



DNA Barcoding of Endangered Giant Freshwater Whipray *Urogymnus polylepis* (Bleeker, 1852) from North Kalimantan, Indonesia

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ABSTRACT

Stingrays are a subset of Elasmobranchii, a group of fish species that are characterized by their cartilage-based skeletal systems. Within Indonesia, stingrays have consistently held a position of economic significance, particularly for their high export value. However, the combined pressures of over-exploitation and insufficient conservation initiatives have threatened stingray populations in various Indonesian waters. To aid conservation efforts, it's crucial to precisely identify stingray species. This not only helps in establishing their conservation status but also in preserving the genetic integrity of both the species and their ecosystems. While, several methods exist for fish identification, DNA barcoding stands out as a revolutionary molecular-based technique offering swift, accurate, and definitive organism identification. This research aimed to identify the species of stingrays found in the Sesayap River of North Kalimantan Indonesia and delineate the relationships among these species using phylogenetic trees. To construct the phylogenetic tree, we employed the MEGA11 software, utilizing the neighbor-joining algorithm and the Kimura-2 parameter model with a bootstrap value set at 1000. Our findings identified a single stingray species, *Urogymnus polylepis*. The phylogenetic analysis indicates that this species shares a close genetic relationship with a genetic distance of 0.000-0.002 to the *U. polylepis* found in Thailand. Notably, the conservation status of the *U. polylepis* is currently listed as "Endangered (EN)".

INTRODUCTION

Stingrays, functioning as mesopredators, hold a crucial position within the aquatic ecosystem, connecting diverse trophic levels (Domingues *et al.*, 2019). From a biological perspective, they exhibit characteristics like low reproductive rates, sluggish growth, and delayed sexual maturity, with their reproductive patterns being deeply influenced by the hydrological rhythms of waters they inhabit (Renza-Millán *et al.*, 2019; Roycroft *et al.*, 2019). Consequently, freshwater stingrays find themselves at the mercy of environmental fluctuations. In north Kalimantan's Sesayap River, fishermen frequently catch stingrays using both age-old and contemporary fishing techniques. Sometimes, these rays inadvertently end up in their nets. Their primary harvest value lies in their meat, fins, skin, liver oil, cartilage, teeth, and jaws (Vella *et*

al., 2017). Local residents typically savor these freshwater stingrays either grilled or transformed into salted delicacies, and they are even traded across the border to Malaysia.

Rising demand and consumption of freshwater stingrays has ignited worries about the looming extinction of specific ray species. Past studies suggest that excessive hunting of these creatures considerably heightens the vulnerability of the Elasmobranchii species in the Indonesian marine territories (**Mardhiah *et al.*, 2019**). Amplifying these concerns is the diminishing water quality of the Sesayap River, a primary stingray fishing zone in north Kalimantan. The River's degradation can be traced back to its frequent use as a transport route by speedboats, timber, and coal company ships, leading to significant pollution affecting the resident stingrays. Excessive hunting, coupled with aquatic contamination and the ever-present threat of climate change, can erode the genetic diversity of freshwater stingrays (**Then *et al.*, 2022**). A dip in a population's genetic diversity compromises its adaptability, the vitality of its organisms, and promotes the prevalence of recessive genes (**Beever *et al.*, 2016**). Hence, safeguarding stingrays is of paramount importance to mitigate both species and population declines in the wild. The preliminary step in this mission is the accurate identification of stingray species. This becomes especially pertinent given that three freshwater stingray species have now gained protection in Indonesia and feature on the IUCN's Red List as endangered species (**KKP, 2021**).

Traditionally, the classification of stingray species leans on morphological attributes, such as body contour, disc structure, and color patterns (**Then *et al.*, 2022**). However, solely relying on this approach can yield inconsistent results, especially when distinguishing species with hidden, intricate traits, high adaptability in appearance, or diverse color schemes (**Fontenelle *et al.*, 2021**). This has led to many stingrays being erroneously classified or mislabeled (**Vella & Vella, 2021**). These inaccuracies undeniably impede the conservation and management initiatives aimed at freshwater stingrays. A shift towards molecular techniques offers a solution to these challenges.

DNA barcoding stands out as a potent molecular tool embraced for species identification, revered for its precision, efficiency, and universally acknowledged protocols. Its prowess can be attributed to the roughly 655bp sequence found in the mitochondrial DNA COI (Cytochrome Oxidase Subunit I) gene, which remains largely unchanged. This sequence paves the way for meticulous species identification and lineage tracing across a plethora of taxa. The beauty of DNA barcoding lies in the fact that it eliminates the need for deep taxonomic knowledge (**Priyono *et al.*, 2023**). A slew of research efforts corroborates the efficacy and practicality of leveraging DNA barcoding for stingrays, ranging from pinpointing species identities to dissecting intricate species nuances, evaluating species lineage, gauging biodiversity nuances, discerning population architectures, and pinpointing geographic evolutionary paths (**Ory *et al.*, 2019**; **Rizo-fuentes *et al.*, 2020**; **Vella & Vella, 2021**).

MATERIALS AND METHODS

Sample collection

During April 2023, fifteen samples of giant freshwater whipray were collected from Sesayap River, north Kalimantan (Fig. 1, 2). The Sesayap River ranks among the five major Rivers flowing through north Kalimantan. The collection process involved the assistance of local fishers. Each specimen was meticulously documented. A portion of muscle tissue was excised from five of these stingray and placed in a sterile 1.5ml tube, which was filled with 96% ethanol to ensure preservation over an extended period (**Gaffar *et al.*, 2021**). Subsequently, both the preserved muscle samples were transported to the Central of Life Science Laboratory at Universitas Borneo Tarakan in north Kalimantan, Indonesia. For the purpose of further study, the muscle tissue samples preserved in ethanol were stored at a temperature of -20°C .

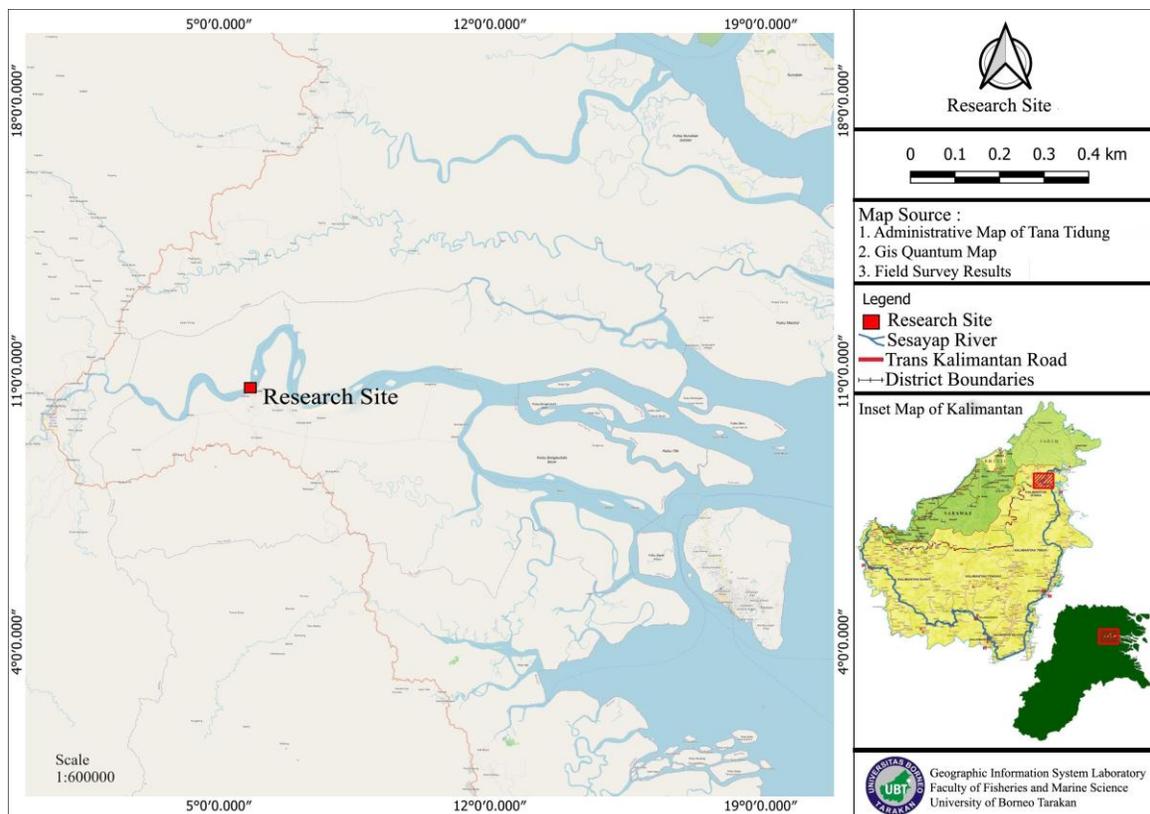


Fig. 1. Research site



Fig. 2. Habitat of stingray at Sesayap River, north Kalimantan, Indonesia

DNA extraction, gene amplification, sequencing and sequence analysis

We implemented the TianGen kit (tissue protocol) to extract genomic DNA from each of the stingray muscle samples, which were each approximately 50 to 100 milligrams in weight. The resulting genomic DNA was then diluted to a total volume of 100 μ L per specimen. The amplification of the COI mitochondrial region was achieved using the primers *Fish FI* 5'TCAACCAACCACAAAGACATTGGCAC3' and *Fish RI* 5'TAGACTTCTGGGTGGCCAAAGAATCA3', as outlined by **Ward *et al.* (2005)**. The polymerase chain reaction (PCR) procedure was undertaken in a 15 μ L, incorporating 7.5 μ L of Quick Taq® HS DyeMix PCR Kit from Toyobo, 2 μ L of the extracted genomic DNA, 1 μ L of each primer, and 3.5 μ L of nuclease free water. These reactions occurred in an Eppendorf X50s Thermal Cycler. The PCR regimen included an initial denaturation at 95°C for 1min., succeeded by 30 cycles alternating between 95°C for 15sec., 50.8°C for 15 sec., and 72°C for 10 sec., concluding with a final extension at 72°C for 2min. The amplified PCR outputs of the stingray were then electrophoresed on 1% agarose gels, stained with FloroSafe DNA Stain provided by 1st BASE. Subsequently, COI sequence reactions were discerned in both forward and

reverse orientations, adhering to the standard procedure with the ABI Big Dye Terminator version 3.1 cycle sequencing kit from Applied Biosystems. The mix contained 5 to 7 μ L of purified PCR product and 0.8 μ L of either primer per reaction. The resultant sequence-reaction products were then introduced into an ABI 3500 Genetic Analyzer, and also by Applied Biosystems, and the amplicons underwent sequencing in both forward and reverse directions.

Information processed by the sequencing analysis software underwent validation through the use of sequence scanner software (Applied Biosystems genetic analyzer instruments). DNA segments within the COI region were examined with DNA Baser (DNA Sequence Assembler v4 (2013)), a tool utilized to generate consensus fragments (Gaffar & Sumarlin, 2021). Following this, each stingray's consensus sequence was translated for the detection of the stop codon, employing the vertebrate mitochondrial code. The COI sequences were subsequently reformatted into fasta files and aligned utilizing ClustalW, part of the MEGA11 software (Kumar *et al.*, 2018). Species identification through sequence similarity search was performed on public databases, namely BLASTn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Using the MEGA 11 software, the genetic distance was determined among sequences and reconstructed the phylogenetic tree as mentioned by Kimura (1980) and Tamura *et al.* (2021). The evaluation of phylogenetic relationships was undertaken employing a neighbor-joining (NJ) algorithm and the Kimura-2 parameter model with 1000 bootstrap replication. For comparison and outgroup, sequences at Table (1) were acquired from GenBank (www.ncbi.nlm.nih.gov).

Table 1. Specimens and sequences used in the phylogenetic analysis for mtCOI gene fragment

Species name	Country	GenBank accession number
<i>Urogymnus polylepis</i>	Thailand	OR395291.1
<i>Urogymnus polylepis</i>	Thailand	MH908734.1
<i>Urogymnus asperrimus</i>	Australia	KC250636.1
<i>Urogymnus asperrimus</i>	India	KT766194.1
<i>Himantura granulata</i>	India	KF899471.1
<i>Himantura granulata</i>	Malaysia	MF039700.1
<i>Brevitrygon imbricata</i>	Saudi Arabia	KU317893.1

RESULTS

Molecular identification of stingrays

In general, the freshwater stingrays observed in this study exhibited certain distinctive characteristics. They possess an oval, flattened body shape that tapers to a point. Their dorsal coloration is a muted brownish-gray, while the underside of their pectoral fins and belly is white. Dark brown spots can be noticed along their posterior edges. They have relatively small eyes that do not protrude outward, and their tails are long, slender, and whip-like in appearance (Fig. 3).

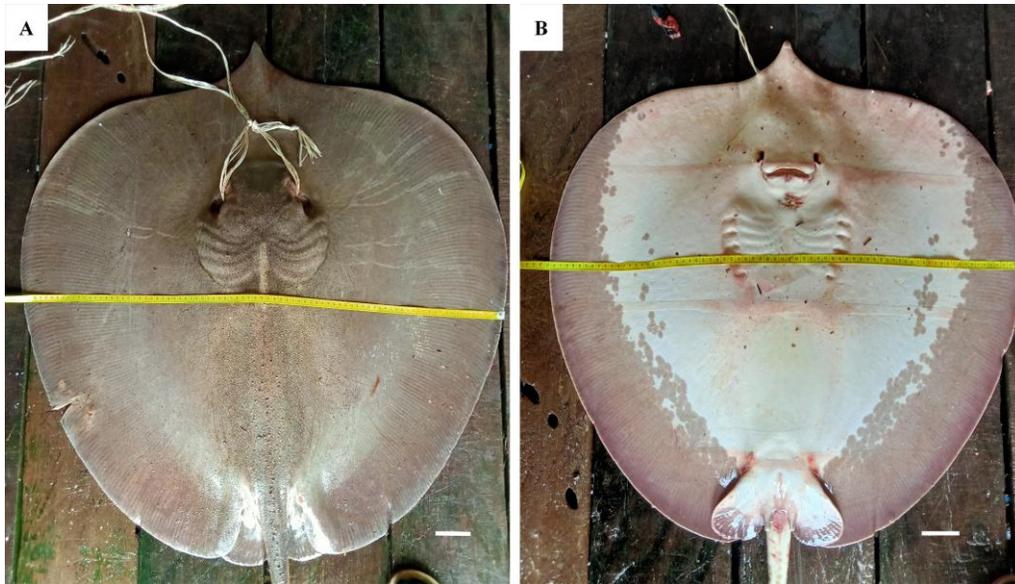


Fig. 3. Giant freshwater whipray from Sesayap River, north Kalimantan showing: **a)** Dorsal side and **b)** Ventral side (Bar Scale = 5 cm)

BLASTN analysis revealed that specimens labeled KUPI01 to KUP105 were identified as the species *Urogymnus polylepis* (Bleeker, 1852), linked with the accession number OR395291.1. The sequences KUPI01 to KUP105 that were successfully identified in BLASTN have nucleotide base lengths in the following respective order: 679, 702, 673, 691, and 683bp. Remarkably, there's a sequence similarity of 100%. This significant percentage suggests that the sample sequences closely mirror the species sequences cataloged in the database, marking the pinnacle of analytical results across all samples. Therefore, based on the BLASTN outcomes, it's conclusive that specimens KUPI01 to KUP105 belong to the *Urogymnus polylepis* species. Detailed BLASTN results for stingray samples collected from the Sesayap River in north Kalimantan can be referenced in Table (2).

Table 2. BLASTN analysis results showing homology matches between stingray samples and the GenBank database

Sample code	Species	Local name	Common name	% Identity	Query cover (%)	GenBank accession no.
KUPI01	<i>Urogymnus polylepis</i>	Mud Stingray	Giant Freshwater Whipray	100	93	OR395291.1
KUPI02	<i>Urogymnus polylepis</i>	Mud Stingray	Giant Freshwater Whipray	100	90	OR395291.1
KUPI03	<i>Urogymnus polylepis</i>	Mud Stingray	Giant Freshwater Whipray	100	94	OR395291.1
KUPI04	<i>Urogymnus polylepis</i>	Mud Stingray	Giant Freshwater Whipray	100	91	OR395291.1
KUPI05	<i>Urogymnus polylepis</i>	Mud Stingray	Giant Freshwater Whipray	100	92	OR395291.1

The length of the sequences analyzed in the phylogenetic construction was 616bp. The resultant phylogenetic portrayal of stingrays from the Sesayap River in north Kalimantan can be viewed in Fig. (4).

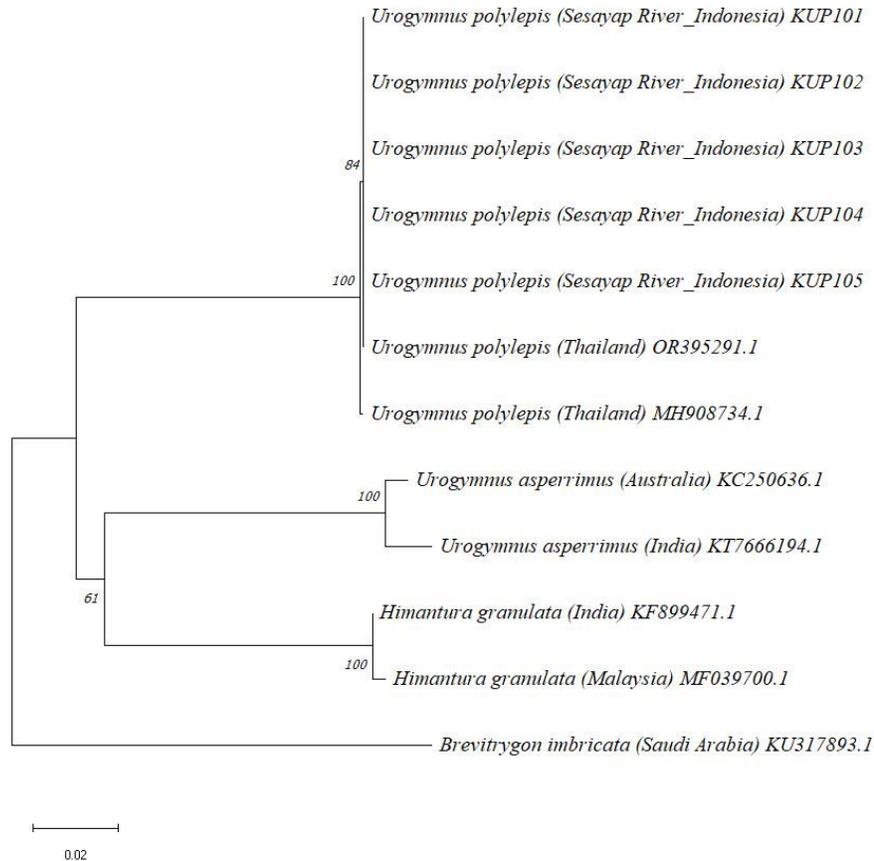


Fig. 4. Stingray phylogenetic tree generated by MEGA11

The analysis of genetic distance was performed using the MEGA11 software. From the analysis, it was evident that the genetic distance among the stingray samples, labeled KUPI01 - KUPI05 of the *Urogymnus polylepis* species from the Sesayap River in north Kalimantan, showed no variation; their genetic distance was zero. However, when comparing these samples to *U. polylepis* from Thailand OR39529.1, a slight genetic distance of 0.002 was observed. When contrasting *U. polylepis* from both Indonesia and Thailand to other stingray species, the intraspecies genetic distances ranged between 0.137 & 0.182 (Table 3).

Subsequent to the stingray identification, the findings were analyzed concerning their conservation status by referencing the IUCN (International Union for Conservation of Nature and Natural Resources) website. The IUCN also advocates for the restriction of trade involving endangered species by endorsing an international agreement, specifically the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The conservation status for stingray species is presented in Table (4).

Table 3. The genetic distance of stingray with sequences from GenBank

Species	Species											
	1	2	3	4	5	6	7	8	9	10	11	12
1												
2	0,000											
3	0,000	0,000										
4	0,000	0,000	0,000									
5	0,000	0,000	0,000	0,000								
6	0,000	0,000	0,000	0,000	0,000							
7	0,002	0,002	0,002	0,002	0,002	0,002						
8	0,146	0,146	0,146	0,146	0,146	0,146	0,149					
9	0,151	0,151	0,151	0,151	0,151	0,151	0,153	0,017				
10	0,137	0,137	0,137	0,137	0,137	0,137	0,139	0,138	0,145			
11	0,141	0,141	0,141	0,141	0,141	0,141	0,144	0,134	0,140	0,003		
12	0,182	0,182	0,182	0,182	0,182	0,182	0,180	0,190	0,204	0,182	0,187	

Notes:

1. KUPI01 *Urogymnus polylepis* (Sesayap River, North Kalimantan, Indonesia)
2. KUPI02 *U. polylepis* (Sesayap River, North Kalimantan, Indonesia)
3. KUPI03 *U. polylepis* (Sesayap River, North Kalimantan, Indonesia)
4. KUPI04 *U. polylepis* (Sesayap River, North Kalimantan, Indonesia)
5. KUPI05 *U. polylepis* (Sesayap River, North Kalimantan, Indonesia)
6. OR395291.1 *U. polylepis* (Thailand)
7. MH908734.1 *U. polylepis* (Thailand)
8. KF899471.1 *Himantura granulata* (India)
9. MF039700.1 *H. granulata* (Malaysia)
10. KC250636.1 *U. asperrimus* (Australia)
11. KT766194.1 *U. asperrimus* (India)
12. KU317893.1 *Brevitrygon imbricate* (Saudi Arabia)

Table 4. Stingray conservation status based on IUCN and CITES

Specimen code	Species	IUCN status	CITES	Threat to humans
KUPI01 - KUPI05	<i>Urogymnus</i> <i>polylepis</i>	Endangered (EN)	Not evaluated	Venomous

DISCUSSION

Molecular identification techniques are applied to stingrays to ascertain their exact species. Based on sequencing data, the five stingray samples labeled as KUPI01 through KUPI05 were confirmed to be *Urogymnus polylepis*, also known as the Giant Freshwater Whipray. They showcased a remarkable sequence similarity of 100%. This lofty percentage suggests an identical match of our sample sequences with the species data archived in the database, ranking as the pinnacle BLASTN analysis result for each sample. Essentially, the higher the score, the closer the resemblance between the investigated sequence and the reference

sequence in the database. According to **Yang et al. (2014)**, a similarity range of 98-100% with species registered in GenBank suggests that the examined specimens can be confidently identified as that species due to their high resemblance. Moreover, this likeness between the sample and the database sequences is further quantified by the similarity in the percentage values. A similarity score as high as 100% typically denotes that the compared species are identical (**Bhattacharjee et al., 2012**). Thus, drawing from the BLASTN results, it's clear that specimens KUPI01 to KUPI05 belong to the *Urogymnus polylepis* species. The sequencing outcomes were subsequently used to craft a phylogenetic tree. The absence of matching species from Indonesia in the BLASTN results suggests that the nucleotide data from this study represent the first records based on the COI gene.

The phylogenetic tree constructed in this research incorporates five sequences, bolstered by the addition of sequence data sourced from GenBank. In total, we assessed seven specimens of *U. polylepis*, two specimens each of *H. granulate* and *U. asperrimus*, and a lone outgroup specimen *B. imbricata*. For constructing an accurate phylogenetic tree, an outgroup species from GenBank is essential. We selected the stingray species *B. imbricata* as our outgroup for the Dasyatidae family, following the guidelines of **Froese and Pauly (2021)**. This outgroup species offers a reference point, aiding in the delineation of species within the primary group, or ingroup. With the outgroup in place, it facilitates a more robust classification of relationships among individuals and species (**Jamil, 2019**). The reconstruction of the phylogenetic tree employed the neighbor-joining method, supplemented with the Kimura-2 parameter model and a bootstrap value set at 1000.

Upon analyzing the phylogenetic tree of stingray samples from the Sesayap River in north Kalimantan, we discerned three primary clades within the Dasyatidae family. The *U. polylepis* defines the first clade, *H. granulate* stands out in the second, and the third is characterized by *U. asperrimus*. Further details revealed that the *U. polylepis* clade includes sequence codes OR395291.1 and MH908734.1, both originating from Thailand and obtained from GenBank. Intriguingly, the phylogenetic tree, constructed using the neighbor-joining method, confirms that all specimens, regardless of whether they are from Indonesia or Thailand, group together in one clade. This lack of differentiation between locations or populations insinuates a mixed population. Concerning the *H. granulate* clade, it comprises sequences KF899471.1 from India and MF039700.1 from Malaysia. On the other hand, the *U. asperrimus* clade is marked by sequences KC250636.1 (Australia) and KT766194.1 (India).

The phylogenetic tree, showcased in Fig. (4), has a scale of 0.020, suggesting that for every 100 nucleotide sequences, there are two distinct bases in each branch. We can deduce species identities through the unique branching patterns forming distinct groups on the stingray's phylogenetic tree. The bootstrap value, ranging between 1-100%, reflects the repetition accuracy from 1000 iterations for branching determinations. Since the bootstrap value approaches 100%, the stability and accuracy of the phylogenetic tree's branching increases (**Soltis & Soltis, 2003**). In our study, a bootstrap value of 100 at each branch implies a high degree of accuracy in our clade delineations.

The stingray samples KUPI01 through KUPI05 from Sesayap River have a genetic distance of 0.000, indicating that these five sequences are from species closely related to one another. For the genetic distance in *U. polylepis* in Indonesia and Thailand had diversity about 0.000 and 0.002. This tiny genetic distance, less than 2%, underscores their likely classification as the same species (Nei, 1972). The *U. polylepis* stingray, present in both Kalimantan and Thailand, exhibits a marked genetic connection. This relationship can be traced back to the stingray's impressive ability to thrive in diverse environments from freshwater and estuaries to coastal waters (Campbell *et al.*, 2023). The similarities might also arise from comparable aquatic conditions. Past studies have highlighted the environment's pivotal influence on the genetic diversity of fish (Leeuwen *et al.*, 2018). Additionally, the merging of the Southeast Asian landmass into Sundaland during the ice age paved the way for these stingrays to migrate between the waters of Kalimantan and Thailand. This genetic exchange has been sustained over centuries, passing inherited traits from one generation to the next (Kurniawan *et al.*, 2022). When comparing the Sesayap River stingray sequences with those from other regions, the genetic differences vary between 0.146 and 0.182. This suggests that for every 1,000 nucleotide sequences, there are 146 bases that differ. In addition, the most distant genetic comparison is with KU317893.1 *B. imbricata* from Saudi Arabia, with a divergence of 0.182, meaning that out of every 1,000 nucleotide sequences, 182 bases differ.

U. polylepis is currently classified as 'Endangered' (EN), as indicated by Grant *et al.* (2021). This designation, conferred by the International Union for Conservation of Nature (IUCN), denotes a species that faces a markedly elevated risk of extinction in the wild. Such an assessment is derived from various metrics, including the rate of decline, current population size, geographic distribution, and the fragmentation of both population and habitat. On the trade front, the convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) categorizes this stingray species as 'Not Evaluated'. This implies that the species has not undergone evaluation against trade criteria and is, for now, considered appropriate for international trade. Accurate species identification serves dual purposes. For one, it ensures transparency in trade and offers precise information to consumers. Secondly, it plays a pivotal role in the efficient management and conservation of marine resources. Given the potential for misidentification—which can distort species-specific catch data—relying on DNA analysis emerges as a precise and reliable identification tool. The study exploring the genetic identification of the *U. polylepis* stingray from the Sesayap River in north Kalimantan, Indonesia, based on the COI mitochondrial gene, represents a pioneering effort in this domain. This groundbreaking discovery could significantly inform the basis management and conservation efforts for the species. Moreover, the genetic data accumulated will be instrumental in arranging the COI library of *U. polylepis* within Indonesia.

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