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COMMENT

DNA contamination and taxonomic diversity hinder eDNA research on freshwater stingray species: Comment on Lim & Then (2022)

Norli Fauzani Mohd Abu Hassan Alshari[1,#], Muhamad Hanif Iryani Bin Adnan[2,#], Jamsari Amirul Firdaus Jamaluddin[2], Sébastien Lavoué[2,*], Siti Azizah Mohd Nor[1]

[1]Institute of Marine Biotechnology, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia
[2]School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia

ABSTRACT: Recently, Lim & Then (2022; Endang Species Res 48:43–50) used an environmental DNA (eDNA) PCR-based method for detecting the presence of an endangered species of freshwater stingray, *Fluvitrygon kittipongi* (Dasyatidae), in the Pahang River basin, east Peninsular Malaysia. For that, they designed a species-specific pair of primers to amplify a 196 base pair (bp) fragment of the cytochrome *c* oxidase I (COI) gene. Lim & Then (2022) reported positive detection of this species along an upstream stretch of the Pahang River during the inter-monsoon and the southwest monsoon seasons, with the PCR-amplified eDNA sequences identical to the reference COI sequences of *F. kittipongi* collected from the Perak River basin, west Peninsular Malaysia. Here, we argue that such positive results are likely the consequence of a DNA contamination because the 196 bp COI fragments of *F. kittipongi* from the Pahang River and the Perak River differ from each other by 4 nucleotide substitutions. The source of contamination in Lim & Then (2022) could have been the Perak samples the authors handled to develop the primers. We also briefly discuss the potential impact of the presumed co-occurrence of freshwater stingrays of the genus *Fluvitrygon* in the Pahang River on the specificity of the *F. kittipongi*-specific primers designed by Lim & Then (2022).

KEY WORDS: *Fluvitrygon kittipongi* · environmental DNA · eDNA · Contamination · *Fluvitrygon signifer* · Conservation

Lim & Then (2022) developed an environmental DNA (eDNA) PCR-based method aiming to detect the presence of the endangered freshwater stingray *Fluvitrygon kittipongi* (Dasyatidae) in Peninsular Malaysia. For that purpose, Lim & Then (2022) designed an oligonucleotide primer pair (FkitF1 and FkitR1) for amplifying a 196 base pair (bp) (244 bp including both primer sequences) fragment within the mitochondrial cytochrome *c* oxidase I (COI) gene (Fig. 1A). They used 650 bp COI 'barcode' sequences determined from 3 specimens of *F. kittipongi*, 2 collected from the Perak River (west Peninsular Malaysia) and one from an unknown locality (COI GenBank accession number: MG792100). These 3 sequences are identical to each other. Lim & Then (2022) tested the specificity of their primers against *Urogymnus polylepis*, another species of freshwater stingray occurring in Peninsular Malaysia, but one which is only distantly related to *F. kittipongi*. The primers were tested neither against the 2 other species of *Fluvitrygon* that

[#]These authors contributed equally to this work
[*]Corresponding author: microceb@hotmail.com

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have been reported from Peninsular Malaysia (*F. signifer* and *F. oxyrhynchus*) nor against the population of *F. kittipongi* from the Pahang River. Lim & Then (2022) then used their primers to detect the eDNA of *F. kittipongi* at 14 localities along the main course of the Pahang River. Positive PCR amplifications at the stations S5 to S9 (in Lim & Then 2022) were confirmed by DNA sequencing of the PCR products showing a 100% nucleotide similarity with the 3 COI reference sequences.

As part of an ongoing study by our research group (unpubl. data), we collected 3 specimens of freshwater stingrays from the Pahang River: 2 specimens (CMR23 and CMR24; the latter is shown in Fig. 1B) were purchased from the fish market of Maran and 1 specimen (TAB92; Fig. 1C) from the morning market of Temerloh. Maran and Temerloh are located immediately downstream from Station S14 of Lim & Then (2022). The morphological characteristics of these specimens correspond to those of *F. kittipongi* according to the description of this species provided by Vidthayanon & Roberts (2005). We determined their COI 'barcode' haplotypes (GenBank accession numbers: OP459307–OP459309) and found that they are identical to each other, but they differ from the COI haplotype of *F. kittipongi* of the Perak River by an uncorrected genetic distance of ~1.5%. Such genetic differentiation between populations of a freshwater fish species from the west and east Peninsular Malaysia has already been observed (e.g. de Bruyn et al. 2013).

Using the primers and the PCR thermal cycle profile of Lim & Then (2022), we then attempted to amplify the 196 bp COI fragment from our 3 specimens. Despite noting 1 nucleotide difference between the reverse primer FkitR1 and its target sequence in the Pahang River specimens' COI haplotype (Fig. 1A), we succeeded in amplifying this fragment for all our specimens, and the PCR products were sequenced in both directions. The comparison of the 196 bp fragments from the Pahang and Perak Rivers uncovered 4 unambiguous nucleotide differences (Fig. 1A), corresponding to a ~2% uncorrected genetic distance for this fragment.

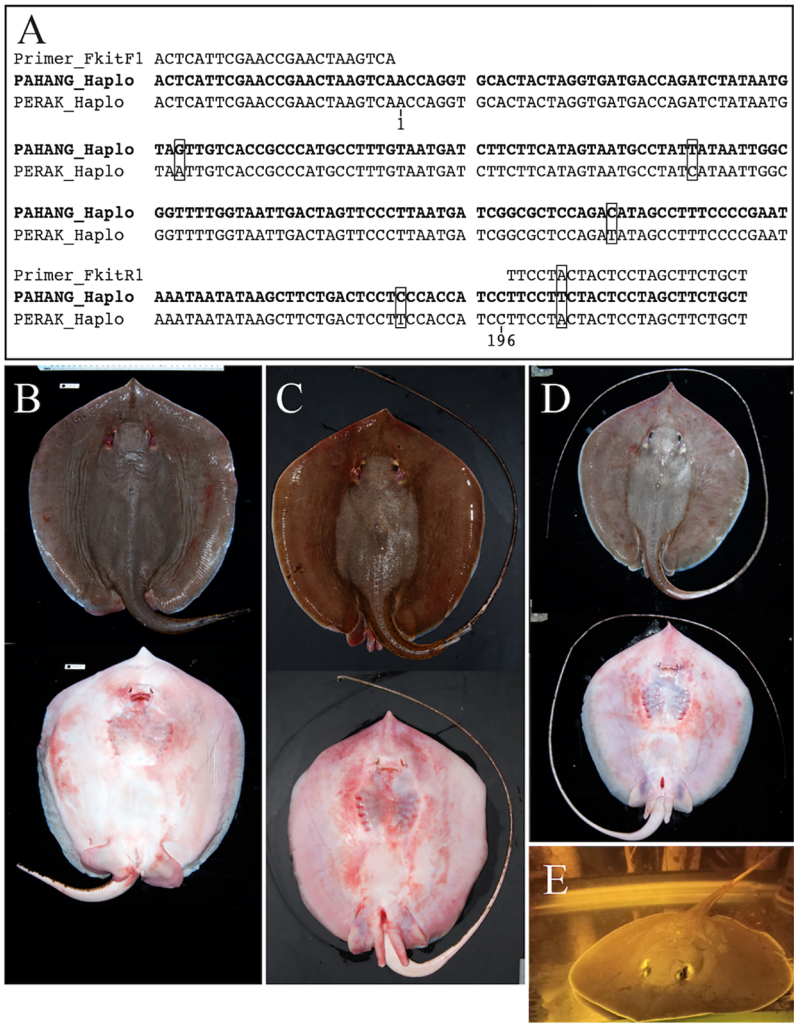

Fig. 1. (A) Alignment of the COI haplotypes of Pahang specimens ('PAHANG_Haplo' highlighted in bold) and Perak specimens ('PERAK_Haplo') of the 196 bp eDNA fragment examined by Lim & Then (2022), flanked by the 2 primers (FkitF1 and FkitR1); vertical boxes indicate nucleotide differences. (B–D) Dorsal (top) and ventral (bottom) views of freshwater stingray specimens of *Fluvitrygon*: (B) specimen CMR24, [USMFC (1) 00008], female, 34.5 cm disc width, tail cut and stings removed; purchased in Maran, Pahang River; identified as *Fluvitrygon kittipongi*; (C) specimen TAB92 [USMFC (1) 00006], male, 23.0 cm disc width, stings removed; purchased in Temerloh, Pahang River; identified as *Fluvitrygon kittipongi*; (D) specimen TAB95 [USMFC (1) 00007], 23.0 cm disc width, stings removed; purchased in Temerloh, Pahang River; this specimen differs from specimens of *Fluvitrygon kittipongi* CMR23 (not shown), CMR24 and TAB92 by a distinct dorsal white margin band and a tail more than twice as long as the disc width. (E) Antero-dorsal view of a live specimen AAK01, estimated ~30.0 cm disc width, stings removed; captured and kept live by a local fisherman in Kampar, Perak River; this specimen differs from specimens CMR23, CMR24 and TAB92 by a distinct dorsal white margin band and a tail more than twice as long as the disc width (latter character not shown on photo)

Therefore, the 100% similarity between the haplotype of *F. kittipongi* from the Perak River and the eDNA fragments amplified from water samples of the Pahang River, as reported by Lim & Then (2022), is puzzling. We think that the most likely explanation for this result is a DNA contamination from a Perak sample previously handled by these authors. DNA contamination is a recognized concern in PCR-based eDNA studies because of the small quantity of degraded DNA contained in environmental water samples (Sepulveda et al. 2020). Such contamination would have been impossible to notice for Lim & Then (2022) without reference sequences from the Pahang River.

Beside the contamination issue, the reported occurrence of 2 other species of freshwater stingray of the genus *Fluvitrygon*, *F. signifer* and *F. oxyrhynchus*, in the Pahang River (see references in Lim & Then 2022), may complicate the development of a species-specific eDNA approach to independently monitor the presence of *F. kittipongi* or any other 2 species of *Fluvitrygon*. Using the mitochondrial NADH2 gene and specimens collected in Borneo, Naylor et al. (2012) showed that the 3 species of *Fluvitrygon* form a monophyletic group, in which the morphologically similar *F. kittipongi* and *F. signifer* are sister species, exhibiting low genetic differentiation between them. Therefore, any species-specific pair of primers designed for the eDNA monitoring of one of these species in the Pahang River should first be tested against any other co-occurring species of *Fluvitrygon* to ensure their specificity.

As a final remark, we would like to stress that the taxonomy of *Fluvitrygon* in Peninsular Malaysia is still poorly resolved. During our research, we observed intriguing morphological variability in specimens of *Fluvitrygon*, questioning their taxonomic status. Beside the 3 specimens from the Pahang River reported above that we identified as *F. kittipongi*, we acquired another specimen of stingray (TAB95; USMFC (1) 00007) from the fish market of Temerloh (Pahang River). This 4th specimen displayed characteristics of both *F. kittipongi* (having strong denticulation on the central part of the dorsal disc and a mid-dorsal pearl organ) and *F. signifer* (having a distinct dorsal white margin band and a tail more than twice as long as the disc width) (Fig. 1D). We found that its COI sequence (GenBank number: OP459310) is identical to the haplotype of *F. kittipongi* from the same river. We also sequenced the COI gene of a freshwater stingray specimen (AAK01; specimen not preserved; Fig. 1E) from the Perak River basin, again intermediate in appearance to *F. kittipongi* and *F. signifer*. This sequence (GenBank number: OP459311) is identical to the haplotype of *F. kittipongi* from the Perak River as determined by Lim & Then (2022). As noted by Lim & Then (2022), there is still no published comprehensive specimen-based comparative study on freshwater stingrays of the genus *Fluvitrygon* in Peninsular Malaysia using morphological and/or genetic evidence. Such taxonomic study is urgently needed to determine how many species of *Fluvitrygon* occur in Peninsular Malaysia by describing their morphological characteristics, and to estimate their degree of genetic differentiation. This information is fundamental to further assess the distribution and conservation status of these highly threatened freshwater fishes through eDNA approaches.

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