

# Integrative Identification of *Melanoides tuberculata* from Jalgaon, Maharashtra India Using DNA Barcoding

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## ABSTRACT

Accurate identification of freshwater gastropods is often challenging due to morphological plasticity and the presence of cryptic species. The present study employs an integrative approach combining morphological analysis and mitochondrial cytochrome c oxidase subunit I (COI) gene-based DNA barcoding to identify a freshwater gastropod from Jalgaon, Maharashtra, India. Morphological examination revealed diagnostic shell features consistent with *Melanoides tuberculata*. Molecular analysis of a ~600 bp COI fragment followed by BLASTn search showed high similarity with *M. tuberculata*, with the closest match (OR594407.1) exhibiting 99.84% identity and complete query coverage. Phylogenetic analysis using the Neighbor-Joining method (Kimura 2-parameter model) placed the sequence within a well-supported *M. tuberculata* clade, forming a monophyletic group with reference sequences. Genetic distance analysis indicated low intraspecific divergence and clear distinction from related taxa. The findings confirm the identity of the specimen as *M. tuberculata* and demonstrate the reliability of COI-based barcoding in gastropod taxonomy. The generated sequence contributes valuable regional molecular data and supports future studies in biodiversity, phylogeny, and ecological monitoring.

**Keywords:** COI barcode, freshwater gastropod, *Melanoides*, molecular taxonomy, Thiaridae, Jalgaon, Maharashtra, India

## INTRODUCTION

Freshwater gastropods represent one of the most diverse groups of molluscs and play an important role in aquatic ecosystems, contributing to nutrient cycling, food webs, and ecological balance. Specific identification of gastropod species is essential for biodiversity assessment, ecological monitoring, and conservation studies. However, morphological identification of gastropods can often be difficult due to phenotypic plasticity, shell variation due to environmental factors as per the habitat and the occurrence of cryptic species within closely related taxa. In recent decades, DNA barcoding has emerged as a reliable molecular tool for species identification and taxonomic studies. The mitochondrial cytochrome c oxidase subunit I (COI) gene has been widely adopted as the standard barcode marker for animals because it is conserved across taxa while still exhibiting sufficient variability to distinguish species.

The genus *Melanoides* Olivier, 1804 (Family Thiaridae) includes cosmopolitan and endemic species, occurs in freshwater. Comprises morphologically plastic freshwater gastropods with wide geographic distribution, often resulting in taxonomic ambiguity. DNA barcoding using the mitochondrial COI gene has proven effective in resolving cryptic diversity within thiarid snails. DNA barcoding technique was used to identify many gastropods families which can help to overcome identification of morphological problems. Davison *et al.*, (2009) found that DNA barcoding method must ideally have the ability to deliver accurate species identification and act as effective tool for molecular taxonomy. Barcoding method of snail identification is more useful when there was conjunction with other method of identification. Thiaridae family includes freshwater snails mostly have turreted thick shells occurs in various sizes with rounded whorls with smooth or sculptured structure according to the species.

They have ovate aperture which is closed by corneous concentric or paucispiral operculum structure. The members of this genus were reproduced by parthenogenesis and in some by sexual reproduction. About 30 species were recorded, many species within this genus shows significant variations in shell shape, ribbing and pigmentation. Snails of this family were present in South America, North America and some parts of Africa. Some dominant species like *M. tuberculata* are native to Asia and India also. As they enter in to new environment some species adapts themselves according to nature and leads to alteration in phenotypic characters (Jihad *et al.*, 2022). The freshwater snail *Melanoides tuberculata* (family Thiaridae) is widely distributed in tropical and subtropical freshwater habitats. This species is known for its ecological adaptability and has been reported as an invasive snail in many regions worldwide. Additionally, it can act as an intermediate host for several parasitic trematodes, highlighting its ecological and medical significance. Although traditional taxonomy relies mainly on shell morphology, morphological similarities among species within the genus *Melanoides* may lead to misidentification. Therefore, integrating molecular techniques such as COI-based DNA barcoding with classical morphology provides a more robust framework for species identification. To Study of morphological changes in

shells the freshwater gastropods were considered as good models as they adapt themselves according to the environment it may result in expression of new phenotypes (Catalan *et al.*, 2023). Sorenson *et al.*, 2004 suggests that Identification of species with only morphological characters is problematic due to plasticity of species and to overcome this problem other methods like molecular analysis can be used with combination of morphological studies which gives us identification of specific species. The present study reports molecular evidence of a *Melanoides tuberculata* population from Jalgaon, Maharashtra, India, based on COI sequence analysis.

## MATERIALS AND METHOD

### Material Examined

One freshwater gastropod specimen collected from a lentic freshwater habitat in Jalgaon, Maharashtra, India. Tissue preserved in 95% ethanol for molecular analysis. The specimen was studied as per morphological characters of shell, identified as per taxonomic key.

### Taxonomic Account

Kingdom: Animalia  
Phylum: Mollusca  
Class: Gastropoda  
Order: Sorbeoconcha  
Family: Thiaridae  
Genus: *Melanoides*  
Species: *tuberculata*

### *Melanoides tuberculata* (Muller, 1774)

**Shell** :- dark brown in colour with dark red brown dots and longitudinal flames, right handed (Dextral), cone like (turreted) not too much slender or elongated, planoconvex with 9 whorls, surface had texture of vertical ribs and spiral striae (grooves) this striae were raised on upper whorls and flatter on lower whorls, lines between two successive whorls (sutures) were slightly pushed inward, aperture pear shaped which is wide at one end and narrow at other, inner peristome is thicker than that of outer peristome, umbilicus closed, callus well-formed which was shiny, smooth and translucent, height of shell : 33 mm, width of shell : 11 mm, aperture height : 11mm and aperture width : 7mm.



**Ventral View**

**Dorsal View**

**Aperture**



**Height of the shell**



**Width of the shell**

### **Molecular Analysis DNA Barcoding using universal primers of CO1**

#### **Genomic DNA Isolation**

Genomic DNA was isolated from the tissues using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions. Tissues were placed in a 1.5 ml microcentrifuge tube. 180 µl of T1 buffer and 25 µl of proteinase K was added and incubated at 56°C in a water bath until the tissue was completely lysed. After lysis, 5 µl of RNase A (100 mg/ml) was added and incubated at room temperature for 5 minutes. 200 µl of B3 buffer was added and incubated at 70°C for 10 minutes. 210 µl of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into NucleoSpin® Tissue column placed in a 2 ml collection tube and centrifuged at 11000 x g for 1

minute. The NucleoSpin® Tissue column was transferred to a new 2 ml tube and washed with 500 µl of BW buffer. Wash step was repeated using 600 µl of B5 buffer. After washing the NucleoSpin® Tissue column was placed in a clean 1.5 ml tube and DNA was eluted out using 50 µl of BE buffer.

#### **Agarose Gel Electrophoresis for DNA Quality check**

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1 µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5 µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of

the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system.

### PCR Analysis

PCR analysis was done by using the mixtures of 5µL of 2X Phire Master Mix and 4µL of D/W. 0.25µL of Forward Primer and Reverse Primer was used with 1µL of DNA. Sequencing was carried out in (5' → 3') direction. Primers used for forward sequence was LCO (GGTCAACAAATCATAAAGATATTGG) and for Reverse sequence was HCO (TAAACTTCAGGGTGACCAAAAAATCA). PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

### PCR amplification profile for COX1

Amplification was done using following steps- Denaturation at 98°C was done for 30 seconds followed by 10 cycles of denaturation at 98°C for 5 seconds, annealing at 45°C for 10 seconds and elongation at 72°C for 15 seconds. The process was continued with 30 cycles of denaturation at 98°C for 5 seconds, annealing at 50°C for 10 seconds and elongation at 72°C for 15 seconds and final elongation at 72°C for 60 seconds done with indefinite holding at 4°C.

### Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 4 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

### ExoSAP-IT Treatment

ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications. Five micro litres of PCR product is mixed with 0.5µl of ExoSAP-IT and incubated at 37°C for 15 minutes followed by enzyme inactivation at 85°C for 5 minutes.

### Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The Sequencing PCR mix consist of 6.6µL of D/W, 1.9µL of 5X Sequencing Buffer, 0.3µL of Forward Primer, 0.3µL of Reverse Primer, 0.2µL Sequencing Mix and Exosap treated PCR product 1µL.

### Sequencing PCR amplification

Initial denaturation was done at 96°C for 2 min, followed by 30 cycles of denaturation at 96°C for 30 seconds, annealing at 50°C for 40 seconds and elongation at 60°C for 4 minutes. Cooling was done indefinitely at 4°C.

### Post Sequencing PCR Clean up

The mixture was prepared by adding 5 µl D/W, 0.1 µl 125mM EDTA, 1 µl 3M sodium acetate pH 4.6 and 44 µl 100% ethanol and properly mixed. 50 µl of this mix was added to each well in the sequencing plate containing sequencing PCR product. Vortex by Mix mate vortex and incubated at room temperature for 30 minutes. Spun at 3700 rpm for 30 minutes. Decanted the supernatant and add 50 µl of 70% ethanol to each well. Spun at 3700 rpm for 20 minutes. Decanted the supernatant and repeated 70% ethanol wash. Decanted the supernatant and air dried the pellet. The cleaned, air-dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

### Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond et al., 2010). Genomic DNA was extracted and a partial fragment (~600 bp) of the mitochondrial COI gene was amplified and sequenced. The obtained sequence was subjected to BLASTn analysis against the NCBI nucleotide database.

## RESULTS

### Morphological Identification:

The examined specimen exhibited characteristic shell features of *Melanoides tuberculata*, including an elongated conical shell with multiple whorls, a pointed spire, and a narrow aperture. The shell surface



## DISCUSSION

Accurate identification of gastropod species is crucial for biodiversity studies, ecological monitoring, and taxonomic research. Traditional morphological identification may sometimes be unreliable due to morphological plasticity and the presence of closely related species with similar shell characteristics. DNA barcoding using mitochondrial COI sequences has become an effective tool for resolving such taxonomic ambiguities.

In the present study, the COI gene sequence showed complete similarity with sequences of *Melanoides tuberculata*, confirming the molecular identity of the specimen. The phylogenetic analysis further supported this identification by clustering the query sequence within the *M. tuberculata* clade.

Previous studies have demonstrated that the COI gene provides reliable species-level resolution for many gastropods and other aquatic invertebrates. The combination of morphological examination and molecular barcoding improves the accuracy of species identification and helps detect cryptic diversity.

The minimal genetic divergence observed among *M. tuberculata* sequences suggests high genetic similarity within this species, whereas greater divergence from other gastropods indicates clear species boundaries. Therefore, the integration of molecular and morphological approaches provides strong evidence for the correct taxonomic placement of the specimen.

## CONCLUSION

The present study successfully employed COI-based DNA barcoding to confirm the taxonomic identity of a freshwater gastropod specimen. Both morphological observations and molecular analyses consistently identified the specimen as *Melanoides tuberculata*. Phylogenetic analysis and genetic distance assessment further validated this identification by revealing close evolutionary relationships with previously reported *M. tuberculata* sequences and the formation of a well-supported monophyletic clade.

The analyzed sequence, including partial gene regions and comparisons with complete mitochondrial genome data, demonstrated a high level of conservation characteristic of *M. tuberculata*. Minor

sequence variations observed are likely attributable to intraspecific variation. The combined use of BLAST similarity searches and phylogenetic inference provided robust evidence for accurate taxonomic confirmation, highlighting the reliability of mitochondrial markers in species identification and evolutionary studies.

Although no significant genetic divergence was observed to suggest novelty, the sequence generated in this study represents valuable molecular data for *M. tuberculata* from the Jalgaon district. Such regional genetic records are crucial for understanding species diversity, distribution patterns, and evolutionary relationships of freshwater gastropods across different geographical regions.

In nut-shell, this study underscores the importance of integrating classical taxonomy with molecular approaches and contributes to the growing reference database of freshwater molluscs, thereby supporting future research in biodiversity assessment, phylogenetics, and ecological monitoring.

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**Conflict of Interest:** The authors declare no conflict of interest in relation to this research.

**Data Availability Statement:** Not applicable.

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