong giant catfish and may be particularly important given detection probability is certainly low.

Second, when the species is present at the sampling site a sufficient quantity of its DNA has to be released at the time of sampling. Density of the released DNA depends on the excretion rates of the target species, which varies among species (e.g. Klymus et al., 2015), life stages (for example, the release rate was 3–4 times higher in adult than in juvenile fish, Maruyama et al., 2014), and metabolic rates. No data is available on the DNA excretion rates of the Mekong giant fish, but the experiment performed in the ponds of the Srichiangmai Fisheries Research Centre (above) shows that the giant catfish releases sufficient DNA for eDNA detection. However fish density in such aquaculture ponds is usually much higher than fish density in natural environments. Future work to quantify DNA shedding rates for different genera or functional groups is needed to improve the accuracy, sensitivity and precision of eDNA surveys in the field.

Finally, released eDNA has to be collected in water samples. The probability of this occurring depends on the concentration of released eDNA, its persistence/degradation rate, its vertical, lateral, and longitudinal spatial distribution, and on the sampling strategy (Pilliod et al., 2014). The amount of eDNA at a site can be positively correlated with the target species density (Takahara et al., 2012; Mahon et al., 2013; Eichmiller, 2014; Moyer et al., 2014), yet the ratio of fish density to eDNA concentration is not consistent among different species or taxa (Kelly et al., 2014). Maruyama et al. (2014) showed that the half-life of fish eDNA in tanks after removal of live fish was 6.3 h, and Dejean et al. (2011) showed that detection probability was null two weeks after removal of animals stocked in ponds. The primary factors which would cause a marked difference in these rates in the Mekong when compared to temperate systems are temperature, UV light exposure, and levels of microbial digestion of shed DNA. Given the well-established influence of environmental factors on DNA degradation, we would expect persistence rates to be lower in a tropical setting. Moyer et al. (2014) used experimental ponds to demonstrate that for every 1.02 °C increase in temperature, the per-L-sample probability of eDNA detection decreased by 1.67 times. It is unclear whether this relationship would hold in a dynamic river system, where nearly all environmental variables and their influence on each other are markedly different than in ponds. The obvious and likely most influential difference is the high variability in water volume at any given site as a function of river velocity, discharge, and channel morphology.

Although the volume of water collected in eDNA studies is usually low (less than 2 L) it has been demonstrated that this volume exerts a significant and positive influence on detection probability. Schultz and Lance (2015) modelled the sensitivity of eDNA methods based on data from carp (*Hypophthalmichthys* spp.) and showed that increasing sampling volume from 2 to 3 L contributes the most to increasing sensitivity for field surveys. The logistical challenges presented by the need to filter very large volumes of water to detect rare species in large rivers warrant additional research, and there is plenty of room to adapt/improve field methods to function in this context. In addition, the velocity and discharge of a river or sampling site will also influence the movement and concentration of eDNA once it is shed, and eDNA can persist in detectable levels over relatively short distances in streams, e.g. few km for fish (Civade et al., 2016).

Mekong giant catfish is thought to be benthopelagic (Poulsen et al., 2002) so it is interesting to note that the positive detection occurred in the water column sample and not in the water collected at the bottom of the deep pool. Perhaps the water column offers the best detection probability because it includes water that has been mixed from different depths. In a system with complex bathymetry like the Mekong it is possible for unique currents and upwelling to exist at the scale of a single sampling site. The sampling strategy for the Mekong giant catfish was based on the filtration of 10 L of flowing water at each sampled depth, with a total of 30-L per sampling point ($n = 2$ per site) within each pool, and a total of 60-L per pool. To improve upon our limited results new studies have to be designed to evaluate the efficiency of the sampling strategy according to the expected concentration of Mekong giant catfish eDNA in the river under different animal density and river discharge scenarios.

Finally when eDNA is successfully collected within the water sample, its detection will depend on several factors (e.g. Success of DNA extraction, PCR amplification sensitivity, Wilcox et al., 2013; Ficetola et al., 2015). Sample collection, extraction, dilution, PCR, and sequencing methods can all influence results (Deiner et al., 2015; Schultz and Lance, 2015; Eichmiller et al., 2016). In summary, the obtained result – the detection of the Mekong giant catfish in one of six sampled locations – has to be considered as the demonstration of the potential use of the eDNA technique to search for an extremely rare fish species in a large tropical river, but not as a full Mekong giant catfish survey. In order to increase chances to retrieve DNA from the target species in the future, improvements at different steps of the protocol (e.g. eDNA sampling effort and strategy) should be considered. For instance, an integrative filtration strategy, as proposed by Valentini et al. (2016), could be used.

4.2. Perspectives for the conservation of the giant catfish and other Mekong fauna

Ban Plan Tung, the site where we detected Mekong giant catfish eDNA, is close to the only known spawning site of the species. Annual ceremonial fishing around the spawning season occurred at this site until the early 2000s (Hogan, 2013a). Population genetics studies have shown that giant catfish migrate at least 1000 km from the Tonle Sap in Cambodia upstream to the only known spawning site near Ban Plan Tung, Thailand (Ngamsiri et al., 2007). Unfortunately, this site is upstream of the first mainstream dam being constructed on the lower Mekong near Xayaburi, Lao PDR. Several mainstream dams have already been built in China, but these are upstream of the known distribution of the Mekong giant catfish. It is unclear whether mitigation measures that are associated with the construction of the Xayaburi dam will allow passage of Mekong giant catfish, and other large migratory fishes. Once the dam has been completed future eDNA surveys would allow us to see if the known spawning site upstream of the infrastructure is still occupied. eDNA sampling could be also be used to monitor the 234 fish conservation zones (FCZs) or "no-take" fish sanctuaries that have been established throughout Lao PDR. Targeted eDNA surveying could assess whether Mekong giant catfish utilise these conservation zones and we could maximise the efficient distribution of FCZs throughout the country for more effective species conservation (Baird and Flaherty, 2005).

Given the limited availability of baseline biodiversity data (i.e. little *a priori* knowledge of species composition) for many locations in the Mekong catchment, including the FCZs, eDNA metabarcoding (Taberlet et al., 2012) would be a particularly useful tool for this region. This approach, based on high throughput sequencing, has recently been developed and validated for the monitoring of aquatic biodiversity of fishes and amphibians (Valentini et al., 2016). It allows obtaining a list of taxa present in the sampled environment. Simmons et al. (2016) applied the term "passive molecular surveillance" to this metabarcoding approach and underlined its capability of detecting unexpected invasives. Unfortunately, fish species from tropical river systems as the Mekong are not well represented in public reference databases such as Genbank® and further sequencing efforts need to be carried out to alleviate this pitfall. Alternatively, in the absence of species identification, it is possible to work with Molecular Taxonomic Units (MOTUs) and to monitor those MOTUs in space and time. The discovery of undescribed fish taxon will probably motivate research for taxonomists and ecologists. We propose that such multi-species approaches, combined with species-specific assays as used in this study, have the additional capacity to detect rare and secretive species. We emphasise that accurate interpretation of results and avoidance of false negatives requires thorough analysis of detection probabilities and sensitivities under specific sampling scenarios and molecular workflows. We expect this approach will be increasingly utilised by freshwater conservationists to improve knowledge of aquatic biodiversity, particularly in poorly understood systems such as the Mekong.

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