

MOLECULAR IDENTIFICATION OF EVOLUTIONARILY SIGNIFICANT GANGES RIVER DOLPHINS (*PLATANISTA GANGETICA*) USING COMPLETE MITOCHONDRIAL CYTOCHROME-B GENE SEQUENCES

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A stranded Platanista gangetica carcass was molecularly identified using a 359 bp mitochondrial cytochrome-b gene fragment. DNA was extracted and sequenced, with phylogenetic analysis confirming its evolutionary placement among South Asian isolates. The generated sequence (GenBank: PQ212750) highlights the subspecies' genetic distinctiveness and conservation urgency. This study reinforces the critical need for molecular tools in species verification and emphasizes integrated strategies to conserve this evolutionarily significant freshwater cetacean endemic to the Ganges-Brahmaputra-Meghna river system.

Introduction

The Ganges River dolphin (*Platanista gangetica*), locally known as the susu, is a critically endangered and evolutionarily distinct cetacean, representing the only surviving species of the family Platanistidae^{1,2}. Its phylogenetic placement among riverine odontocetes including the Amazon (*Inia geoffrensis*), La Plata (*Pontoporia blainvillei*), and Yangtze River dolphins (*Lipotes vexillifer*) has long sparked taxonomic and evolutionary discourse due to deep morphological divergences and limited molecular data^{5,13,37}. Recent advancements in molecular phylogenetics have refined our understanding, yet evolutionary relationships within this group remain partially unresolved, underscoring the need for further integrative studies encompassing genomics, paleobiology, and biogeography. The South Asian river dolphins of the genus *Platanista* are critically endangered, with the genus being a unique representative of the family Platanistidae^{3,4,26}. This iconic freshwater species is currently

represented by two subspecies: the Indus River dolphin (*P. gangetica minor*), restricted to the Indus River system of India and Pakistan, and the Ganges River dolphin (*P. gangetica gangetica*), which inhabits the Ganges, Brahmaputra, Meghna, and Karnaphuli–Sangu rivers across India, Bangladesh, Nepal, and Bhutan^{2,10,26–28}. Despite the Ganges being a vital and sacred river for over 600 million people (The World Bank Report No. STEP705, 2011)⁴⁰, it faces severe anthropogenic pressures that jeopardize its biodiversity. The Gangetic dolphin, as the largest mammal of the Ganges River, contends with numerous threats, including pollution, dam construction, barrages, reduced water levels, and illegal hunting for oil and meat, the latter of which is used in catfish fishing operations²⁹. These enduring threats have led to a significant reduction in both the dolphin's distribution range and population size^{26,27}. Consequently, the species is classified as “Endangered” on the IUCN Red List^{1–3} and is protected under Schedule I of the Indian Wildlife (Protection) Act, 1972, as well as listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and Appendix II of the Convention on Migratory Species (CMS). Furthermore, the taxonomic classification

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of *Platanista* remains a subject of ongoing debate due to its complex evolutionary history^{5,13-15,37,41}.

The Ganges River dolphin, a critically endangered freshwater cetacean, is an emblem of the rich aquatic biodiversity of the Ganges River Basin. This species, endemic to the rivers of the Indian subcontinent, faces numerous challenges that threaten its survival, including habitat degradation, pollution, and anthropogenic pressures¹⁸. Despite concerted conservation efforts, the stranding of *P. gangetica* carcasses along the Ganges River, particularly in Balagarh, West Bengal, highlights ongoing and emerging management challenges and illustrates the importance of further research. Recent studies have revealed a worrying trend in the population dynamics of the Ganges River dolphin. A comprehensive assessment of dolphin abundance and prey availability in the upper Ganges, indicating a decline in population due to habitat degradation and human activities³⁹. Similarly, it has been observed a potential range decline in the Sundarbans, further emphasizing the adverse effects of habitat loss and pollution on dolphin populations²¹. Its unique evolutionary trajectory has been illuminated through phylogenetic analyses offering crucial insights into its genetic distinctiveness and deep divergence within the Cetacea clade³⁶. However, this evolutionary legacy is increasingly imperilled by a mosaic of ecological, anthropogenic, and climatic threats. Another research emphasized the species' ecological specificity, particularly its reliance on select prey species, making it highly sensitive to changes in aquatic food webs²³. Researcher outlined key stressors, including habitat fragmentation, hydrological alterations, and pollution³². Comprehensive assessments documented alarming population declines, underscoring the urgent need for targeted conservation frameworks^{9,35}. Human-induced pressures continue to erode dolphin habitats, as illustrated by Wakid (2009)⁴⁸ in the Brahmaputra and Bashir et al. (2010)³⁹ in the Ganga, while socio-economic dynamics, explored by Choudhury et al. (2010)^{1,2}, complicate conservation efforts. Dolphin strandings are on the rise, highlighting the inadequacy of current management systems. Regionally, assessed population statuses and anthropogenic threats in the Sundarbans and Turag River, respectively^{3, 19, 20}. Additionally, anatomical investigations by provide a physiological context vital for understanding the species' vulnerabilities. Despite these endeavors, recent studies^{4,7,10,15,22,30}. It has been emphasize persistent gaps and call for molecular tools, habitat modeling, and integrative monitoring to inform evidence-based

conservation. In this context, the present study aims to advance the molecular identification of *P. gangetica* and reinforce its evolutionary and conservation significance.

The stranding of *P. gangetica* carcasses in Balagarh, West Bengal, serves as a stark reminder of the urgent need for improved management and research efforts. Understanding the factors contributing to these strandings, including environmental changes, human impacts, and potential disease outbreaks, is key to creating effective conservation strategies. The synthesis of current research highlights the importance of continued monitoring, habitat protection, and community engagement in ensuring the survival of this critically endangered species. In conclusion, the Ganges River dolphin faces numerous challenges that require a comprehensive strategy for conservation. The recent strandings of dolphin carcasses in Balagarh underscore the pressing need for targeted research and management strategies to address the ongoing threats to this iconic species. By integrating ecological, genetic, and conservation perspectives, we can develop effective solutions to ensure the long-term survival of *P. gangetic* and preserve the biodiversity of the Ganges River Basin.

Materials and Methods

Specimen Collection and Identification: On May 12, 2022, a carcass of *P. gangetica* (Ganges River dolphin) was discovered stranded on the shores of the River Ganga at Sabuj Dweep, Balagarh, Hooghly, West Bengal, India (23.151932N, 88.450037E). The specimen was initially identified through its external morphological characteristics. For precise species identification, a single dead dolphin specimen was selected for detailed molecular analysis. Muscle tissue and fin clips were meticulously excised and preserved in 95% ethanol to maintain the integrity of the genetic material for subsequent molecular characterization.

DNA Extraction from Collected Tissue and Fin Clips: DNA extraction was performed using a modified phenol-chloroform-isoamyl alcohol protocol as described by Sambrook et al. (1989). The preserved muscle tissue and fin clips were homogenized, and lysis was achieved with a buffer containing proteinase K. The lysate underwent sequential phenol-chloroform-isoamyl alcohol extraction to isolate DNA, effectively removing proteins and other contaminants. The quality and integrity of the extracted DNA were assessed using 0.9% agarose gel electrophoresis, allowing visualization of DNA bands under UV light. After conducting a quality assessment, we aliquoted the DNA samples and stored them at -20°C to facilitate further genetic analyses.

Amplification of Cytochrome b Gene by Polymerase Chain Reaction (PCR): The cytochrome b (Cytb) gene was amplified from the extracted DNA using Polymerase Chain Reaction (PCR). The primers employed were F- AAAAAGCTTCCATCCAACATCTCTCAGCATGATGAAA and R- AAAGTGCAGCCCCTCAGAATGATATTTGTCCTCA. PCR was conducted in a total volume of 30 µl, which included 3 µl of 10X assay buffer (100 mM Tris-HCl, pH 8.3; 15 mM MgCl₂; 50 mM KCl; 0.1% BSA), 0.6 µl of each dNTP (GeNei, Bangalore), 1 µl of each forward and reverse primer, 0.2 µl of Taq DNA polymerase (GeNei, Bangalore), and 2 µl of extracted DNA. The PCR thermal cycling conditions were set as follows: an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of 30 seconds at 94 °C for denaturation, 1 minute at 55 °C for annealing, and 1.5 minutes at 72 °C for extension, concluding with a final extension at 72 °C for 10 minutes and a hold at 4 °C. Amplified products were confirmed via electrophoresis on 1.5% agarose gels.

DNA Sequencing and Analysis of Cytochrome b Gene: The PCR products were subjected to Sanger dideoxy sequencing at Barcode Biosciences (BBS), Bengaluru. Sequencing aimed to determine the nucleotide sequence of the Cytb gene. The obtained sequences were analyzed using BioEdit software for initial data handling and alignment. To confirm species identity, sequence comparisons were made with known sequences in the GenBank database using BLAST software. This comparative analysis enabled accurate species identification based on sequence homology.

Data Analysis (Phylogenetic Tree Construction and Genetic Distance Analysis): Phylogenetic relationships among dolphin species were assessed using the sequences obtained from this study, compared with related species available in the NCBI database¹⁷. Phylogenetic tree construction was performed using MEGA 11 software, employing the Neighbor-Joining (NJ) method to visualize evolutionary relationships. Genetic distances were calculated using the Kimura-2 parameter model, providing insight into the genetic divergence among species and supporting species identification through molecular data.

Structure of a Reference Database: To aid in the precise identification of specimens, sequence data from this study were compiled and stored in a reference database on GenBank. This database includes validated barcode data and serves as a reference for species identification. Gene sequences of approximately 359 bp from the 5' regions of mitochondrial genes, specifically Cytb, were utilized for molecular taxonomy. The neighbor-joining method was used to clarify the family connections among dolphins,

allowing for a detailed study of genetic differences within and between dolphin species.

Results

Molecular Confirmation and Phylogenetic Placement of *Platanista Gangetica*: The complete mitochondrial cytochrome-b (Cytb) gene of *Platanista gangetica* was successfully amplified and sequenced using species-specific primers. The resultant 359 bp sequence, submitted to GenBank under the accession number PQ212750, was used to assess phylogenetic affinities and evolutionary distinctiveness within the order Cetacea. To delineate the evolutionary position of the Ganges River dolphin, a comprehensive phylogenetic tree was constructed using the Neighbor-Joining (NJ) method with bootstrap replicates in MEGA 11. The analysis included Cytb sequences from representative freshwater and marine cetacean taxa, including *Sousa plumbea*, *Orcaella brevirostris*, *Balaenoptera acutorostrata*, *Kogia sima*, and various *Platanista gangetica gangetica* isolates from different geographic locales (Bangladesh, USA, China, and Belgium), alongside the newly sequenced Indian isolate.

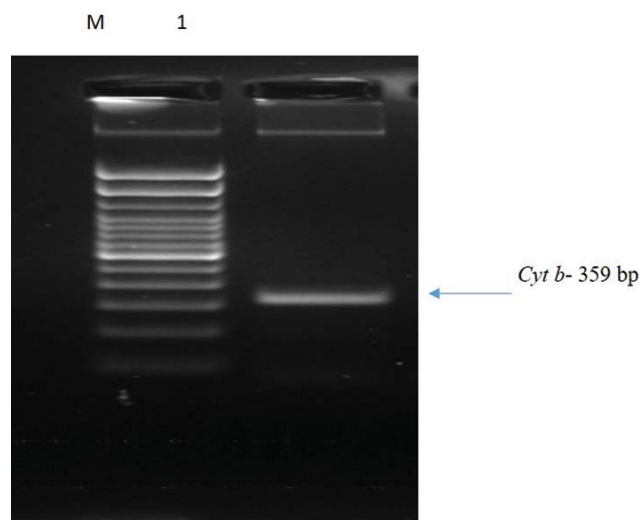


Fig. 1. PCR amplification of the mitochondrial cytochrome-b gene from *P. gangetica*

Nucleotide Composition of the Cytochrome b Gene: The nucleotide composition of the cytochrome b (Cytb) gene from the Ganges Dolphin was analyzed to elucidate the genetic characteristics of the specimen collected from the River Ganga. The PCR amplification yielded a 359 bp product, using the forward primer F- AAAAAGCTTCCATCCAACATCTCTCAGCATGATGAAA and the reverse primer R- AAAGTGCAGCCCCTCAGAATGATATTTGTCCTCA. The sequence data of the Cytb gene revealed the following nucleotide composition: the GC content was calculated to be 45.7%, indicating a

balanced ratio of guanine (G) and cytosine (C) bases. The sequence was compared against known databases to confirm its identity, and the nucleotide sequence comprised a consistent pattern typical for the species. The obtained sequence was meticulously examined and confirmed through sequence alignment and comparison with reference sequences from the GenBank database, ensuring accuracy in species identification. The nucleotide analysis utilized standard methods for calculating GC content and sequence alignments. The sequence showed a high similarity to previously documented sequences of *P. gangetica*, underscoring the reliability of the Cytb gene as a marker for molecular identification. The findings from this study contribute to a more detailed understanding of the genetic makeup of *P. gangetica* and validate the use of the Cytb gene in molecular taxonomy.

Phylogenetic Tree Topology, Bootstrap Support and Analysis of *Platanista gangetica* and Related Dolphin Species from the Ganga River: As illustrated in Figure 1, the phylogenetic reconstruction reveals a robust monophyletic clustering of *P. gangetica* sequences, distinctly separated from other delphinid and mysticete lineages. The Indian isolate (PQ212750) forms a coherent subclade with Bangladeshi isolates (e.g., OB200944, MW287994) and the Belgian isolate (AF304070), with moderate to high bootstrap support values (93% and 59%). This clustering signifies genetic coherence across geographically distributed populations of *P. gangetica*, while still highlighting regional divergence.

The Indian isolate's distinct placement and proximity to South Asian counterparts validate its taxonomic identity as *P. gangetica*, a subspecies uniquely endemic to the Ganges-Brahmaputra-Meghna River system. The presence of *P. gangetica* sequences from China and the USA clustering slightly apart with bootstrap support values of 41% and 99%, respectively, may reflect either historical dispersal, misidentified samples, or captive/reintroduced individuals, underscoring the necessity for further molecular verification. The basal placement of species like *Sousa plumbea* (India) and *Orcaella brevirostris* (India) further accentuates the divergence between marine and freshwater cetacean lineages.

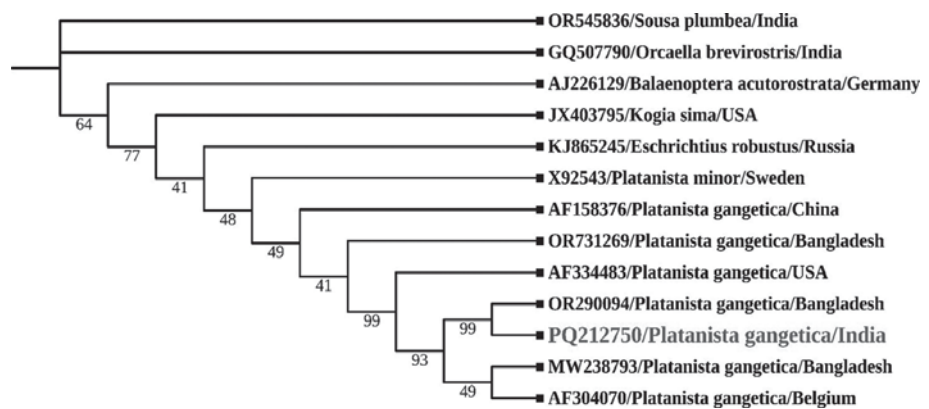


Fig. 2. Phylogenetic analysis of *P. gangetica* and related cetacean species based on mitochondrial cytochrome-b (Cytb) gene sequences

The distinct monophyletic grouping of *P. gangetica* isolates, along with the strong bootstrap support and clear genetic partitioning from other cetacean species, establishes the unique evolutionary trajectory of the Ganges River dolphin. The Indian isolate (PQ212750) contributes a vital data point affirming the endemic genetic structure within Indian river systems, reinforcing the subspecies' evolutionary and conservation significance. These results solidify the mitochondrial Cytb gene as an effective molecular marker for species-level identification and phylogenetic inference among odontocetes. Furthermore, the genetic distinctiveness of *P. gangetica* substantiates its designation as an Evolutionarily Significant Unit (ESU), demanding targeted conservation and protection under both national and international biodiversity frameworks.

Neighbor-Joining (NJ) phylogenetic tree depicting the evolutionary relationships among *P. gangetica* isolates from various geographic regions and representative cetacean species based on complete mitochondrial cytochrome-b gene sequences. The tree was constructed using MEGA 11 with bootstrap replicates; bootstrap values $\geq 40\%$ are indicated at the nodes. The newly sequenced Indian isolate of *P. gangetica* (accession number PQ212750, highlighted in purple) clusters within a monophyletic clade comprising Bangladeshi, Belgian, and Chinese *Platanista* isolates, reflecting shared ancestry and regional genetic continuity. Outgroup taxa include marine and riverine cetaceans such as *Sousa plumbea*, *Orcaella brevirostris*, *Balaenoptera acutorostrata*, *Kogia sima*, and *Eschrichtius robustus*, supporting the evolutionary distinctiveness of *Platanista*. This molecular phylogeny underscores the unique evolutionary lineage of the Ganges River dolphin and highlights its status as an Evolutionarily Significant Unit (ESU), warranting dedicated conservation strategies.

Discussion

The stranding of a Ganges River dolphin (*P. gangetica*) carcass on the shores of the Ganga River at Balagarh, West Bengal, demonstrates the urgent conservation challenges faced by this endangered species. The event highlights the pressing need to understand the ecological dynamics and anthropogenic pressures impacting the survival of *P. gangetica* in its natural habitat. This incident provides an opportunity to reassess current management practices and research strategies to mitigate further decline. From a genetic and phylogenetic perspective, the Ganges River dolphin represents a unique evolutionary lineage within the cetaceans, was elucidated^{36,16}. Their studies, utilizing mitochondrial cytochrome b and nuclear gene sequences, have confirmed the distinct evolutionary trajectory of *P. gangetica*. This unique genetic makeup, however, is increasingly threatened by habitat fragmentation, pollution, and human interference, necessitating an urgent re-evaluation of conservation protocols.

The successful amplification and phylogenetic analysis of the mitochondrial cytochrome b (Cytb) gene from *P. gangetica* confirms its distinct evolutionary identity within the order Cetacea. The Indian isolate (GenBank: PQ212750) forms a well-supported monophyletic cluster with Bangladeshi and Belgian counterparts, indicating genetic cohesion across the subcontinent while preserving regional divergence. The observed phylogenetic topology reinforces *P. gangetica* as a genetically discrete and endemic lineage of the Ganges-Brahmaputra-Meghna River system. Its separation from marine taxa such as *Sousa plumbea* and *Orcaella brevirostris* underscores the freshwater lineage's ancient divergence. These findings validate the Cytb gene as a reliable molecular marker for species delineation and support the designation of *P. gangetica* as an Evolutionarily Significant Unit (ESU), meriting urgent conservation prioritization. The genetic insights presented here enrich our understanding of the species' evolutionary heritage and reinforce the necessity of safeguarding its dwindling populations within fragmented riverine habitats. This study's findings align with previous research indicating the distinct evolutionary path of riverine cetaceans in the Ganga River^{9,10,32-34}.

The Ganges River dolphin is not only a key indicator of riverine health but also a critical component of the aquatic ecosystem. Its presence reflects the overall biodiversity of the Ganga River Basin, which is home to numerous species, many of which are endemic or endangered.^{12,38} The dolphin's role as a top predator helps maintain the ecological balance by regulating fish

populations and contributing to the nutrient cycle within the river system¹¹. The genetic distinctiveness of *P. gangetica* highlighted in this study reflects its specialized adaptation to the unique environmental conditions of the Ganga River. This specialization underlines the species' importance in the trophic dynamics of the river, where it helps regulate fish populations and contributes to the overall health of the aquatic ecosystem. The distinct genetic identity of *P. gangetica* revealed by Cytb gene analysis reinforces the importance of conserving this species to preserve the genetic diversity of the Ganga River's aquatic fauna. The genetic divergence between *P. gangetica* and other dolphin species underscores the need for targeted conservation measures that protect not only the species but also its unique genetic heritage¹⁷. Given the critically endangered status of *P. gangetica*, conservation efforts must prioritize the protection of its habitat, mitigation of threats such as pollution and habitat fragmentation, and the implementation of species-specific management plans. The findings of this study provide a molecular basis for such conservation strategies, offering insights into the genetic health of the population that can guide future conservation actions¹¹.

Furthermore, the presence of *P. gangetica* serves as an indicator of the ecological integrity of the Ganga River. The river dolphin's sensitivity to environmental changes, such as water quality and habitat availability, makes it a valuable bioindicator for assessing the health of the river ecosystem^{22,23}. The decline or stranding of dolphin carcasses, as observed in Balagarh, West Bengal, signals potential ecological distress, necessitating immediate conservation interventions to mitigate further degradation of the river environment. The decline of *P. gangetica* populations, as evidenced by recent strandings, signals potential ecological imbalances that could cascade through the entire river ecosystem. Such imbalances may lead to a reduction in biodiversity, as the loss of apex predators often triggers a trophic cascade, resulting in altered species composition and ecosystem functions^{10,33}.

The conservation of the Ganges River dolphin requires an integrative approach that addresses both the species-specific needs and the broader environmental issues affecting the Ganga River Basin. The dolphin is recognized as a flagship species for aquatic biodiversity conservation in India¹⁰. Protecting *P. gangetica* entails safeguarding its habitat from the multifaceted threats of industrial pollution, agricultural runoff, dam construction, and unregulated fishing practices¹⁻⁶. Recent research emphasizes the importance of identifying and prioritizing key habitats for the species' survival. Das et al. (2024)⁹

have highlighted the need for focused conservation efforts in smaller river stretches within the Ganga River Basin, which are often overlooked in broader conservation strategies. The stranded dolphin carcass at Balagarh highlights the urgent need to implement effective habitat protection measures, including the establishment of protected areas and community-based conservation initiatives. The decline in *P. gangetica* populations, as evidenced by the stranding of carcasses, poses significant risks to the Ganga River ecosystem. The loss of this apex predator could lead to imbalances in the river's food web, potentially resulting in the overpopulation of certain fish species and the decline of others. This disruption of the trophic structure could have cascading effects on the river's biodiversity and the services it provides to human communities, such as fisheries and water quality maintenance. Moreover, the conservation of *P. gangetica* is intertwined with the broader goal of preserving the ecological health of the Ganga River. Protecting this species requires addressing the root causes of habitat degradation, including pollution, unsustainable fishing practices, and water diversion, all of which have far-reaching impacts on the entire riverine ecosystem²¹.

The stranding of *P. gangetica* at Balagarh underscores critical gaps in riverine biodiversity management amid escalating anthropogenic pressures on the Ganga River ecosystem^{31,39}. Urban runoff, industrial effluents, and agricultural pollutants compromise water quality, disrupt trophic dynamics, and threaten the ecological viability of endemic fauna, including the Ganges River dolphin. Molecular insights from this study provide a valuable genetic baseline to monitor population health and detect early signs of genetic bottlenecks (Wakid, 2009). To address the multifactorial threats facing *P. gangetica*, conservation frameworks must integrate genetic research, ecological assessments, and socio-economic interventions, especially involving riparian communities³⁹. Further population-level studies across the Brahmaputra and Karnali basins are essential to elucidate genetic structure and inform adaptive conservation²⁵. A holistic approach rooted in transdisciplinary collaboration is vital to ensure the long-term survival of this riverine sentinel species. Future research should focus on understanding the complex interactions between *P. gangetica* and its environment, including studies on habitat use, population genetics, and the impact of environmental changes on dolphin behaviour and health. Advanced genetic tools, as employed could provide deeper insights into the population structure and connectivity of *P. gangetica* populations across the Ganga River Basin^{8, 30}. In addition,

there is a need for comprehensive studies on the ecological roles of the Ganges River dolphin within the riverine ecosystem, as well as on the socio-economic impacts of its conservation⁶. Understanding the links between biodiversity conservation and human livelihoods is crucial for developing sustainable conservation strategies that benefit both the species and the people who depend on the river.

Conclusion

The stranding of a Ganges River dolphin at Balagarh serves as a stark reminder of the fragility of this species and its habitat. The incident underscores the urgent need for targeted conservation efforts, informed by rigorous scientific research and supported by effective management strategies. The molecular characterization of *P. gangetica* using the Cytb gene provides essential insights into the genetic uniqueness and evolutionary significance of this critically endangered species. The findings underscore the importance of targeted conservation efforts to preserve the genetic diversity and ecological role of the Ganges River dolphin within the Ganga River ecosystem. As such, the conservation of this species should be a top priority in the broader context of riverine ecosystem management in India and beyond. Protecting *P. gangetica* is not just about preserving a species; it is about maintaining the health and biodiversity of one of the world's most important river ecosystems. Addressing the management challenges and research needs identified in this study is crucial for ensuring the long-term survival of *P. gangetica* and maintaining the ecological integrity of one of India's most iconic rivers.

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