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Molecular Phylogeny and DNA Barcoding of the Narrowly Endemic Freshwater Snail *Bithynia gonensis (Mollusca: Bithyniidae)* from Türkiye

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Abstract

Objective: *Bithynia gonensis* D. A. Odabaşı, 2024 is a narrowly endemic freshwater snail restricted to the Gönen Stream in northwestern Türkiye. Despite the reported diversity of Bithyniidae in Türkiye, species identifications have largely relied on morphologic characteristics without molecular support. This study aimed to investigate the phylogenetic position of *B. gonensis* using mitochondrial COI barcode data.

Materials and Methods: Specimens were collected from the type locality, and genomic DNA was extracted from foot tissue. A partial fragment of the mitochondrial cytochrome c oxidase subunit I gene was amplified using modified LCO/HCO primers. Sequences were aligned and analyzed in MEGA X, and phylogenetic relationships were inferred using the neighbor-joining method based on uncorrected p-distances with 1000 bootstrap replicates.

Results: The analyses revealed that *B. gonensis* forms a distinct and well-supported monophyletic lineage. It appears as a sibling group to a clade comprising *Bithynia* sp. from Lake Eğirdir and *B. zeta* Glöer & Pešić, 2007, with moderate bootstrap support. The species shows clear genetic divergence from B. tentaculata and B. cettinensis.

Conclusion: This study provides the first molecular data for a bithyniids species from Türkiye and supports the distinct taxonomic status of *B. gonensis*. The findings emphasize the importance of integrating molecular approaches into the taxonomy of Turkish freshwater gastropods.

Keywords Bithynia · COI · freshwater snail · phylogeny · Türkiye

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Introduction

The *phylum Mollusca* represents one of the most diverse groups in the animal kingdom, with approximately 86,000 described species and potentially up to 200,000 species yet to be discovered (Rosenberg, 2014; Bouchet *et al.*, 2016). In Türkiye, freshwater gastropod diversity includes more than 100 taxa (MolluscaBase, 2024a).

Within the endemic taxa, the family *Bithyniidae* is represented by two genera, *Bithynia* Leach, 1818, and *Pseudobithynia* Glöer & Pešić, 2006, in Turkiye and Europe. The number of fossil and extant taxa belonging to these two genera is estimated to be ~390 (Molluscabase, 2024b; 2024c). In particular, the Mediterranean basin is highlighted as the geographic region with the greatest species diversity for these two genera. (Glöer & Bössneck, 2007; Glöer & Pešić, 2007; Glöer, 2019; Glöer *et al.*, 2007, Odabaşı *et al.*, 2024). The reported species in Türkiye include 17 species, i.e. *Bithynia tentaculata*, *Bithynia leachii*, *Bithynia pseudemmericia*, *Bithynia phialensis*, *Bithynia badiella*, *Bithynia pesicii*, *Bithynia yildirimii*, *Bithynia timmii*, *Bithynia kayrae*, *Pseudobithynia pentheri*, *Pseudobithynia yildirimii*, *Pseudobithynia adiyamanensis*, *Pseudobithynia guldeni*, *Pseudobithynia cocussica*, *Bithynia alii*, *Bithynia gonensis* and *Pseudobithynia mardinensis* which are mostly native for the region (Şahin and Yıldırım, 2007; Glöer and Georgiev, 2012; Odabaşı and Arslan, 2015; Odabaşı & Odabaşı, 2017; Glöer and Yıldırım, 2006; Odabaşı *et al.*, 2013; Gürlek, 2017; Gürlek, 2018; Akay *et al.*, 2024; Odabaşı *et al.*, 2024; Gürlek, 2025).

Morphologic characters are the most common features during descriptions of freshwater snails, especially for Bithyniidae in Türkiye. The shell morphology is generally informative only at the family level in Bithyniidae, whereas male anatomical characteristics (penis and associated appendages) are considered more reliable for generic and species-level differentiation (Glöer, 2019). Nevertheless, the identification of bithyniids species based solely on structure may be problematic, particularly among closely related taxa with overlapping characters. In Türkiye, most recognized species exhibit distinct morphologic features and restricted geographic distributions, which facilitate their identification in many cases. However, species with similar morphologic characteristics may still be misidentified when structure is used as the sole diagnostic criterion. A good example of this problem is when both species, *B. tentaculata* and *B. leachii*, are frequently reported from various locations, including the freshwater basins of the Marmara Region of Türkiye. According to the two-species concept of Schütt (1987), both species inhabit Central Europe and the northern Alps (Glöer *et al.*, 2007). Another limitation of relying solely on morphologic characteristics is the potential for misidentifications, which may lead to reports of species occurring far outside their known distribution ranges; for example, records of *B. pseudemmericia* and *B. pesicii* from the Thrace region of Türkiye (Şahin *et al.*, 2017) may reflect such taxonomic uncertainties. Consequently, detailed morphologic comparisons supported by molecular data are needed to verify the identity of these populations.

The lack of molecular data represents a major limitation in resolving the taxonomy and phylogeny of Turkish bithyniids. To address this gap, the present study investigates the phylogenetic position of the recently described endemic species *Bithynia gonensis* using mitochondrial COI sequence data, providing the first molecular insight into the group in Türkiye.

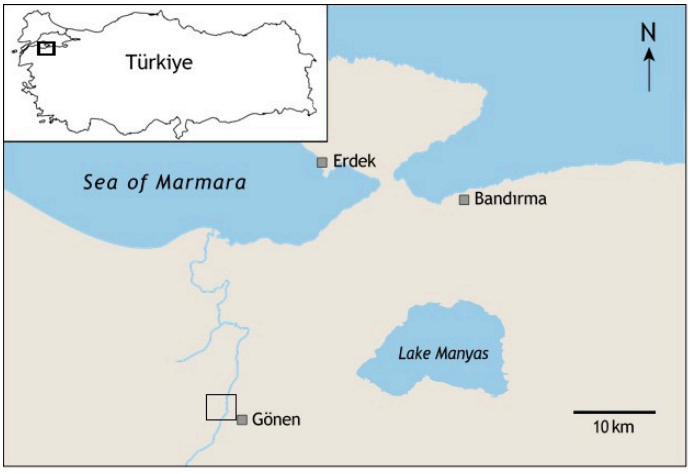
Materials and Methods

Study Area

Sampling was conducted in the Gönen Stream, located in northwestern Türkiye (Figure 1), which is recognized for its remarkable ecological diversity (Arı, 2019). The sampling site corresponds to the type locality of *B. gonensis*, which is known only from this region.

Figure 1

The sampling location and type locality of B. gonensis (Odabaşı et al. 2024).



Sample Collection and Preservation

A total of 10 individuals were collected from the sampling site using a hand net. Specimens were fixed in 80% ethanol in the field and subsequently transferred to 96% ethanol after 24 hours to ensure proper DNA preservation (Pešić *et al.*, 2019).

DNA Extraction, PCR, and Sequencing

Following the manufacturer's instructions, genomic DNA was extracted from the foot tissues using the EurX GeneMATRIX Tissue & Bacterial DNA Kit. PCR amplification of the mitochondrial cytochrome c oxidase subunit I (COI) barcode region was carried out using the LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' (Folmer *et al.*, 1994) and COR722b 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Wilke & Davis, 2000). Each 35 µl reaction mixture was prepared with 3 µl of template DNA, 1 µl PCR buffer, 1.5 mM of MgCl2, 0.2 mM of dNTP mix, 0.3 µM of each primer, 2 U of FIREPol® DNA Polymerase, and PCR grade water. The cycling profile (Szarowska, 2006) consisted of an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 45 seconds, annealing at 57°C for 45 seconds, and extension at 72°C for 60 seconds, culminating in a final extension at 72°C for 5 minutes and a final hold at 4°C. The amplification products were visualized under UV light using ethidium bromide staining following electrophoresis on a 1.5% agarose gel prepared with 1 TAE buffer, running at 100 V for 90 minutes. The resulting amplicons were bidirectionally sequenced using the Sanger method on an ABI 3730XL DNA Analyzer (Applied Biosystems, USA). Finally, the obtained sequence reads were assembled into consensus sequences using the CAP contig assembly algorithm within the BioEdit software.

Data Analysis

For the molecular data analyses, the COI gene region was successfully sequenced for two *B. gonensis* specimens, and the resulting nucleotide data were aligned using the ClustalW algorithm implemented in MEGA X (Kumar *et al.*, 2018). The generated sequences were deposited in GenBank under accession numbers PZ347683 and PZ351246. To infer phylogenetic relationships, a tree was constructed in MEGA X using the neighbor-joining (NJ) method (Saitou and Nei, 1987) based on uncorrected p-distances. The analysis used 1000 bootstrap replicates and the pairwise deletion option. Uncorrected p-distances were deliberately selected to calculate interspecific genetic distances (with 1,000 bootstrap replicates in MEGA X), as they have been demonstrated to yield higher accuracy for species delimitation than corrected substitution models such as the Kimura 2-parameter or Tamura-Nei (Srivathsan and Meier, 2012). The reference sequences retrieved from the GenBank database are listed in Table 1. *Bythinella austriaca* (JQ639858) and *Bythinella micherdzinskii* (JQ639854) sequences were used as outgroup taxa to root the phylogenetic tree.

Table 1

List of GenBank COI sequences of the species used in the molecular analysis of B. gonensis.

Species	COI GenBank Numbers	References
Bithynia tentaculata (Linnaeus, 1758)	MN013036-MN013037	Pešić *et al.*, 2019
Bithynia zeta Glöer et Pešić, 2007	MN013038-MN013041	Pešić *et al.*, 2019
Bithynia cettinensis Clessin, 1887	MN013042	Pešić *et al.*, 2019
Bithynia sp. – Egridir Lake, Tr	FJ160291	Benke *et al.*, 2009
Bithynia sp. – Poso Lake, Indo.	KY574006	Van Bocxlaer *et al.*, 2018
Bithynia funiculata Walker, 1927	KY118598	Kulsantiwong *et al.*, 2013
Bithynia funiculata Walker, 1927	KY118601	Kulsantiwong *et al.*, 2013
Bithynia siamensis goniomphalos (Morelet, 1866)	KY118603	Kulsantiwong *et al.*, 2013
B. siamensis goniomphalos	KY118610	Kulsantiwong *et al.*, 2013
B. siamensis goniomphalos	KY118619	Kulsantiwong *et al.*, 2013
B. siamensis goniomphalos	KY118658	Kulsantiwong *et al.*, 2013
Bythinella austriaca (von Frauenfeld, 1857)	JQ639858	Falniowski *et al.*, 2012
Bythinella micherdzinskii Falniowski, 1980	JQ639854	Falniowski *et al.*, 2012
Gabbia pygmaea (Preston, 1908)	KY118682	Kulsantiwong *et al.*, 2013
Gabbia wykoffi (Brandt, 1968)	KY118724	Kulsantiwong *et al.*, 2013
Hydrobioides nassa (Theobald, 1865)	KY118756	Kulsantiwong *et al.*, 2013
Hydrobioides nassa	KY118765	Kulsantiwong *et al.*, 2013

Species	COI GenBank Numbers	References
Wattebledia baschi Brandt, 1968	KY118769	Kulsantiwong *et al.*, 2013
Wattebledia crosseana (Wattebled, 1884)	KY118793	Kulsantiwong *et al.*, 2013
Gabbia wykoffi (Brandt, 1968)	KY118724	Kulsantiwong *et al.*, 2013
Bithynia gonensis D. A. Odabaşı, 2024	PZ347683 PZ351246	This Study

Results and Discussion

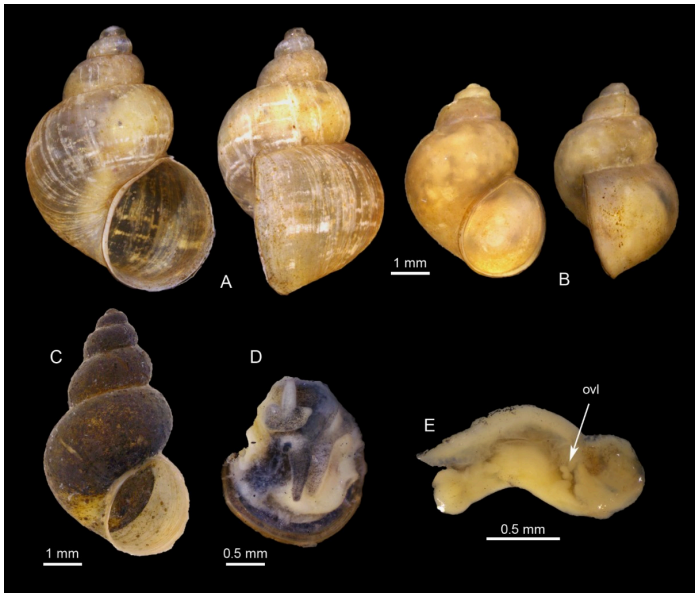
Systematics

The systematic information based on the morphologic characteristics of *B. gonensis* is provided below (Odabaşı *et al.*, 2024) (Figure 2).

Bithynia gonensis D.A. Odabaşı, 2024

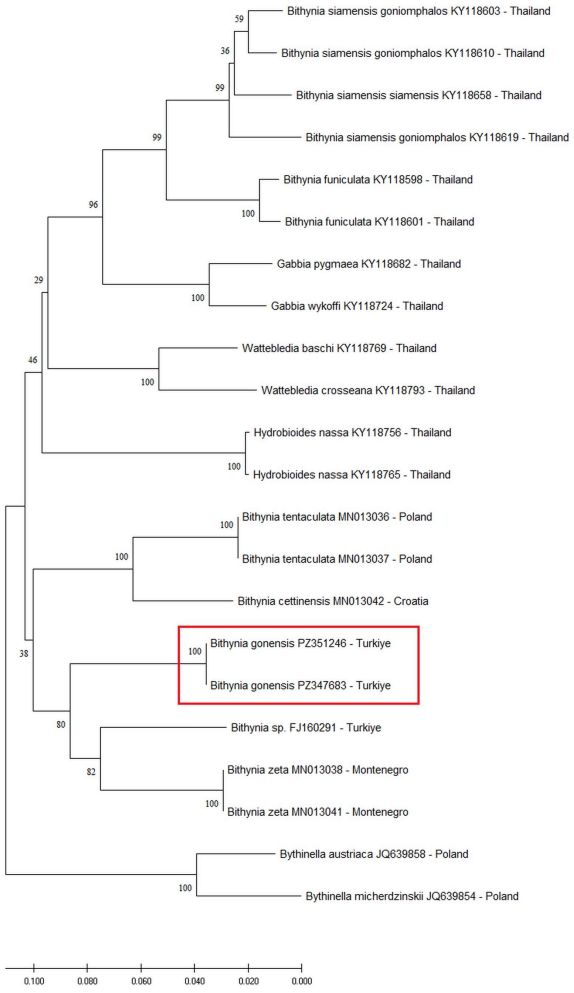
Original Description: Shell is conical, and whorls are swollen. Shell height is up to 7.14 mm and its width is up to 5.14 mm. Sutures are deep, and the body whorl is prominent. The umbilicus is slit-like, and the outer lip of the aperture is sinuate. The head is gray except around the eyes. The penis is small, broad, and ends with a pointed tip. The penial appendix is as long as the penis and close to the penis tip.

Figure 2
Bithynia gonensis. *A – C. Shells of type species: D. Male (head, penis, penial appendix, and tentacle). E. Female reproductive organ (oviductal loop, capsule gland) (COMULM-G 296-297)*



Analysis of mitochondrial COI sequences provided a clear phylogenetic reconstruction for the investigated Bithyniidae taxa (Figure 3). According to the resulting tree, the Turkish endemic *Bithynia gonensis* (PZ347683 and PZ351246) separates as a well-defined, independent monophyletic clade. Rather than grouping with common European taxa, this species emerged as a sister lineage, with good statistical backing (80% bootstrap), to the group containing *Bithynia* sp. from Lake Eğirdir (FJ160291) and *Bithynia zeta* from Montenegro (MN013038, MN013041). Furthermore, isolates of *B. tentaculata* and *B. cettinensis* from Poland and Croatia clustered into a separate clade with strong support (99% bootstrap). Overall, this tree topology explicitly supports the taxonomic validity of *B. gonensis* as a distinct species.

Figure 3
Tree based on neighbor-joining analysis of pairwise genetic p-distances. Bootstrap values for 1000 replicates are shown above branches on the tree (scale: 1% p-distance).



Based on the tree topology, the Turkish endemic *Bithynia gonensis* (PZ347683 and PZ351246) groups in proximity (80% bootstrap) to a *Bithynia* sp. isolate (FJ160291) previously collected from Lake Eğirdir (Benke *et al.*, 2009). Historical surveys indicate that *Bithynia pseudemmericia* is the sole bithyniid inhabiting this lake (Yıldırım, 2004) and the broader Antalya basin (Schütt, 1964; Geldiay & Bilgin, 1969; Özbek *et al.*, 2004; Glöer & Yıldırım, 2006; Kılıçarslan & Özbek, 2010; Zeybek *et al.*, 2012), suggesting that the specimen from Lake Eğirdir, for which a sequence was deposited in GenBank by Benke et al. (2009), most likely belongs to *B. pseudemmericia*. Yet, the distinct branching of *B. gonensis* confirms that it is an independent lineage, refuting any conspecificity with *B. pseudemmericia*. In addition, these molecular results call into question the traditional morphology-based taxonomy of Turkish bithyniids. While *B. tentaculata* has been widely recorded from freshwater habitats throughout Türkiye (Odabaşı *et al.*, 2024), those records remain unverified by DNA data. We provide clear evidence of a deep genetic separation between *B. gonensis* and actual *B. tentaculata* reference sequences from Poland (MN013036, MN013037), as the latter robustly clustered (99% bootstrap) with the Croatian *B. cettinensis* (MN013042).

Given the small sample size, the results should be considered preliminary. Nevertheless, they provide an important first step toward a molecular framework for Turkish Bithyniidae. Although based on a limited dataset, the observed genetic differentiation is consistent with the species' restricted distribution and supports its recognition as a distinct endemic lineage.

Conclusion

Turkish freshwater systems host 17 recognized bithyniid taxa (Glöer & Yıldırım, 2006; Glöer and Georgiev, 2012; Odabaşı *et al.*, 2013; Odabaşı & Arslan, 2015; Odabaşı & Odabaşı, 2017; Gürlek, 2017; Gürlek, 2018; Gürlek *et al.*, 2019; Akay *et al.*, 2024; Odabaşı *et al.*, 2024; Gürlek, 2025); yet, their classification has historically depended entirely on morphological criteria. Such morphology-driven taxonomy can mask cryptic evolutionary relationships, meaning that records of species, including *Bithynia leachii*, *B. phialensis*, and *B. badiella*, urgently require genetic confirmation (Odabaşı, 2024). To resolve these traditional limitations, we successfully sequenced the newly described *B. gonensis*, contributing its very first DNA barcode to GenBank. This milestone creates a reliable molecular starting point for studying the area's malacofauna. Future research must increasingly adopt these molecular tools to accurately reflect the evolutionary diversity of the family Bithyniidae in Türkiye and to correct persistent morphologic misidentifications.

Peer Review Externally peer-reviewed.
Conflict of Interest The authors have no conflict of interest to declare.
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