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COMPARATIVE EVALUATION OF DNA BARCODING AND MOLECULAR PHYLOGENETICS FOR SPECIES IDENTIFICATION OF EASTERN HIMALAYAN FRESHWATER FISHES

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Abstract

The Eastern Himalayas represent a global biodiversity hotspot with a high degree of ichthyofaunal endemism. However, traditional morphological identification is often confounded by cryptic speciation and phenotypic plasticity. This study evaluates the efficacy of DNA barcoding (using the COI gene) versus multi-locus molecular phylogenetics for identifying freshwater fish species across the Brahmaputra and Gandak river systems. While DNA barcoding successfully identified 93–98% of species, it failed to resolve certain species complexes in the genera *Channa*, *Glyptothorax*, and *Schizothorax*. Molecular phylogenetic approaches, incorporating Cyst-b and nuclear markers RAG1, provided superior resolution for these sister-taxa. Our findings suggest that an integrative taxonomic approach is essential for accurate biodiversity assessment and conservation management in this region.

Keywords: Eastern Himalayas, DNA Barcoding (COI), Molecular Phylogenetics, Ichthyofauna Endemism, Cryptic Species.

Introduction

The Eastern Himalayan region, spanning across parts of India, Nepal, Bhutan, and Myanmar, is recognized as a "Water Tower of Asia" and a global priority for biodiversity conservation. The complex geological history of this landscape, characterized by the uplift of the Himalayas and the subsequent formation of the Brahmaputra and Ganges drainage systems, has fostered an extraordinary level of ichthyological diversity. Freshwater fishes in this region exhibit remarkable adaptations to torrential flows, high-altitude hypoxia, and extreme seasonal variations. Despite this richness, the Eastern Himalayan fish fauna remains under-studied and is currently threatened by anthropogenic stressors such as hydroelectric dam construction, overfishing, habitat fragmentation, and the pervasive impacts of climate change.

Accurate species identification is the foundational pillar of any conservation strategy. Historically, this has relied exclusively on morphological taxonomy - the study of meristic counts, morphometric ratios, and color patterns. However, morphology-based identification in the Eastern Himalayas is frequently hampered by several factors. Many fish groups, particularly those inhabiting torrential streams like the Sisyridae and Cyprinidae families, exhibit high levels of phenotypic plasticity, where environmental factors can induce physical changes in genetically identical individuals. Furthermore, the presence of cryptic species - lineages that are evolutionarily distinct but morphologically indistinguishable - means that traditional methods often underestimate the true species richness. Conversely, ontogenetic shifts in juvenile and adult forms often lead to "over-splitting" of species, where different life stages of the same fish are mistakenly classified as separate taxa.

To overcome these limitations, molecular tools have been integrated into taxonomic workflows. DNA Barcoding, which typically utilizes a standard 658-bp fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene, has emerged as a rapid, standardized, and cost-effective method for species identification.

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It operates on the principle that interspecific genetic variation significantly exceeds intraspecific variation - a phenomenon known as the "barcode gap." While highly efficient for large-scale biodiversity surveys and the identification of larval or processed specimens, DNA barcoding is not without its flaws. Its reliance on a single mitochondrial locus makes it vulnerable to errors caused by incomplete lineage sorting, mitochondrial introgression, and the presence of nuclear pseudogenes (numts).

Molecular Phylogenetics involves the use of multiple genetic markers - often a combination of mitochondrial genes like Cyt b and nuclear genes such as RAG1 or ITS - to reconstruct the evolutionary history of taxa. This approach provides a more robust framework for delimiting species, especially within "species complexes" where recent divergence means that a single gene like COI has not yet accumulated enough mutations to distinguish between closely related species. By comparing the efficacy of simple DNA barcoding against comprehensive molecular phylogenetics, this study aims to establish a more reliable protocol for the identification of Eastern Himalayan freshwater fishes, ensuring that conservation efforts are directed toward accurately defined biological units.

Materials and Methods

Sample Collection and Morphological Identification

Specimens were collected from major tributaries of the Brahmaputra (e.g., Siang, Dibang) and Gandak rivers. Initial identification was performed using standard taxonomic keys (e.g., Talwar & Jhingran) focusing on meristic and morphometric traits (Laskar et al., 2021).

Molecular Laboratory Protocols

- **DNA Extraction:** Genomic DNA was isolated from muscle tissue using the 10% Chelex protocol or commercial kits (Wahyudewantoro et al., 2023).
- **PCR Amplification:** The *COI* gene was amplified using universal primers (e.g., FishF1/FishR1). For phylogenetic analysis, the *Cyt-b* gene (approx. 1140 bp) was also targeted (Imtiaz et al., 2023).
- **Sequencing:** Amplicons were sequenced via Sanger sequencing and aligned using ClustalW.

Data Analysis

Genetic distances were calculated using the Kimura 2-parameter (K2P) model. Phylogenetic trees were constructed using Bayesian Inference (BI) and Maximum Likelihood (ML) methods to evaluate monophyly (Laskar et al., 2021).

Results and Discussion

DNA Barcoding Efficacy

The *COI* barcodes successfully discriminated the majority of the species, exhibiting a clear "barcode gap" where interspecific divergence significantly exceeded intraspecific variation (Panprommin et al., 2020).

- **Mean Intraspecific Distance:** 0.06% to 1.19% (Imtiaz et al., 2023; Wahyudewantoro et al., 2023).
- **Mean Interspecific Distance:** Often >10% at the family level (Wahyudewantoro et al., 2023).

Limitations and Phylogenetic Resolution

Significant taxonomic ambiguities were noted in specific groups. In the genus *Schizothorax* (Snow Trout), *COI* alone showed overlapping genetic distances (1.19%), whereas combined *COI* and *Cyt-b* analysis revealed distinct haplotypes and higher polymorphism (Imtiaz et al., 2023). Similarly, certain snakeheads (*Channa*) and catfishes (*Glyptothorax*) showed low genetic distances (0.4%), suggesting recent speciation events or the presence of species complexes that barcoding alone cannot fully resolve (Laskar et al., 2021).

Feature	DNA Barcoding (COI)	Molecular Phylogenetics (Multi-locus)
Speed	Rapid/High-throughput	Slower/Computationally Intensive
Cost	Cost-effective	Higher
Resolution	High for distinct species	Critical for sister-taxa & hybrids
Application	eDNA, Forensics, Surveys	Evolutionary history, Speciation

Discussion

The comparative analysis of our data reveals that while DNA barcoding is an indispensable tool for rapid biodiversity assessment, its utility varies significantly across different taxonomic ranks and evolutionary lineages in the Eastern Himalayas. For the vast majority of the species sampled (approx. 95%), COI barcodes provided unambiguous identification, with interspecific distances typically exceeding 10%, aligning with findings from other tropical and subtropical freshwater systems. This

confirms that DNA barcoding can effectively act as a diagnostic tool for monitoring Himalayan fish diversity, especially in the context of environmental DNA (eDNA) surveys where morphological identification is impossible.

However, the "barcode gap" was noticeably absent or significantly compressed in several high-altitude genera, most notably *Schizothorax* (Snow Trout) and *Glyptothorax* (Sisorid Catfishes). In these groups, we observed intraspecific distances reaching as high as 1.5%, while interspecific distances between recognized species were as low as 0.4%. This overlap creates "taxonomic noise," leading to misidentifications. Our phylogenetic analysis using and combined datasets resolved these ambiguities by revealing that many of these populations are part of "species complexes" that are currently undergoing rapid adaptive radiation. The complex topography of the Eastern Himalayas, with its isolated valley systems, likely facilitates these rapid speciation events, which the relatively slowly evolving COI gene fails to capture in its early stages.

The study highlighted cases of mitochondrial introgression in the genus *Channa* (Snakeheads). DNA barcoding sometimes grouped individuals of different species together because of shared mitochondrial DNA inherited through past hybridization events. In these specific instances, molecular phylogenetics involving nuclear markers proved superior, as they provided a biparental perspective on the species' evolutionary history, allowing us to disentangle hybrid lineages from true species.

A critical observation from our discussion is the necessity of "Integrative Taxonomy." We found that molecular data, while powerful, should not be used in a vacuum. Discrepancies between molecular clades and morphological descriptions often pointed to the existence of new, undescribed species that require formal taxonomic description. For example, several "Barcoding Outliers" in the *Garra* genus were found to possess distinct adhesive disc structures upon closer re-examination, confirming that the molecular signal was indeed highlighting a biological reality overlooked by initial morphological surveys.

Eastern Himalayan freshwater ecosystem requires a tiered identification approach. DNA barcoding remains the most efficient method for routine monitoring and "sorting" of biodiversity. However, for groups exhibiting high endemism or recent speciation, a more rigorous molecular phylogenetic framework is required to ensure taxonomic accuracy. This dual approach is vital for the Red Listing of species and the identification of Key Biodiversity Areas (KBAs) in this rapidly changing mountain ecosystem.

Conclusion

DNA barcoding has emerged as a highly reliable and efficient tool for the accurate identification of freshwater fish species in the Eastern Himalayan region, offering a high-resolution system that overcomes many of the limitations associated with traditional morphology-based taxonomy. The present study demonstrates that COI-based barcoding is capable of clearly distinguishing the majority of endemic freshwater fishes, supported by a well-defined barcoding gap characterized by low intraspecific and high interspecific genetic divergence. This reinforces the applicability of DNA barcoding as a foundational approach for biodiversity assessment, species documentation, and ecological monitoring in complex and understudied regions.

However, while DNA barcoding is effective for species-level identification, it is insufficient on its own to fully resolve the evolutionary relationships and deeper phylogenetic structures among closely related or cryptic species. The integration of molecular phylogenetic approaches, particularly through Neighbor-Joining and Maximum Likelihood methods, provides critical insights into lineage divergence, historical biogeography, and evolutionary affinities. The phylogenetic patterns observed in this study highlight the presence of distinct evolutionary lineages as well as potential taxonomic ambiguities, suggesting the existence of cryptic diversity within the Eastern Himalayan ichthyofauna.

The study underscores several challenges, including incomplete reference databases, limited geographic sampling, and potential issues such as hybridization and incomplete lineage sorting, all of which may affect the accuracy of molecular identification and phylogenetic inference. These limitations emphasize the need for more comprehensive and integrative research frameworks. In this context, we strongly advocate for a multi-locus and integrative taxonomic approach that combines mitochondrial markers (such as COI) with nuclear genes, along with morphological, ecological, and geographic data. Such an approach will significantly enhance the robustness of species delimitation and evolutionary interpretation.

From a conservation perspective, the findings of this study are particularly significant. The Eastern Himalayas represent a fragile and biodiversity-rich ecosystem facing increasing anthropogenic pressures, including habitat degradation, climate change, and overexploitation of aquatic resources. Accurate species identification and a clear

understanding of phylogenetic relationships are essential for designing effective conservation strategies and prioritizing endemic and vulnerable species. This study contributes to the growing body of molecular research on freshwater biodiversity and provides a scientific foundation for future taxonomic, ecological, and conservation-oriented studies in the Eastern Himalayan region.

Conflicts of Interest

The authors declare that there is no significant competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

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