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Short Communication

Preliminary genetic assessment of geographically distant populations of the giant freshwater stingray, *Urogymnus polylepis* (syn. *Himantura chaophraya*), calls for taxonomic re-examination

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Abstract

The giant freshwater stingray, previously classified as *Himantura chaophraya* Monkolprasit & Roberts 1990, has been re-named as *Urogymnus polylepis*. Populations of this species have been reported in Indonesia, Myanmar, Bangladesh, and India, while the populations in Thailand have been reported in three rivers draining the Central Plains and the Mekong River. The presence of two different names in Thai documents has led to confusion in legislative references. To clarify the difference between these names, we employed molecular markers to examine genetic differentiation among geographically distant populations. Our findings revealed a large genetic divergence between the populations in Thailand and *U. polylepis* of the Indian Subcontinent and Greater Sunda Islands. This level of differentiation is worth a re-examination of biological characteristics of these stingrays. The additions of samples from Indonesian islands, Myanmar, Bangladesh, and India, along with the examination of more molecular markers should allow the clarification of the taxonomic relations of *U. polylepis* and *U. chaophraya*. Such efforts will benefit conservation protection of all giant freshwater stingray populations throughout their distribution ranges.

Keywords: COI, *Himantura chaophraya*, genetic differentiation, population, *Urogymnus polylepis*

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1. Introduction

The giant freshwater stingray was first described as *Trygon polylepis* (Bleeker, 1852) from the Java Sea and is currently assigned as *Urogymnus polylepis* (Last, Naylor, & Manjaji-Matsumoto, 2016), though it originally lacked detailed morphological description. Ostensibly similar species have since been reported in different geographic regions. In Thailand, a population of the giant freshwater stingrays was described in 1990 as *Himantural chaophraya* based on specimens collected from the Chao Phraya River, approximately 100 km upriver from the Gulf of Thailand. This description included detailed morphological characteristics (Monkolprasit & Roberts, 1990). However, the presence of both names, *Himantural chaophraya* and *Urogymnus polylepis*, in many Thai documents has led to species identification confusion. This ambiguity could create legal loopholes in the enforcement of wildlife protection law, as this species is listed as a protected species under the Wildlife Conservation and Protection Act B.E. 2562 (2019) (WARPA).

Further populations of this species have been reported in Peninsular Malaysia (Iqbal, Setiawan, Windusari, Yustian, & Zulkifli, 2020), Borneo (Windusari, Iqbal, Hanum, Zulkifli, & Yustian, 2020), Indonesia (Iqbal, Yustian, Setiawn, Nurnawati, & Zulkifli, 2020), India (Sen, Dash, Kizhakudan, Chakraborty, & Mukherjee, 2021), and Myanmar (Grant *et al*., 2022). Furthermore, a COI sequence submission in GenBank (Accession No. MZ363899) indicated the presence of this species in Bangladesh. Since geographic isolation may influence genetic distinctiveness among stingray populations (Khudamrongsawat *et al*., 2017; Sezaki *et al*., 1999), we examined the haplotype diversity of existing giant freshwater stingray populations. The objective of this study was to examine patterns of geographic differentiation among these populations using cytochrome c oxidase subunit I (COI) as a genetic marker. A preliminary genetic comparison of geographically distant populations may help clarify taxonomic uncertainty surrounding the named taxa that has arisen in the relative sparsity of morphological data. This, in turn, could support conservation efforts of the potentially threatened species or species group.

2. Materials and Methods

There were 14 samples of the giant freshwater stingrays collected from the Mae Klong (MK) and Bangpakong (BPK) Rivers of Central Thailand; one sample from the Mekong River (gfs_Mekong) in Bueng Kan Province, Northeast Thailand; single samples of *U. polylepis* from the Siak River, Sumatra (SU1), and the Citarum River in Java (JKT280218), Indonesia (Appendix 1). All tissue samples were collected and stored at the Veterinary Medical Aquatic Animal Research Center, Faculty of Veterinary Medicine (VMARC), Chulalongkorn University, Thailand. DNA samples were extracted using a commercial DNA extraction kit (NucleoSpin-Macherey-Nagel, Germany). Primers for COI amplification were obtained from Ward, Zemlak, Innes, Last, and Hebert (2005). Fragments of COI were amplified following standard PCR protocol. Successfully amplified products were cleaned using NucleoSpin gel and PCR clean-up kit (Macherey-Nagel, Germany) and sequenced using the BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.) on an ABI 3730xl capillary sequencer, and sequenced.

Sequence alignment and correction was done using MEGA X (Kumar, Strecher, Li, Knyaz, & Tamura, 2018). Final alignments of COI were 653 base pairs (bp). All sequences were deposited in the NCBI database (Appendix 1). Additional 4 COI sequences from GenBank (MK978688, MK978689, MZ363899, MZ363900) obtained from samples in India and Bangladesh were included for phylogenetic analyses. A phylogenetic tree was constructed based on COI fragments of 622 bp using maximum likelihood (MEGA X). The selected model for ML was HKY (bootstrap support values = 1,000 iterations). A Median-Joining haplotype network (Bandelt, Forster, & Röhl, 1999) based on COI fragments was also constructed to visualize population differences between stingray populations from Thailand and others using PopART (ver. 1.7 for Windows) (Leigh & Bryant, 2015). Intraspecific genetic distance for populations in Thailand and for populations in Indonesia, India, and Bangladesh as well as interspecific genetic distances among Thailand populations and elsewhere were calculated using Kimura 2-parameter (K2P) using bootstrap support values of 1,000 iterations performed in MEGA X.

3. Results

Of the 17 samples examined, there were five different haplotypes and 21 polymorphic sites that consisted of 5 singletons and 16 parsimony informative sites (Table 1). The sample from the Mekong River differed from the Central Thailand samples by one base. Two samples of *U. polylepis* from Java and Sumatra showed an identical haplotype and differed from the Central and Northeast Thailand samples by several bases.

With the addition of GenBank COI sequences of *U. polylepis* from Bangladesh and India to the analysis, all examined samples described under *U. polylepis* and *chaophraya* formed a monophyletic group. This group is separated in two lineages consisting of group A (samples from Java + Sumatra + India + Bangladesh) and group B (samples from the Central Plains and Mekong Drainage populations in Thailand) (Figure 1). The COI haplotype network also showed two distinctive groups, which corresponded to the phylogenetic tree (Figure 2). Genetic variation within the populations of the Indonesian islands, India, and Bangladesh was 0.69% (±0.22%) and among those in central Thailand and Mekong River was 0.11% (±0.05%). The average genetic distance between these two groups and within groups was 3.24% and 0.27%, respectively.

4. Discussion

Molecular markers serve as valuable tools for ichthyologists to resolve taxonomic confusion among species complexes and to uncover cryptic species (White & Last, 2012). Our preliminary study employed the use of COI, a barcoding region for the identification of fishes (Ward *et al*., 2005), which distinguished the giant freshwater stingray populations in Thailand and Mekong River currently designated as *U. chaophraya* (syn. *Himantura chaophraya*) and the populations from Sumatra, Java, Bangladesh, and India recognized as *U. polylepis*. COI sequence divergence in

Table 1. COI haplotypes and nucleotide diversity of *U. polylepis* syn. *U. chaophraya* from central Thailand, Mekong River, and rivers in Indonesia

Sample	Polymorphic site																					
	1 ..	37	93	166	179	184	246	250	311	322	334	349	395	478	499	518	521	553	562	601	604	613
	T	A	A	C	G	T	T	C	A	C	T	T	G	C	G	T	T	G	T	G	A	C
MK310317-1M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BPK2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BPK050116-1F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BPK171215-1M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MK080317-2F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MK091109-1F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MK101109-1M	.	.	**C**	.	.	.	**G**	.	**T**	.	.	.	.	.	.	.	.	.	.	.	.	.
MK190316-1M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MK201115-2F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MK201115-3M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MK211015-1F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MK221015-1F	.	.	.	.	**C**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MK250509-1F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MK250316-1M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
GFS_MEKONG	.	**G**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
JKT280218	.	.	.	**T**	.	**C**	.	**T**	.	**T**	**C**	**C**	**A**	**T**	**A**	**C**	**C**	**A**	**C**	**A**	**G**	**T**
SU1	.	.	.	**T**	.	**C**	.	**T**	.	**T**	**C**	**C**	**A**	**T**	**A**	**C**	**C**	**A**	**C**	**A**	**G**	**T**

White shade = samples from central Thailand and Mekong River in Thailand side; Gray shade = samples from Indonesian island

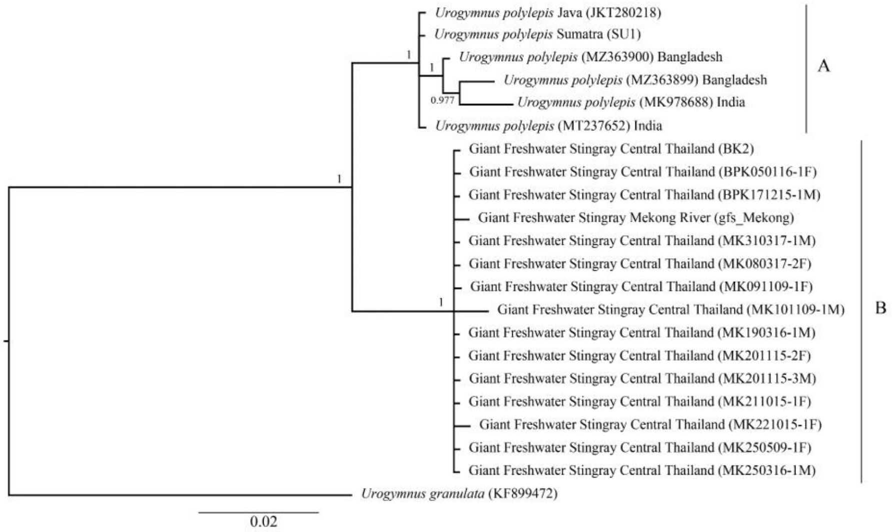

Figure 1. Maximum Likelihood phylogenetic tree of (A) COI for *Urogymnus polylepis* (syn. *Himantura chaophraya*-Giant Freshwater Stingray in central Thailand)

different populations of a single taxon was generally less than 2% for most of fishes (Ward, 2009), but different populations of some taxa presently recognized as conspecific may show sequence divergence greater than 2% (Cerutti-Pereyra *et al*., 2012), which depends on the evolution of different lineages (Ward, 2009). Our study revealed substantial genetic differentiation (> 2%) among populations of the giant freshwater stingrays, which likely indicates the possibility of species differentiation similar to the previous study using cytochrome b sequences and amino acid sequences that

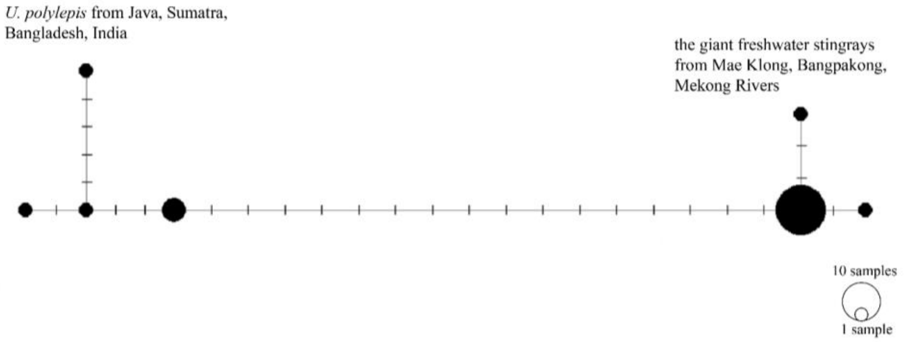

Figure 2. The COI median-joining haplotype network *U. polylepis* populations from Indonesian islands, Bangladesh, India and the giant freshwater stingray (syn**.** *Himantura chaophraya*) populations from central Thailand and Mekong River

reported marked genetic differentiation between *H. chaophraya* in Thailand and India (Sezaki *et al*., 1999). Although a fixed distance threshold for species delimitation is not specified, some proposals have been provided. Herbert, Stoeckle, Zemlak, and Francis (2004) suggested a threshold for a species as 10x the average genetic distance within the groups of study. Based on our data (10 x 0.27% = 2.7%), the populations in this study likely represent distinct species. Alternatively, it has been considered that a higher threshold (ratio of the average genetic distance between groups and within groups greater than 15) should be set for taxonomic re-examination (Ward, Holmes, White, & Last, 2008). Using the latter threshold (3.24/0.27 = 12.00) the current study fell short of this criterion. Additional samples from various geographic locations should provide more powerful analyses. Nevertheless, considering the high genetic distance between groups, we suggest a thorough examination of the giant freshwater stingray populations across their distribution range.

This finding suggests that giant freshwater stingrays are genetically isolated into two groups, with a possible relation to geography. Historical geological events in Southeast Asia and surrounding regions may have contributed to their geographic separation. Analyses of historical biogeography revealed multiple invasions and diversification of Asian freshwater stingrays gives an estimated divergence time between *U. polylepis* and the closely related brackish stingray species, *U. granulata* and *U. lobistoma* as approximately 41 million years ago (Kirchhoff, Hauffe, Stelbrink, Albrecht, & Wilke, 2017). The uplifting Tibetan Plateau causing shallow coastal areas (Royden, Burchfiel, & van der Hilst, 2008), movement of the Indian Subcontinent closer to Southeast Asia during the mid-Eocene (Klaus, Schubart, Streit, & Pfenninger, 2010), and sea level fluctuation in Sunda (Carpenter *et al*., 2011) could have allowed movement as well as isolation of several freshwater species (Bolotov *et al*., 2022; Klaus *et al*., 2010; Yamanoue *et al*., 2011) as well as of marine maskrays, the *Neotrygon kuhlii* complex, which revealed haplotype differentiation between populations in the Indo-Pacific region and India Ocean (Puckridge, Andreakis, Appleyard, & Ward, 2013).

The lack of samples collected at type localities and other locations such as Myanmar (Grant *et al*., 2022) for genetic analyses further obscured taxonomic confirmation for both species. A genetic examination of *U. polylepis* in Malaysia using COI sequences as well as information from this study (cited as GenBank accession numbers) also showed the grouping of populations in Mainland Southeast Asia with those in Malay Peninsula and Borneo and also revealed geographic separation between this group and individuals from Java and Sumatra (Arshaard, Yusof, & Nor, 2024). An integrated effort to discern the taxonomic and phylogenetic relationships of *U. chaophraya* and *U. polylepis* will require both extensive morphological examination and evaluation of ecological characteristics, such as geographic range and breeding seasonality. The morphology of *H. chaophraya* has been described in detail (Monkolprasit & Roberts, 1990) but may require additional investigation while that of *U. polylepis* (Bleeker, 1852) has not yet been comprehensively examined. Few ecological characteristics have been described for the populations in Chao Phraya and Mae Klong Rivers in Thailand and have revealed differences in age and growth rates of these populations (Phomikong, Seehirunwong, & Juatagate, 2019) while these characteristics in other geographic populations remain undescribed and may provide valuable support for taxonomic clarification.

5. Conclusions

We recognized genetic differentiation between different geographic populations of the giant freshwater stingrays. Additional samples throughout the distribution range of this species or groups of species with the use of multiple genetic markers and extensive morphological examination will provide taxonomic clarification. Understanding the taxonomic position of the geographic populations of the giant freshwater stingrays will strengthen the regulation of the Wild Animal Reservation and Protection Act in Thailand to protect our natural resources at present and in the future.

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Appendix 1. Voucher collection of all samples from this study

Sample code	Date	Sex	Location	COI GenBank accession number
JKT280218	28 Feb 2018	Female	Java	MH908732
SU1	28 Nov 2017	male	Sumatra	MH908733
BPK2	7 Jan 2013	Unknown	Central Thailand Bangpakong River	MH908734
BPK050116-1F	5 Jan 2016	Female	Central Thailand Bangpakong River	MH908735
BPK171215-1M	17 Dec 2015	Male	Central Thailand Bangpakong River	MH908736
MK080317-2F	8 Mar 2017	Female	Central Thailand Mae Klong River	MH908737
MK091109-1F	9 Nov 2009	Female	Central Thailand Mae Klong River	MH908738
MK101109-1M	10 Nov 2009	Male	Central Thailand Mae Klong River	MH908739
MK190316-1M	19 Mar 2016	Male	Central Thailand Mae Klong River	MH908740
MK201115-2F	20 Nov 2015	Female	Central Thailand Mae Klong River	MH908741
MK201115-3M	20 Nov 2015	Male	Central Thailand Mae Klong River	MH908742
MK211015-1F	21 Oct 2015	Female	Central Thailand Mae Klong River	MH908743
MK221015-1F	22 Oct 2015	Female	Central Thailand Mae Klong River	MH908744
MK250509-1F	25 May 2009	Female	Central Thailand Mae Klong River	MH908745
MK250316-1M	25 Mar 2016	Male	Central Thailand Mae Klong River	MH908746
MK310317-1M	31 Mar 2017	Male	Central Thailand Mae Klong River	MH908747
gfs_Mekong	22 Aug 2018	-	Northeastern Thailand Bueng Kan Province	OP492156