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REPLY COMMENT

# Population genetics of freshwater stingray require investigation to confirm DNA contamination: Reply to Alshari et al. (2023)

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ABSTRACT: Alshari et al. (2023; Endang Species Res 50:311–313) were able to collect 3 *Fluvitrygon kittipongi* specimens from the Pahang River, which were not available to us at the time of our study (Lim & Then 2022; Endang Species Res 48:43–50). The cytochrome *c* oxidase I (COI) sequences (or haplotypes) of these 3 stingrays were identical to each other but differed from the haplotypes of our Perak River specimens. The result of 100% similarity between COI sequences of our Perak River rays and the eDNA water samples isolated from Pahang River in our study was suggested by Alshari et al. (2023) as DNA contamination in our study, which is plausible. However, further population genetics studies would be necessary to ascertain that the Perak River haplotype does not occur in stingrays of the Pahang River.

KEY WORDS: Haplotype · *Fluvitrygon* · eDNA · Pahang River · Perak River · Malaysia

Alshari et al. (2023) presented additional evidence that was not available to us at the time our paper (Lim & Then 2022) was published, specifically the mitochondrial cytochrome *c* oxidase I (COI) sequences of 3 individuals of freshwater stingray *Fluvitrygon kittipongi* from the Pahang River that our eDNA barcoding study intended to detect. The sequences of the 3 Pahang rays (Pahang haplotype) were shown to be identical to each other but displayed 4 nucleotide differences when compared against sequences from our Perak River rays (Perak haplotype) and the eDNA supposedly recovered from the Pahang water samples. This evidence was presented as a strong case for eDNA contamination in our study, specifically from the Perak River ray individuals during handling of the Pahang River water samples.

While the results presented by Alshari et al. (2023) are important and highly useful to highlight the presence of a different COI haplotype in Pahang River rays, the evidence for definite contamination in our study based on 3 individuals is not robust (T. Kajita pers. comm.). Without population genetic studies using multiple *F. kittipongi* samples across multiple rivers (in the context of our work, both Pahang and Perak Rivers), it is difficult to ascertain that the Perak haplotype does not occur in the Pahang River. A phylogeographic study on freshwater snakehead fish *Chana striata* showed multiple shared haplotypes of the ND5 mitochondrial gene between individuals sampled in rivers in both eastern and western Peninsular Malaysia (Tan et al. 2012). Such a possibility of shared COI haplotypes cannot be ruled out for *F. kittipongi* without in-depth studies. The finding of 1

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stingray specimen from the Pahang River that possessed features of both *F. kittipongi* and *F. signifer* but with Pahang COI haplotype of *F. kittipongi* (Specimen coded TAB95 [USMFC (1) 00007] in Alshari et al. 2023), provides compelling support for the need to study local morphotypes and haplotypes of the *Fluvitrygon* species not only in the Perak and Pahang Rivers but across other rivers where these species have been reported to occur. The potential for hybridization in *F. kittipongi* should also be further investigated, since this has also been reported for other freshwater stingrays (S. J. Caballero pers. comm.).

Alshari et al. (2023) additionally highlighted that it is important to test primer specificity for species-specific detection of co-occurring *Fluvitrygon* species within a single river, specifically *F. kittipongi, F. signifer* and *F. oxyrhynchus* in the Pahang River (and by extension other rivers within Peninsular Malaysia). The need for primer specificity testing is due to possibly low genetic differentiation among the 3 species, as shown using the mitochondrial NADH2 gene for Borneo samples (Naylor et al. 2012; highlighted in Alshari et al. 2023). To date there are no available COI sequences in the NCBI GenBank or the Barcode of Life Database for the 2 other species, *F. signifer* and *F. oxyrhynchus* — this critical information gap prevented the ideal testing (as suggested by Alshari et al. 2023) of the COI primer that we developed.

In conclusion, we are not able to definitively rule out the possibility of contamination in our study and we concur with Alshari et al. (2023) on the need to clarify the taxonomic status of not only *F. kittipongi* (our focal species) but also of the other 2 congeners *F. signifer* and *F. oxyrhynchus*. Future DNA barcoding effort and eDNA metabarcoding applications could include sequencing of the mitochondrial 12S rRNA gene to allow testing of eDNA samples using MiFish, a set of universal PCR primers designed for fish (Miya et al. 2020), and investigation of other more useful non-COI barcode regions for freshwater stingrays (Garcia et al. 2015).

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LITERATURE CITED

Alshari NFMAH, Adnan MHIB, Jamaluddin JAMF, Lavoué S, Nor SAM (2023) DNA contamination and taxonomic diversity hinder eDNA research on freshwater stingray species: Comment on Lim & Then (2022). Endang Species Res 50:311–313

Garcia DA, Lasso CA, Morales M, Caballero SJ (2016) Molecular systematics of the freshwater stingrays (Myliobatiformes: Potamotrygonidae) of the Amazon, Orinoco, Magdalena, Esequibo, Caribbean, and Maracaibo basins (Colombia–Venezuela): evidence from three mitochondrial genes. Mitochondrial DNA Part A 127:4479–4491

Lim KC, Then AY (2022) Environmental DNA approach complements social media reports to detect an endangered freshwater stingray species in the wild. Endang Species Res 48:43–50

Miya M, Gotoh RO, Sado T (2020) MiFish metabarcoding: a high-throughput approach for simultaneous detection of multiple fish species from environmental DNA and other samples. Fish Sci 86:939–970

Naylor GJP, Caira JN, Jensen K, Rosana KAM, White WT, Last PR (2012) A DNA sequence-based approach to the identification of shark and ray species and its implications for global elasmobranch diversity and parasitology. Bull Am Mus Nat Hist 367:1–262

Tan MP, Jamsari AFJ, Siti Azizah MN (2012) Phylogeographic pattern of the striped snakehead, *Channa striata* in Sundaland: ancient river connectivity, geographical and anthropogenic signatures. PLOS ONE 7:e52089

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