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Original Research

DNA Barcoding and Phylogenetic Placement of Selected Freshwater Nepomorpha (Hemiptera: Heteroptera) from Southern India Based on *COI* Sequences

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ABSTRACT

Aquatic Hemipterans are ecologically significant predators in freshwater ecosystems, yet species-level identification is often challenging due to morphological similarity and life-stage variation. This study employed mitochondrial cytochrome c oxidase subunit I (*COI*) barcoding to identify taxa and evaluate phylogenetic relationships among four freshwater bug specimens (*Laccotrephes maculatus* (n=1), *Ranatra filiformis* (n=1), *Diplonychus rusticus* (n=1), Notonectidae sp. (n=1)) collected from three suburban lakes in Mysore, India. Preliminary identification using taxonomic keys was followed by *COI* amplification, sequencing (~550 bp), and Maximum Likelihood phylogenetic analysis. Genomic DNA was extracted using the QIAGEN DNeasy Ultra Clean Microbial Kit (Cat. No. 12224-50) according to the manufacturer's protocol. The nucleotide divergence was highest in *L. maculatus*, followed closely by *R. filiformis,* and lowest in *D. rusticus.* Phylogenetic clustering corroborated morphological identifications, with three taxa grouping with regional conspecific isolates, while the Notonectidae specimen clustered with Kenyan isolates. Overall, the findings demonstrate the effectiveness of *COI* barcoding in resolving taxonomic ambiguities and elucidating evolutionary relationships among freshwater hemipterans, highlighting its utility as a complementary tool to traditional morphology-based identification in biodiversity assessments.

Keywords: Aquatic hemipterans, *COI*, maximum likelihood tree, phylogenetic analysis

INTRODUCTION

Aquatic insects constitute the dominant group of invertebrate fauna in most freshwater ecosystems and display remarkable diversity in morphology, development, physiology, and ecology (Dijkstra et al., 2014). Among the hemi-metabolous insects, the Heteroptera, or true bugs, represent a significant and diverse component of the global aquatic insect fauna (Polhemus & Polhemus, 2008). Within this group, the true water bugs, classified under the infraorder Nepomorpha, one of the seven infraorders within the suborder Heteroptera (Insecta: Hemiptera) are of considerable economic importance, as nearly all members, except for some Corixidae, are predatory (Schuh & Slater, 1995). With over 4,656 species distributed across 326 genera and 20 families, aquatic Heteroptera are noted for their ability to occupy an exceptionally wide range of habitats, extending from marine and intertidal zones to arctic and high alpine regions (Polhemus & Polhemus, 2008). In India, approximately 350 species of aquatic and semi-aquatic hemipterans have been identified (Thirumalai, 2013; Jehamalar & Chandra, 2020), with over 157 species from infraorder: Nepomorpha (Basu & Subramanian, 2017). Due to their widespread abundance in freshwater ecosystems, their importance as bioindicators of water quality, and their distinctive morphological and ecological adaptations to specialized microhabitats, these groups have long been a central focus of entomological and ecological research (Paripatyadar et al., 2020; Özdamar & Kıyak, 2025). However, due to their highly similar morphology, species identification is often difficult and typically requires the expertise of trained taxonomists. Identifying nymphs or females of certain species can be particularly challenging or even impossible (Park et al., 2011). Given their ecological and conservation significance, correct species identification of aquatic Heteroptera is essential (Whiteman & Sites, 2008). In this context, DNA barcoding has emerged as a widely used and reliable method for species identification (Hajibabaei et al., 2007; Miller et al., 2016). The approach is based on the principle that interspecific genetic variation exceeds intraspecific variation for a given marker, resulting in each species forming a distinct DNA barcode cluster (Hebert et al., 2003). The mitochondrial cytochrome oxidase subunit I gene (*COI*) has been proposed as a standardized region for invertebrate species identification and systematics (Folmer et al., 1994). Records from NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) indicate that DNA barcoding has been performed on aquatic bugs of the infraorder Nepomorpha from various regions of India, with 14 submissions reported (Accession number: KP274068, KP274069, KP274070; PV041409, PVO41412; PV041413, PV041420; PP032035, PP885727, PP885728; PQ436053; KY224979, KY224980, and ON357573). Barcoding studies on aquatic hemipterans from the Mysore region remain limited, even though the district is geographically positioned in a transitional zone where the Eastern and Western Ghats converge toward the Nilgiri Hills, creating a distinctive and ecologically diverse landscape. This strategic setting contributes significantly to the region's varied topography and ecological richness (Rai, 1995). The district is sustained by two major river systems and numerous inland water bodies, which support a wide range of fauna (Prasad et al., 2009; Ramachandra et al., 2017; Abhilash et al., 2023). The objective of present study is to identify species using *COI* barcodes and to assess the phylogenetic relationships of few freshwater bugs.

METHODS

Collection of specimens

Aquatic Hemipterans were collected from three sub-urban lakes of Mysore (Figure 1) *viz* Marasettihalli Lake (12°24′09"N, 76°69′63"E), Janthagalli Lake (12°24′30"N, 76°73′59"E) and Hadinaru Lake (12°17′22"N, 76°74′91"E) using circular pond net (Dalal and Gupta, 2016). The specimens were collected in 90% Ethanol and stored at 4°C at Ecology and Environmental Biology laboratory, Department of Zoology, Yuvaraja's College (University of Mysore) until further laboratory analysis. Before molecular analysis, the specimens were identified based on morphological features such as number of antennal segments, body length, wing shape and color patterns using a stereomicroscope (Labomed CZM4) and following taxonomic keys (Thirumalai, 2004; Jehamalar and Chandra, 2013; Basu et al., 2018) to the lowest possible taxonomical rank *viz* family Notonectidae and genus *Laccotrephes, Ranatra* and *Diplonychus*. The map for this paper was created using QGIS open-source software (version 3.22.11) by Open-Source Geospatial Foundation (OSGeo).

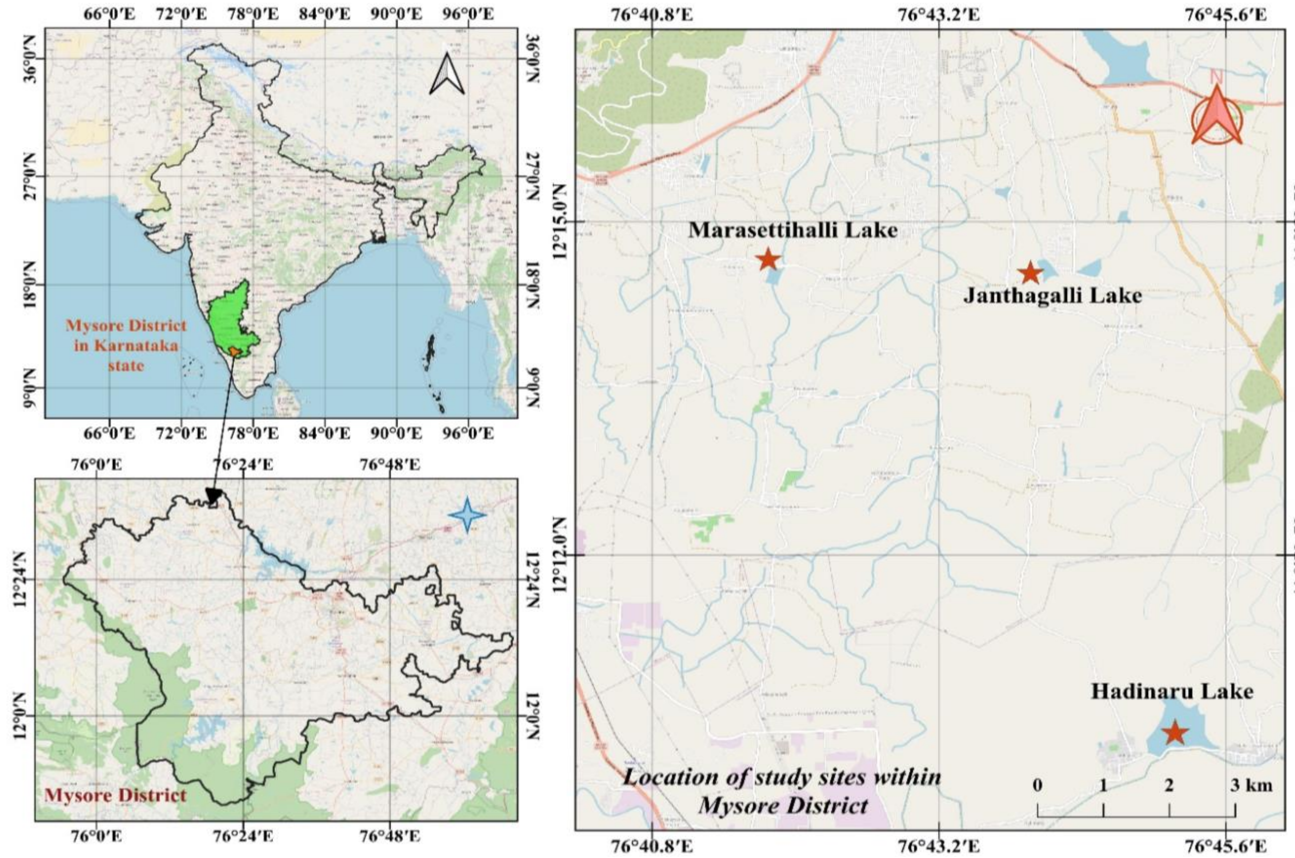

Figure 1. Sampling sites of the studied Hemipterans from Mysore District

DNA extraction, amplification, sequencing and data depository

Genomic DNA was extracted using the QIAGEN DNeasy Ultra Clean Microbial Kit (Cat. No. / ID: 12224-50) following the manufacturer's protocol. PCR amplification of the cytochrome c oxidase subunit 1 (*COI*) mitochondrial gene was performed from single specimen from each taxa using the primers: forward (5′-GGTCAACAAATCATAAAGATATTGG -3′) and reverse (5′-TAAACTTCAGGGTGACCAAAAAA TCA -3′), adapted from Folmer et al. (1994). The reaction was carried out in a total volume of 10µL using a MiniAmp™ Plus Thermal Cycler. The PCR conditions included an initial denaturation at 95°C for 1 minute, followed by 40 cycles of denaturation at 95°C for 1 minute, annealing at 40°C for 1 minute, elongation at 72°C for 1 minute and 5 seconds, and a final extension at 72°C for 3 minutes. PCR products were assessed via 1.0% agarose gel electrophoresis and then purified using the QIAGEN QIAquick PCR Purification Kit (Cat. No. / ID: 28104) to remove contaminants. Sequencing was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), and the products were analyzed on an ABI 3730xL Genetic Analyzer (Applied Biosystems, USA).

Sequence analysis and dataset formation

The generated sample's forward and reverses ABI sequences (.abi) were analyzed using NCBI Reference Sequence (.gb): NC_012817 for *Laccotrephes*; MZ305077.1 for Notonectidae, KY224979.1 for *Ranatra* and MZ363628.1 for *Diplonychus* for chromatogram analysis with Seqscape v3 software (Applied Biosystems, USA). Each forward and reverse sequence was thoroughly annotated, and they were edited by pruning to remove any ambiguous bases and overlapping regions. After validation, the aligned ABI (.abi) sequences were saved as FASTA file (.fst) for further investigation. The confirmation of the species was done using NCBI BLASTn (Johnson et al., 2008) which identified genus *Diplonychus* as *Diplonychus rusticus*, genus *Laccotrephes* as *Laccotrephes maculatus*, genus *Ranatra* as *Ranatra filiformis* as true specimens; the specimen from family Notonectidae was not identified to the species level. Multiple sequence alignment was conducted using ClustalW program in MEGA X (Kumar et al., 2018). The generated four sequences were submitted to the GenBank database, and unique accession numbers were issued (Accession number: PV066062 - PV066065).

Genetic divergence and cluster analysis

For comparability, other *COI* sequences of few closely related Hemipteran species were obtained from the GenBank Database, which were aligned with sequences generated from this study using the MEGA X software package (Kumar et al., 2018); for phylogenetic analysis, the Maximum-Likelihood method was computed using the bootstrap consensus tree derived from 1000 replicates (Tamura & Nei, 1993). Nucleotide diversity (π) was estimated using DnaSP v6 (Rozas et al., 2017).

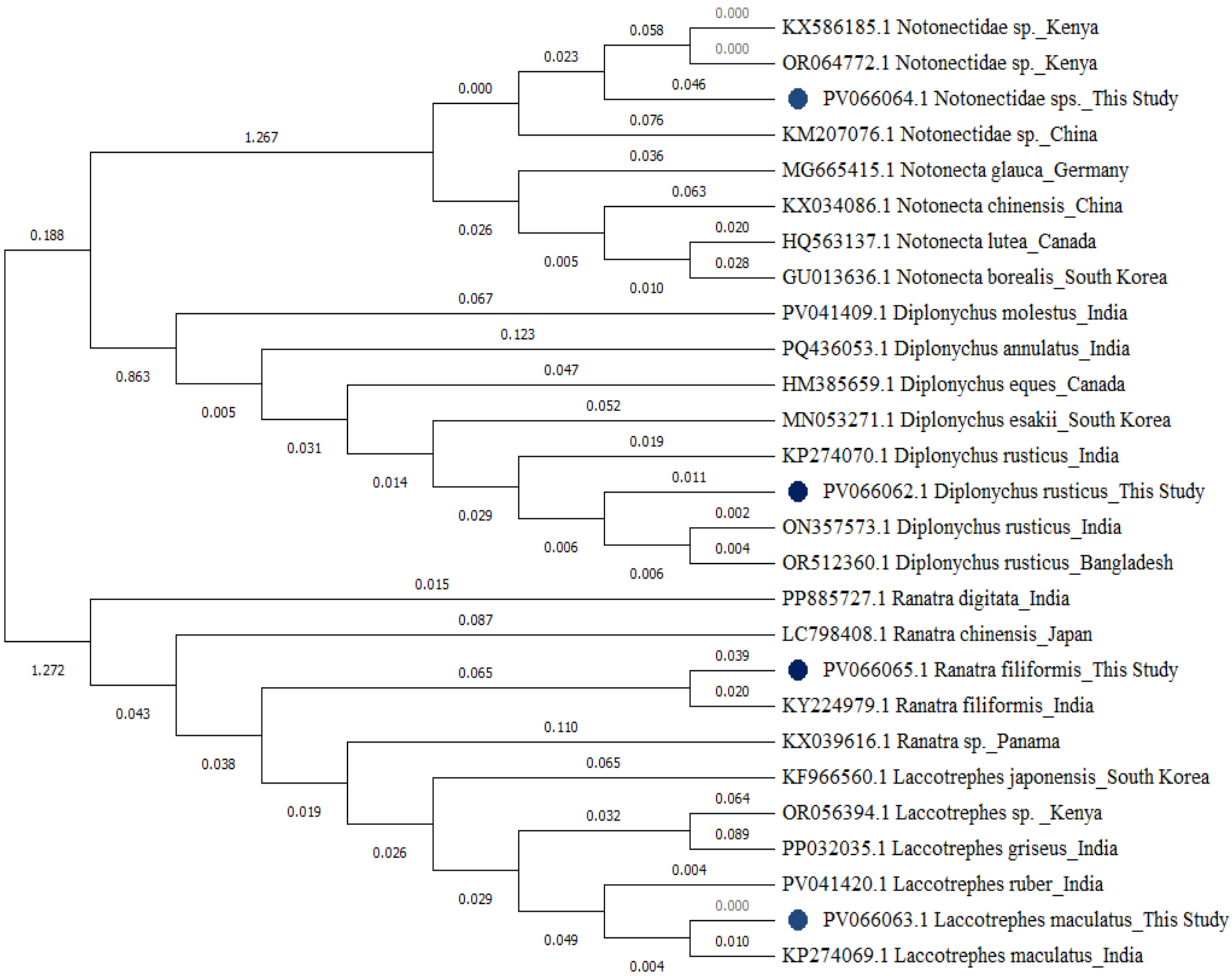

Figure 2. Phylogenetic tree of Hemipteran species based on *COI* sequences. The Maximum-Likelihood (ML) tree was constructed from the alignment of *COI* gene sequences in MEGA X software. The numbers displayed on branches are the bootstrap support obtained through 1000 replications. GenBank sequences are shown with accession numbers. The species from this study are represented by circles along with the NCBI accession no.

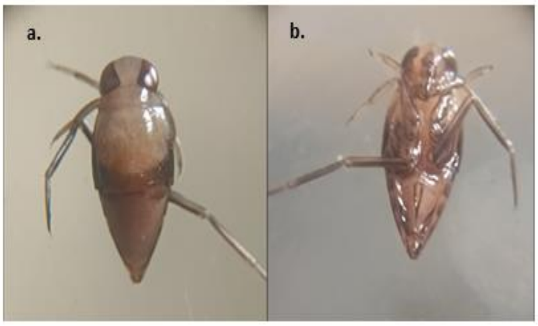

Figure 3. Photographic documentation of the unidentified Notonectidae sp. a) dorsal view, b) ventral view.

Table 1. List of sequences of *COI* gene retrieved from GenBank used for Phylogenetic analysis

Family	Species	Country	GenBank Accession No.
Notonectidae	Notonectidae sp.	Kenya	KX586185.1
	Notonectidae sp.	Kenya	OR064772.1
	Notonectidae sp.	China	KM207076.1
	Notonecta glauca	Germany	MG665415.1
	Notonecta chinensis	China	KX034086.1
	Notonecta lutea	Canada	HQ563137.1
	Notonecta borealis	South Korea	GU013636.1
Belostomatidae	*Diplonychus molestus*	India	PV041409.1
	Diplonychus annulatus	India	PQ436053.1
	Diplonychus eques	Canada	HM385659.1
	Diplonychus esakii	South Korea	MN053271.1
	Diplonychus rusticus	India	KP274070.1
	Diplonychus rusticus	India	ON357573.1
	Diplonychus rusticus	Bangladesh	OR512360.1
Nepidae	*Ranatra digitata*	India	PP885727.1
	Ranatra chinensis	Japan	LC798408.1
	Ranatra filiformis	India	KY224979.1
	Ranatra sp.	Panama	KX039616.1
	Laccotrephes japonensis	South Korea	KF966560.1
	Laccotrephes maculatus	Kenya	OR056394.1
	Laccotrephes griseus	India	PP032035.1
	Laccotrephes ruber	India	PV041420.1
	Laccotrephes maculatus	India	KP274069.1

RESULTS AND DISCUSSION

The sequences of the species we studied exhibited close similarity to those available in the NCBI database, including locally and globally sourced sequence entries (Table 1). The aligned dataset comprised approximately 550 base pairs of the mitochondrial *COI* gene from four hemipteran species: *Laccotrephes maculatus, Ranatra filiformis, Diplonychus rusticus*, and an unidentified Notonectidae sp. The nucleotide diversity (π) was estimated at 0.065, 0.059, and 0.038 for *L. maculatus, R. filiformis,* and *D. rusticus*, respectively. Phylogenetic analysis using *COI* region sequences from closely related species available in GenBank, conducted through the Maximum Likelihood (ML) method, showed a high level of similarity with isolates of species previously identified based on morphological characteristics (Figure 2). At the species level, the deep branches corroborate the taxonomic identification of *Diplonychus rusticus* with isolates from India (ON357573) and Bangladesh (OR512360). *Laccotrephes maculatus* branched into a clade with same isolates from North East India (KP274069). The sequences of *Ranatra filiformis* branched into an isolate of same species from Kerala, India (KY224979). A backswimmer species (Figure 3) collected from Hadinaru Lake (Figure 1) showed a close genetic relationship with an unidentified Notonectidae species submitted by the Molecular Biology and Bioinformatics Unit, International Centre of Insect Physiology and Ecology, Nairobi, Kenya (KX586185), and another unidentified Notonectidae species submitted by the Centre for Biodiversity, Molecular Genetics section, National Museums of Kenya, Nairobi, Kenya (OR064772).

Aquatic Hemipterans play a significant role in freshwater ecosystems forming an integral component of food webs, nutrient cycling, and ecosystem health, serving both as predators and prey, while also helping to maintain the balance and productivity of aquatic habitats (Marschall & Schaefer, 2009). Accurate identification of these taxa is therefore essential for ecological assessment and biodiversity studies. Cryptic insect species identification is a complex task for understanding biodiversity, ecology, and evolutionary processes where conventional taxonomy offers a systematic framework for identifying and categorizing insects, relying on a combination of morphological features, ecological information, and behavior (Ward, 2010). However, morphological similarity and life-stage variation often complicate species-level resolution, particularly within diverse aquatic Hemipteran lineages (Havemann et al., 2018).

DNA barcoding provides a powerful complementary approach by employing standardized genetic markers typically the *COI* gene that are conserved enough to be universally amplified yet variable enough to distinguish between species (Moritz & Cicero, 2004; Caesar et al., 2006; Havemann et al., 2018; Thomsen, & Sigsgaard, 2019). In this study, Maximum Likelihood (ML) analysis of mitochondrial *COI* sequences was conducted to infer phylogenetic relationships of few selected species from infraorder: Nepomorpha. Three main genus-level clades *viz*., *Diplonychus, Ranatra, Laccotrephes* showed strong monophyly with isolates from different realms (Figure 2). Within the *Diplonychus* clade, species from India, Canada, South Korea, Bangladesh cluster closely. For example, *Diplonychus rusticus* sequences from India and Bangladesh cluster tightly with the study's *Diplonychus rusticus* sample (PV066062.1), confirming species identification and evolutionary relatedness. The *Ranatra* clade groups species from Japan, India, Panama, and the study's *Ranatra filiformis* (PV066065.1), with the close genetic similarity between the Indian and study sequences supporting their conspecificity. The *Laccotrephes* clade includes species from Kenya, India, South Korea, and the study's *Laccotrephes maculatus* (PV066063.1) indicating close evolutionary relationships across these geographically diverse populations. However, Notonectidae sp. form a distinct cluster including specimens from Kenya, China, Germany, Canada, and South Korea, with the study's Notonectidae sp. (PV066064.1) grouping closely with Kenyan isolates (Figure 2). This clustering suggests a shared recent common ancestry with the Kenyan isolates, despite geographic separation, where the genetic similarity outweighs geographic distance, which is common in widely distributed aquatic insects where dispersal or historical connectivity can maintain conserved mitochondrial lineages. For example, there is documented taxon overlap in families such as Gerridae (water striders) and Corixidae (water boatmen) have broad, nearly global distributions that include both Afrotropical and Oriental regions (Cano et al., 2018). The wide distribution of many aquatic invertebrates could be elucidated by the passive dispersal of their eggs by migratory water birds (Darwin, 1950) owing to their abundance, global distribution, flight abilities, and migratory behavior (Figuerola et al., 2003; Brochet et al., 2010). The passive movement of adult specimens or their propagules between isolated wetlands can also be dispersed by transport vectors like air, water or animals (Figuerola and Green, 2002; Vanschoenwinkel et al., 2011). Freshwater insects are consistently less represented than terrestrial species when it comes to the number of non-native species compared to native species (Sendek et al., 2022).

Though *COI*-barcoding demonstrated strong effectiveness in confirming morphological identification and revealing phylogeographic affinities, dependence on a single mitochondrial locus has inherent limitations (Solovyeva et al., 2023). Single-locus phylogenies may not fully capture species boundaries due to introgression, incomplete lineage sorting, maternal inheritance, or the presence of nuclear mitochondrial pseudogenes (Bensasson et al., 2001; Song et al., 2008; Harenčár, 2024). Although the observed concordance between molecular and morphological data in this study supports the reliability of species delimitation, future research incorporating multi-locus datasets (e.g., whole mtDNA sequencing) and integrative taxonomic approaches would provide more robust phylogenetic resolution and deeper insights into evolutionary relationships. Overall, the integration of morphological taxonomy with *COI*-based molecular analysis enhances the accuracy of species identification, strengthens barcode reference libraries, and contributes to a more comprehensive understanding of freshwater Hemipteran biodiversity.

CONCLUSIONS

This study provides new *COI* barcode records for four aquatic Hemipteran species (*Diplonychus rusticus, Laccotrephes maculatus, Ranatra filiformis*, and an unidentified Notonectidae sp.) from the study region. Maximum Likelihood phylogenetic analysis confirmed the morphological identifications, with the study sequences clustering closely with conspecific isolates from regional and global databases. Notably, the study's Notonectidae sp. grouped with Kenyan isolates, reflecting conserved mitochondrial lineages across geographic distances. These results enhance regional barcode reference libraries and demonstrate the utility of *COI*-based molecular data in supporting morphological species identification. Future work integrating multi-locus markers and broader taxon sampling would further refine phylogenetic resolution.

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