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Environmental DNA approach complements social media reports to detect an endangered freshwater stingray species in the wild

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ABSTRACT: Environmental DNA (eDNA) barcoding has emerged as an important non-invasive sampling technique for the detection of rare and endangered species that can be difficult to sample. Our objective was to develop a low-cost eDNA barcoding approach for the detection of an Endangered freshwater stingray species *Fluvitrygon kittipongi* in a tropical river system in Peninsular Malaysia. We designed a species-specific primer for a fragment of *F. kittipongi* cytochrome oxidase subunit I mtDNA (244 bp). The effectiveness of this primer to detect the stingray was evaluated using water samples taken from the upper and middle reaches of the Pahang River coupled with social media reports on sightings of *F. kittipongi*. Five of 14 water samples tested showed positive PCR amplification for the targeted species. These results represent the first successful application of eDNA to detect freshwater stingrays in Malaysia. Using a combination of freshly obtained carcass samples, social media reporting and target species eDNA detection, this study provides formal occurrence records of *F. kittipongi* in Malaysia in 3 major watersheds: the Perak, Pahang and Kelantan Rivers.

KEY WORDS: *Fluvitrygon kittipongi* · eDNA · Distribution · Malaysia · Pahang River · Non-invasive sampling

1. INTRODUCTION

A review of available studies suggest that there are at least 10 dasyatid stingray species known to inhabit or enter freshwater habitats of the geopolitical region of Southeast Asia (both mainland and islands): *Brevitrygon heterura* (Bleeker, 1852), *B. imbricata* (Bloch & Schneider, 1801), *Fluvitrygon kittipongi* (Vidthayanon & Roberts, 2005), *F. oxyrhyncha* (Sauvage, 1878), *F. signifer* (Compagno & Roberts, 1982), *Hemitrygon laosensis* (Roberts & Karnasuta, 1987), *Makararaja chindwinensis* Roberts, 2007, *Pastinachus ater* (Macleay, 1883), *P. stellurostris* Last, Fahmi & Naylor, 2010 and *Urogymnus polylepis* (Bleeker, 1852) (Kottelat 2013, Last et al. 2016). Among these, the genera of *Fluvitrygon* and *Makararaja* inhabit freshwater and occasionally explore brackish water or estuaries (Kottelat 2013, Last et al. 2016). Three *Fluvitrygon* species had been reported in Malaysian rivers (Yano et al. 2005, Last et al. 2010, Hasan et al. 2021a) and currently, *F. signifer* and *F. oxyrhyncha* are known from the Pahang River (Yano et al. 2005, Hasan et al. 2021a). Furthermore, captures of *F.* cf *kittipongi* have recently been reported from the Pahang, Kelantan and Perak Rivers by local fisher groups on various social media platforms (Hasan et al. 2021b, K. C. Lim unpubl. data).

All 3 *Fluvitrygon* species occurring in Malaysia are listed as Endangered on the IUCN Red List (Compagno 2016a,b, Vidthayanon & Manjaji 2016) due to geographic range reduction to less than 5000 km^2

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(IUCN 2012). Freshwater stingrays of the family Dasyatidae share many similar characteristics, including slow maturation, a long gestation period and low fecundity (Dulvy et al. 2014). These biological features, coupled with relatively limited riverine habitat suitability and home range, render them vulnerable to localised extinction from habitat loss due to river-based pollution such as domestic waste-water, soil erosion and agricultural/industrial wastes (Compagno 2016a,b, Vidthayanon & Manjaji 2016). The water quality of Malaysian rivers has declined considerably within a 5 yr period from 2012–2017, with 57 monitored rivers being downgraded from the 'clean' to 'slightly polluted' category (DoEM 2017). The degradation of water quality was closely linked to increasing urban land use changes in the forms of urbanization, industrialization and agricultural processes due in part to increased population growth (Camara et al. 2019, Yaakup et al. 2000, How Jin Aik et al. 2021). Without the ability to move to more suitable habitat, responses of aquatic organisms to polluted waters vary from reduction in reproductive capacity to death (Bassem 2020). With the projected increase in Malaysia's population density (Department of Statistics Malaysia 2012) and associated land use changes, the future outlook for Malaysian watershed and riverine systems and the viability of their freshwater stingray populations is not optimistic.

To date, there has been limited scientific effort to elucidate the occurrence and distribution of freshwater stingrays in Malaysian riverine systems. Successful capture of target aquatic wild species that are rare or difficult to sample, including freshwater rays, typically requires considerable labour, costs and logistical planning (Simpfendorfer et al. 2016, Weltz et al. 2017). Freshwater stingrays prefer benthic and turbid habitats (Last et al. 2010), which increases the difficulty of sampling to accurately determine their occurrences and distribution range (Simpfendorfer et al. 2016). Although sampling methods such as hook-and-line fishing, gillnets and traps were useful in capturing freshwater stingrays (Last et al. 2010, Iqbal et al. 2019, Windusari et al. 2019, Hasan et al. 2021a), post-release mortality from these catch methods can be very high, ranging from 20–70% for stingrays (Ellis et al. 2017). This adds an additional ethical concern of lethal sampling for endangered species due to their small population size (Hilton & Richardson 2004). Therefore, the use of non-invasive sampling methods is ideal, as they can be more cost-effective (Davy et al. 2015, Simpfendorfer et al. 2016, Weltz et al. 2017) and prevent further population reduction due to research activities.

The use of internet-based reported sightings posted by various wildlife-focused communities on social media platforms (e.g. Facebook, Instagram, YouTube) has greatly improved the knowledge base on local distributions of many rare species, including freshwater stingrays in Malaysia (e.g. Iqbal et al. 2019, Hasan et al. 2021a). This publicly available source of data can be harnessed additionally to infer relative abundances by locality based on the frequency of reported sightings (Witt et al. 2012); this information can help in prioritising the selection of sites for initial surveys to maximise success of field-based species detection. In the case of published freshwater stingray records in Malaysia, they were based mainly on photographic records with minimal or no direct examination of specimens. Due to possible presence of cryptic species, photo-based species identification alone may not be accurate (Iqbal et al. 2019) and taxonomic identity confirmation through direct or indirect surveys of animals remains an area of research priority (Di Minin et al. 2015).

Environmental DNA (eDNA) barcoding is an emerging technique that has been successfully used in tracking small, rare, or evasive species. The technique relies on detection of traces of DNA of the targeted species (or groups of species, in the case of eDNA metabarcoding) that were released into the environment in the forms of feces, mucous, gametes, shed skin and hair, or carcasses (Pilliod et al. 2013). The lengths of extracted eDNA can be highly variable and are thought to be fragmented as a result of continuous enzymatic digestion, UV radiation, mechanical fragmentation and chemical degradation (Barnes & Turner 2016). These processes happen over a short period of time (Dejean et al. 2011), depending on environmental factors such as temperature, turbidity, acidity and salinity (Tsuji et al. 2017, Harrison et al. 2019). Nevertheless, this technique is a viable non-invasive tool for detecting the presence of endangered species and even abundance within a habitat without the need for lethal sampling. The eDNA barcoding method has been successfully applied elsewhere to determine the presence of endangered elasmobranchs such as skates (Weltz et al. 2017) and sawfish (Simpfendorfer et al. 2016) in Australia. However, tracking of endangered taxa using eDNA barcoding in Malaysia has only been conducted for the southern river terrapin (Wilson et al. 2018).

With this in mind, the present study aimed to explore the use of a low-cost eDNA barcoding approach for detection of a freshwater stingray species in a tropical river system in Peninsular Malaysia; specifically, the roughback whipray *F. kittipongi*. This species has

been recorded in Malaysian Borneo (Last et al. 2010) but not formally in Peninsular Malaysia. We harnessed the power of internet-based social media reports available on this species to determine the site selection for field-testing the approach. We designed species-specific primers that target a short fragment of the cytochrome oxidase subunit I (COI) mtDNA sequence of *F. kittipongi* and evaluated the effectiveness of the primers on water samples taken from a selected river site in Peninsular Malaysia.

2. MATERIALS AND METHODS

2.1. Primer design and testing

In the designing of a species-specific primer, an initial search in GenBank (www.ncbi.nlm.nih.gov) showed one available reference COI sequence for *F. kittipongi* (accession number: MG792100) from an unspecified location in Malaysia. We also directly acquired DNA material from freshly dead *F. kittipongi* specimens. Specifically, 2 specimens obtained from Sungai Perak (Fkit1, male, disc width (DW) 23.6 cm, collected 1 February 2015; Fkit2, female, DW 23.0 cm, collected 19 June 2015) were acquired from local fishers. For primer testing, tissue samples of 2 *U. polylepis* specimens collected earlier from Malaysian Borneo (Upol1, female, DW 190 cm, Mukah, 4 April 2016; Upol2, male, DW 120 cm, Sandakan, 25 August 2018) were used. This closely related species was used for the primer specificity test since samples of other *Fluvitrygon* species could not be obtained during the sampling period. Tissue samples of all specimens were preserved in absolute ethanol before subsequent molecular procedures.

DNA was extracted from all tissue samples using G-spin™ Total DNA Extraction Mini Kit (iNtRON Biotechnology). The COI gene was amplified by PCR using the universal primers FishF2 (5′-TCG ACT AAT CAT AAA GAT ATC GGC AC-3′) and FishR2 (5′-ACT TCA GGG TGA CCG AAG AAT CAG AA-3′) (Ward et al. 2008). PCR amplification was performed using a 20 µl reaction mix containing 2 µl of 10× PCR buffer, 0.5 µl of dNTPs mixture (2.5 mM each), 1 µl of 10 pmol primer (both primers), 1.25 unit of *Taq* DNA polymerase (iNtRON Biotechnology), 1 µl of 50 pg to 1.0 µg DNA templates and molecular-grade water. The PCR cycles comprised 2 min initial denaturation at 94°C, followed by 30 cycles of 20 s at 94°C, 20 s at 50°C, 1 min at 72°C and a final extension of 5 min at 72°C. The PCR products were examined using 1% agarose in Tris-acetate-EDTA (TAE) buffer prior to Sanger sequencing service at Apical Scientific. The obtained sequences were reviewed manually, edited and trimmed using ChromasPro version 1.5 software (Technelysium). The edited sequences were then deposited in GenBank with accession numbers MZ976812–MZ976815 for Upol1, Upol2, Fkit1 and Fkit2 respectively.

The new primer pair for eDNA analysis was designed using Primer-BLAST in NCBI GenBank (www.ncbi.nlm.gov/tools/primer-blast/) (Ye et al. 2012). Both the reference sequence and DNA sequences from the *F. kittipongi* specimens were used to search for target amplicons of small sizes between 70 and 300 bp because eDNA will often be degraded. The designed primer was tested for specificity and the PCR cycle parameters were optimized using extracted DNA from tissue samples of obtained *F. kittipongi* specimens (Fkit1 and Fkit2) and closely related species of *U. polylepis* (Upol1 and Upol2). A negative control was included in each PCR run. The primer test PCR was performed using a 25 µl reaction mix containing 12.5 µl of MyTaq Red Mix (Meridian Bioscience), 1 µl of 10 pmol primer (both designed primers), 1 µl of 50 pg to 1.0 µg DNA templates and molecular-grade water. MyTaq Red Mix is a mixture of MyTaq DNA polymerase, dNTPs, $MgCl_2$ and enhancers at optimal concentrations, and it has been shown to work well even in the presence of inhibitors. The PCR cycles comprised 2 min initial denaturation at 94°C, followed by 30 cycles of 20 s at 94°C, 20 s at 3 temperature settings (54, 57 and 60°C), 1 min at 72°C and a final extension of 5 min at 72°C. PCR products were examined using 1% agarose in TAE buffer. Positive amplification of the *F. kittipongi* sequence and zero amplification of the *U. polylepis* sequence suggested the specificity of the designed primer pair.

2.2. Site selection and water sample collection

Selection of a suitable riverine site with known contemporary occurrence of *F. kittipongi* was critical for the field testing of the eDNA method. Although distribution information is highly limited, *F. kittipongi* had been reportedly found in the Pahang, Kelantan and Perak Rivers based on photographic documentation posted by a local fisher-based Facebook group called Ikan Air Tawar Malaysia (Malaysian Freshwater Fish) (Fig. 1). We selected the Pahang River, a large riverine system 459 km in length, for the first eDNA field trial due to a higher frequency of reported sightings of *F. kittipongi* at this site from social media posts.

Photographic identification of *F. kittipongi* was possible based on distinct morphological characteristics on the dorsal surface supplemented by those on the ventral surface when available. Distinct differences between *F. kittipongi* and its congener *F. oxyrhyncha* can be found in the dorsal colouration, where the former shows uniformly brownish colour without distinct patterns while the latter has ornate reticulated patterns. Distinct differences between *F. kittipongi* and *F. signifer* include the presence of a single pearl thorn, broad denticle band, white tail with brown or black spotting (sometimes covering the whole tail) and a dark outer margin on the ventral side in *F. kittipongi*, while *F. signifer* has a whitish edge on the dorsal surface often with a faint reticulate pattern, no pearl thorn, narrow band of denticles (if present) and a uniformly white tail and ventral surface (Vidthayanon & Roberts 2005, Last et al. 2010).

A total of 14 water samples were collected at sites along the upper and middle reaches of the Pahang River and along the Jelai River tributary. Samples from Stns S1–S5 were collected in October 2018 (inter-monsoon period; IM), Stns S6–S9 in August 2019 (southwest monsoon; SW) and Stns S10–S14 in November 2018 (northeast monsoon; NE) (see Fig. 2). Stations were located approximately 10–30 km apart from each other. Environmental parameters, i.e. water temperature, salinity, pH, total dissolved solids, and dissolved oxygen, were measured and recorded at each station. A total of 5 l of surface water per station was collected from the riverbank using plastic bottles that were soaked in a bleach solution and rinsed with distilled water prior to use. The sampling protocol was adapted from Evans & Lamberti (2018). To prevent potential sampling contamination from field boots, clothing and equipment, the plastic bottles were directed upstream during collection. One 'field' negative control (bleach-cleaned sample bottle filled with distilled water) was included in each sampling.

2.3. eDNA analysis and validation

All water samples were subjected to filtration within 24 h of collection, through a 0.45 µm cellulose ester

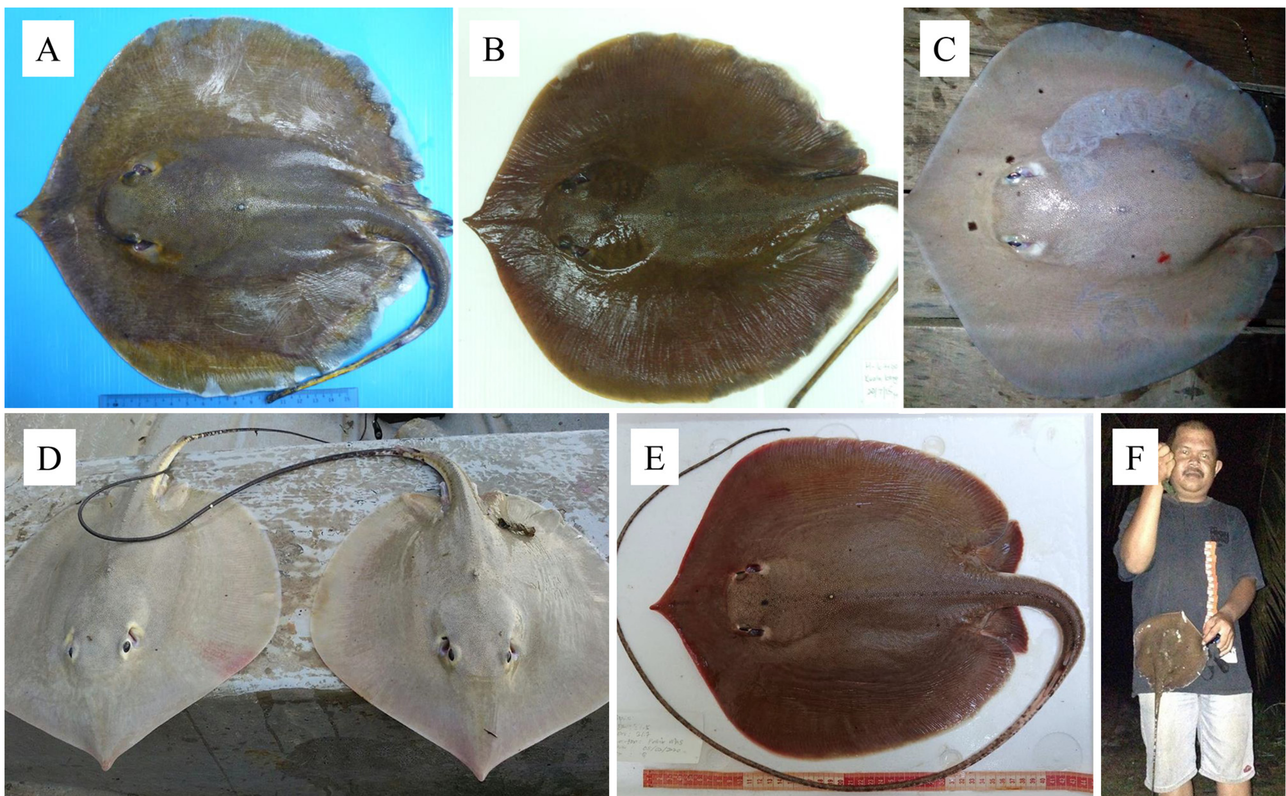

Fig. 1. Roughback whipray *Fluvitrygon kittipongi* collected in the present study and caught by local fishermen or fish anglers posted on Facebook (https://www.facebook.com/groups/158073811005000). (A) Fkit1, (B) Fkit2, (C) Pahang River 20 January 2017 (by Toman Merajuk), (D) Pahang River 30 October 2019 (by Nas Ahmad), (E) Kelantan River 8 February 2020 (by Mohd Saki Noor), (F) Perak River 28 February 2015 (by Shin Chan). Permission to share photos had been obtained from the owners

membrane filter (Whatman) using vacuum filtration. All filter apparatus was cleaned and sterilized with 10% bleach and distilled water between each sample filtration. Due to high turbidity of the sampled water, the membrane filter was replaced every 100–300 ml for each sample until the full volume of 5 l had been filtered. Membrane filters used for each sample were then folded, cut and placed into individual sterilized 50 ml tubes.

DNA extraction from isolates on membrane filters was performed using NucleoSpin Tissue kit (Macherey-Nagel) with slight modification to the manufacturer's instructions. Modifications included increasing the volume of lysis buffer (T1) from 180 µl to 10 ml to immerse the membrane filter entirely and the lysis incubation duration was extended from 1–3 to 24 h. The extracted DNA was quantified using a NanoDrop spectrophotometer. Six PCR replicates were performed for each sample and field negative control (3 using extracted DNA, 3 using PCR product), with the inclusion of a PCR negative control on each run, following the test and optimized parameters under 54°C annealing temperature. The PCR products were examined using 1% agarose in TAE buffer. Positive PCR products were sequenced in both directions using the Sanger sequencing service at Apical Scientific, and the results were compared with our reference sequences for identity confirmation.

3. RESULTS AND DISCUSSION

Our search on Primer-BLAST revealed multiple candidate primer pairs suitable for detection of *Fluvitrygon kittipongi* eDNA. After careful review of each primer pair, we used the FkitF1 (5'-ACT CAT TCG AAC CGA ACT AAG TCA-3') and FkitR1 (5'-AGC AGA AGC TAG GAG TAG TAG GAA-3') sequences, which showed the least amount of matching with other similar species in amplifying a 244 bp fragment of the *F. kittipongi* COI gene. Temperature optimization of the test PCR on the primer pair showed all 3 tested annealing temperatures successfully amplified sequences of *F. kittipongi* but not *Urogymnus polylepis*, suggesting high specificity of the designed primer. The lowest annealing temperature was applied on the extracted eDNA to improve chances of positive amplification, as overall total concentration of extracted DNA was low (936 ng to 42.7 µg; average: 11.6 µg).

Out of 14 water samples, 5 samples from relatively upstream stations showed positive amplification, including one sample taken during IM (Stn S5: 20% positive amplification) and 4 samples during SW (Stns S6–S9; 100% positive amplification) (Fig. 2). None of the samples from the downstream stations taken during NE showed positive amplification (0%). DNA sequences of the PCR products showed 100% match with our Fkit1 and Fkit2 sequences, confirming the presence of *F. kittipongi* DNA traces in the upstream reaches of the Pahang River.

The environmental parameters recorded according to monsoonal seasons are shown in Table 1. The success rate of amplification appeared to be related to monsoon seasonality, where higher success rate of amplification was found in samples taken during the SW monsoon. The east coast of Peninsular Malaysia is generally affected by higher rainfall during NE (November–March) and receives lower rainfall during SW (May–September) (Mohd Akhir & Chuen 2011, Faudzi et al. 2017). Our results suggest that heavy rainfall episodes during NE increased the river flow rate, which likely reduced the persistence and concentration of available DNA traces — a conjecture that is supported by other studies (Akre et al. 2019, Harrison et al. 2019, Curtis et al. 2021).

To date, there is no official publication reporting the presence of *F. kittipongi* in Malaysia. This species was first found in Thailand, Menam Maekhlong near Kanchanaburi by Kittipongs Jarutanin on 30 January 2004 (Vidthayanon & Roberts 2005). It was later assessed by the IUCN in 2007, suggesting possible occurrence in the Pahang River (Vidthayanon & Manjaji 2016). Recent fish surveys in the Pahang River by Zulkafli et al. (2015) and Hasan et al. (2021a) reported the presence of sister species *F. signifer* (in Temerloh district, around Stn S10) and *F. oxyrhyncha* (in Kuala Lipis, around Stn S8) but not *F. kittipongi*. Although Last et al. (2010) reported that *F. kittipongi* was found in Peninsular Malaysia and Borneo, the distribution map suggests official records only from Indonesian Borneo in Kalimantan.

The detection of *F. kittipongi* eDNA represents the first successful application of this non-invasive sampling technique to detect an endangered freshwater stingray in Malaysian rivers. This result most likely reflects a contemporary distribution, as the detection probability of eDNA declines on a scale of days or weeks in tropical freshwater riverine ecosystems (Thomsen & Willerslev 2015). Using a combination of methods, our study presents evidence of one of the first records of *F. kittipongi* in Malaysian rivers, specifically in the Perak River (fresh samples of Fkit1 and Fkit2 and photographic evidence), Pahang River (eDNA and photographic evidence) and Kelantan River (photographic evidence) (see Fig. 1 for photo-

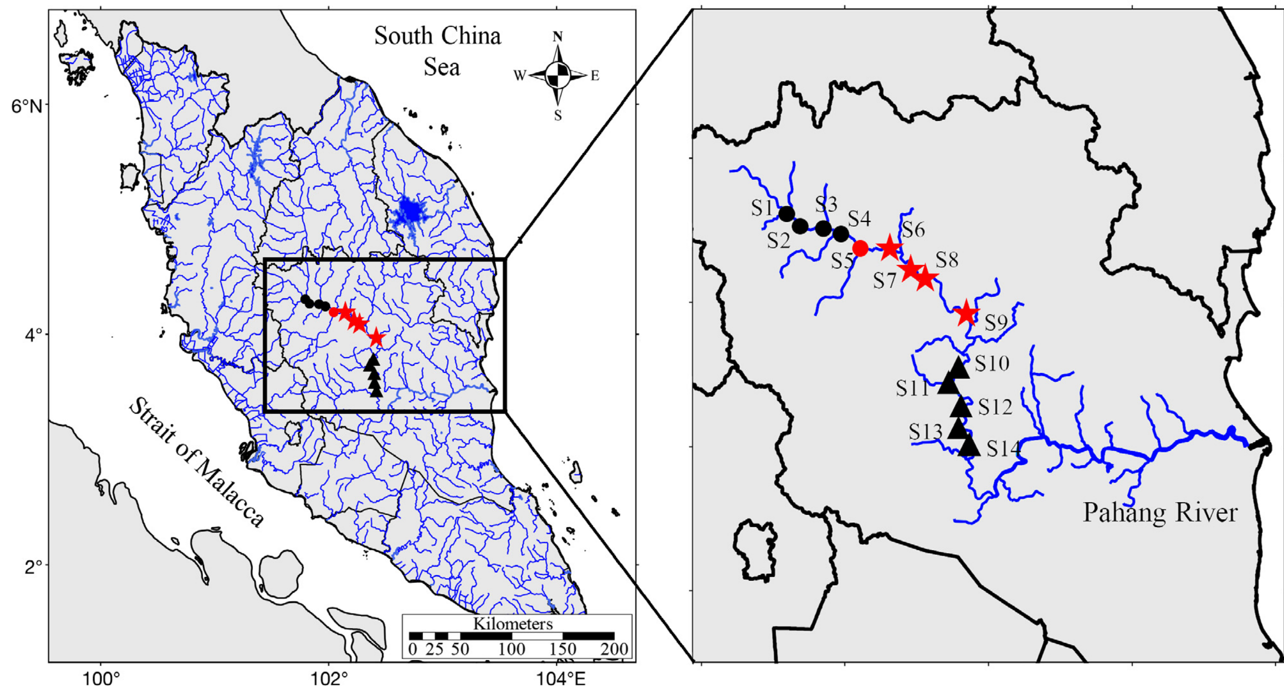

Fig. 2. Sampling stations along the Pahang River (inset map) in Peninsular Malaysia. Blue lines: local riverine networks. Stns S1–S5 (circles) were sampled during the inter-monsoon period; Stns S6–S9 (stars) during the southwest monsoon; and Stns S10–S14 (triangles) during the northeast monsoon. Red symbols: positive detection of eDNA of stingray *Fluvitrygon kittipongi*; black symbols: negative detection

Table 1. Water parameters recorded at the Pahang River in Peninsular Malaysia with respect to monsoon effect (see Section 2.3 and Fig. 2 for sampling station information on Stns S1–S14)

Variable	Inter-monsoon Stns S1–S5	Southwest monsoon Stns S6–S9	Northeast monsoon Stns S10–S14
Temperature (°C)	21.52–22.99	23.40–25.56	20.60–22.79
Salinity (ppt)	0.02–0.04	0.03–0.04	0.01–0.02
pH	6.10–7.14	6.59–6.90	6.91–9.91
Total dissolved solids (g l^{-1})	0.036–0.054	0.047–0.054	0.017–0.036
Dissolved oxygen (mg l^{-1})	7.31–7.84	8.12–8.66	5.70–7.35

graphic evidence). Future studies should continue to evaluate the extent of *F. kittipongi* distribution and abundance in these rivers in the context of anthropogenic threats to their survival, preferably using non-invasive sampling techniques. This scientific knowledge will allow this Endangered species to receive conservation consideration in future river management plans and environmental impact assessments of river-related development projects.

Despite the usefulness of eDNA, there remain challenges associated with the application of the method. As reviewed by Thomsen & Willerslev (2015), these challenges are associated with reliability of the results and inferences drawn as a result of contamination, inhibition, sequencing errors and PCR approaches. For applications used to determine the presence of endangered species, a major challenge lies in the success rate of detection. A study on endangered sawfish in Australia showed about 20% positive detection rate for samples taken from areas with and without prior sawfish records (Simpfendorfer et al. 2016). A similar result was found in our study, with only 36% of the water samples taken along the river showing positive amplification. While non-detection could be due to a number of reasons (discussed below), there is no reason to suspect that *F. kittipongi* does not use the riverine areas of Stns S1–S4 and Stns S10–S14 from a habitat suitability standpoint.

Although amplification success rates appeared to be closely linked to rainfall amounts that affect river flow during sampling of eDNA, further investigation on this seasonality aspect needs to be conducted to maximise success of similar applications in other rivers with no prior stingray records. Other possible factors for low successful amplification include low density of targeted species in the river (Takahara et

al. 2012, Thomsen et al. 2012, Pilliod et al. 2013), presence of PCR inhibitors such as humic acids or humic substances in the river (McKee et al. 2015, Sigsgaard et al. 2015), short duration of DNA persistence (usually less than 5 d) in rivers (Harrison et al. 2019), unpredictable DNA fragments in terms of sections and sizes available during sampling resulting in unsuccessful matches with the primer pairs used (Bylemans et al. 2018, Shogren et al. 2018), and lengthy preparation of highly turbid samples resulting in degradation of DNA (Wilson et al. 2018, Seymour et al. 2018). While most of these other factors were beyond our control, we attempted to ameliorate the issue of PCR inhibitors by using MyTaq Red Mix, which produced encouraging results from our study.

4. CONCLUSIONS

The present study demonstrated the potential of the eDNA barcoding application in determining the occurrence and distribution of fish in tropical freshwater riverine systems, using the Endangered freshwater stingray in a Malaysian river as a case study. Preliminary work presented here shows the feasibility of the approach across other riverine systems in the region. This approach can be highly useful to accurately map the distribution range of endangered freshwater stingrays and other riverine organisms without lethal sampling, thus providing valuable biodiversity information for effective conservation and management plans.

Ethics approval. All stingray specimens in this study were dead upon collection from local fishers. At the time of sampling, no specific research permit was needed for collection of *Fluvitrygon kittipongi* from Peninsular Malaysia or from Sarawak in Malaysian Borneo. Collection permits for tissue from a closely related giant freshwater stingray *Urogymnus polylepis* from Sabah, Malaysian Borneo (used for primer testing), were approved by the Sabah Biodiversity Council (Access License Reference No: JKM/MBS.1000-2/2 JLD.9 [21–23] and Transfer License Reference No: JKM/MBS. 1000-2/3 JLD.4 [18]).

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