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DNA barcode of native apple snail *Pila virescens* (Deshayes, 1824) (Gastropoda: Ampullariidae) from Madura Island, Indonesia

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Abstract. *Ambarwati R, Rahayu DA, Faizah U, Isnaningsih NR. 2025. DNA barcode of native apple snail* Pila virescens *(Deshayes, 1824) (Gastropoda: Ampullariidae) from Madura Island, Indonesia. Biodiversitas 26: 1145-1155. Pila virescens* is a native apple snail species in Indonesia that is currently under severe threats that urgently require our attention. This research is therefore crucial in validating *P. virescens* from Madura Island, using both morphological and molecular data, specifically DNA Barcode Cytochrome Oxidase Subunit 1 (COI). Samples were collected from Tlambah Reservoir, Sampang, Madura. Morphological identification was conducted based on shell characteristics, while molecular characterization was applied based on DNA barcode COI. The 618-base pair (bp) of CO1 gene sequence was used for the analysis of genetic diversity and the reconstruction of a phylogenetic tree. Muscle samples were preserved in pure-grade ethanol and underwent a series of procedures, including DNA extraction, Polymerase Chain Reaction (PCR), and sequencing, all conducted with the utmost care and precision. Sequence data were analyzed using Barcode of Life Data Systems (BOLD) Systems, the Automatic Barcode Gap Discovery (ABGD) web application, and haplotype network analysis. The results showed a high Haplotype diversity (Hd) of 0.09091 and a nucleotide diversity (π) of 0.17676, representing a substantial range of genetic variation. The frequency of parsimony informative sites was recorded at 26.48%, with 18 polymorphic sites identified, leading to a total transition/transversion (ts/tv) ratio of 2.4. Furthermore, the phylogenetic trees had a clear and distinct branching pattern for the cluster *P. virescens* with another ingroup. These results would significantly enhance the understanding of genetic diversity within the *Pila* genus, which is crucial for effective conservation efforts.

Keywords: Cytochrome C Oxidase Subunit 1, freshwater ecosystem, freshwater snail, native biodiversity

INTRODUCTION

Freshwater snails are an important part of the freshwater ecosystem, and this includes approximately 5386 accepted freshwater gastropod extant species (MolluscaBase Eds. 2025). However, its existence is threatened by habitat degradation and loss, as well as the introduction of invasive animals (Strong et al. 2008; Dhiman et al. 2020; Marwoto et al. 2020a).

Ampullariidae J. E. Gray, 1824, is a family of freshwater gastropods consisting of two subfamilies, namely Ampullariinae and Pomaceinae (MolluscaBase Eds. 2024). *Pila* Röding, 1798 is a genus belonging to Ampullariinae, which includes 23 extant species (MolluscaBase Eds. 2021). The *Pila* snails are native to Southeast Asia where several previous researches reported the presence of members of this genus in Southeast Asia, including *Pila ampullacea* (Linnaeus, 1758) (Dharma 2005; Marwoto et al. 2020a; Purnama et al. 2022), *P. scutata* (Mousson, 1848) (Dharma 2005; Low et al. 2013; Ng et al. 2017; Marwoto et al. 2020a, 2020b; Ng et al. 2020), *P. virescens* (Deshayes, 1824) (Marwoto et al. 2020a; 2020b; Ng et al. 2020), *P. celebensis* (Quoy & Gaimard, 1834), *P. turbinis* (I.Lea, 1856), *P. gracillis* (I.Lea, 1856), and *P. pesmei* (Morlet, 1889) (Ng et al. 2020).

The introduction of *Pomacea* spp. has led to a significant decline in the native apple snail population in freshwater habitats and wetlands. Studies by Horgan et al. (2014) and Ng et al. (2017, 2019, 2020) highlight this issue. *Pomacea* spp. are polyphagous and opportunistic (Saveanu et al. 2023), with faster growth (Ismail et al. 2021), as well as the capability to live in clean waters and polluted water (Oliveira et al. 2024). There are three species of *Pila* in Indonesia, namely *P. ampullacea, P. virescens,* and *P. scutata* (Dharma 2005; Marwoto et al. 2020a, 2020b). *Pila ampullacea* is well-known and consumed by the community and used to prepare animal feed compared to the other 2 species, which have not been widely investigated. *Pila virescens* prefers calm waters, including swamps and ponds (Marwoto et al. 2020a), and spreads across Java Island (Marwoto et al. 2020a) but has decreased presence. A case study in Rawa Pening Lake (Central Java) showed that the presence of *P. virescens* had disappeared from the lake after 1988 (Marwoto et al. 2020b). Therefore, various inventory activities need to be carried out to determine the presence of *P. virescens* in other locations.

Inventory activities are performed to determine a species' distribution and characterization. Snail inventory and identification can be carried out based on morphological and molecular characteristics. Molecular characterization for snails can be conducted by DNA Barcoding (Galan et al. 2018; Kannan et al. 2020; Raphalo et al. 2021; Sarhan et al. 2021; Cunha et al. 2023). One of the good markers for DNA barcode freshwater snails is the Cytochrome Oxidase Subunit I (COI) gene (Kulsantiwong et al. 2013; Kannan et al. 2020; Lorencová et al. 2021). COI barcode plays a crucial role in delimiting species (de Araújo et al. 2023), serving as a tool for quick and accurate identification (Galan et al. 2018; Sarhan et al. 2021) while promoting conservation (Mohammadi and Ahmadzadeh 2024) and monitoring endangered species (Schubert et al. 2023).

A population of *P. virescens* was recently rediscovered on Madura Island, approximately 83 years after the first reported presence on the same island (Van Benthem-Jutting 1941). Based on scientific collection deposited in the Museum Zoologicum Bogoriense, this species was also collected in 1972 from Sumenep, Madura. The disappearance in Rawa Pening Lake, Central Java, has been reported (Marwoto et al. 2020b), while its presence in other localities of Indonesia has received less attention. Therefore, this research aimed to validate *P. virescens* from Madura Island based on morphological and molecular data using DNA barcode of Cytochrome C Oxidase Subunit 1.

MATERIALS AND METHODS

Study area and sampling sites

Specimens were collected from Tlambah Reservoir, in Sampang District, East Java, Indonesia (7°01'56.0"S 113°21'35.1"E and 7°01'55.8"S 113°21'38.4"E) (Figure 1) by hand picking. The samples were then preserved in 70% alcohol, while three individuals were preserved in absolute ethanol for molecular analysis. The collection bottles were tightly closed for further laboratory analysis.

Procedures

Identification

Twenty-three specimens were examined; two of them are deposited in the gastropod collections of the Museum Zoologicum Bogoriense (Cibinong); MZB GST.25.601 and MZB GST.25.602, and the rest of specimens were deposited at Taxonomy Laboratory of Universitas Negeri Surabaya. Four individuals were taken for DNA analysis. Identification based on shell morphology was conducted by observing the shell shape, shell sculpture, aperture shape, and shell operculum. This referred to several references, including Dharma (2005), Ng et al. (2019), and Marwoto et al. (2020b). The taxonomic validation was based on MolluscaBase (https://www.molluscabase.org/). Additionally, morphometric measurements of Shell Height (SH), Shell Width (SW), Body Whorl Height (BWH), Aperture Height (AH), Aperture Width (AW), and Spire (S) were carried out using calipers (Figure 2).

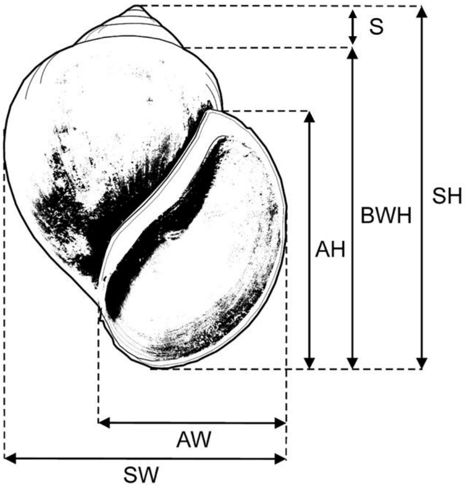

Figure 2. Morphometric measurements of *Pila virescens*

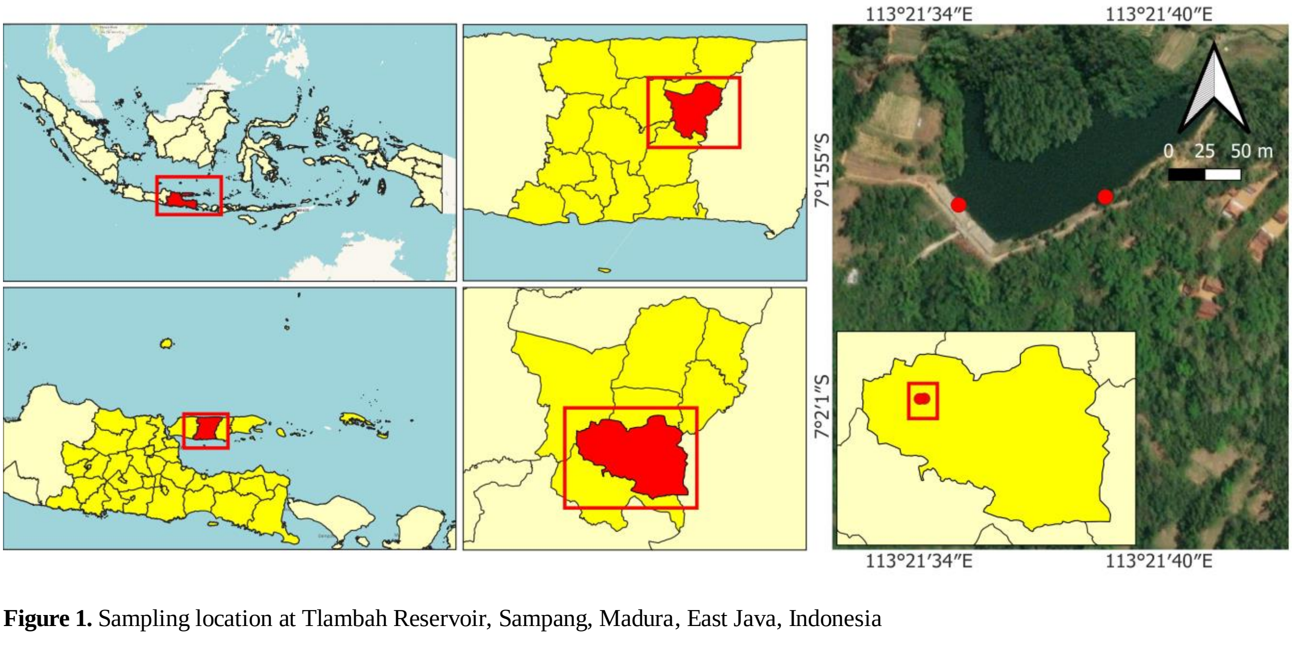

Figure 1. Sampling location at Tlambah Reservoir, Sampang, Madura, East Java, Indonesia

DNA extraction

The extraction of total DNA (whole genome) from muscle samples was performed using the NextPrep Kit, with several modifications. Foot muscle samples from the snails, weighing between 15 and 20 mg, were ground and placed into a 1.5 mL tube. Subsequently, 200 µL of Buffer GT1 was added and mixed thoroughly with a vortex (Pre-lysis Stage). Next, 200 µL of Buffer GT2 and 20 µL of Proteinase K were combined and vortexed (Lysis Stage). The resulting mixture was incubated at 56°C for 10 minutes, with the tube being inverted every 5 minutes. A total of 200 µL of pure ethanol was added to the mixture and vortexed again, then the samples were transferred to a Spin Column and centrifuged at 13,000 rpm for 1 minute. The liquid that passed through was discarded, and 500 µL of W1 buffer was added to the Spin Column, followed by another centrifugation at 13,000 rpm for 1 minute. After removing the flow-through, 700 µL of W2 buffer (containing ethanol) was added to the Spin Column and centrifuged again at 13,000 rpm for 1 minute. The flow-through was discarded once more, and the Spin Column was subjected to an additional 2-minute centrifugation at the same speed. The DNA retained in the Spin Column was then transferred to a new 1.5 mL tube. Finally, 50 to 100 µL of Elution Buffer was added, allowed to sit at room temperature for 1 minute, and then centrifuged at 13,000 rpm for 1 minute.

PCR and sequencing

A segment of about 618 base pairs (bp), corresponding to the COI gene region of mitochondrial DNA (mtDNA), was amplified using Polymerase Chain Reaction (PCR). This amplification applied universal primers LCO1490 (5' `GGT CAA CAA ATC ATA AAG ATA TTG G` 3') and HCO2198 (5' `TAA ACT TCA GGG TGA CCA AAA AAT CA` 3') by Folmer et al. (1994). The PCR implemented hot start methods using a Kapa master mix and two Taq master mixes. The amplification consisted of 35 cycles, each including several steps: initial denaturation at 95°C for 3 minutes, followed by denaturation at 94°C for 45 seconds, annealing at 45°C for 45 seconds, and extension at 72°C for 2 minutes. The process concluded with a final elongation step at 72°C for 10 minutes. Subsequently, the products were analyzed on a 1% agarose gel (prepared with 0.5 g of agarose and 50 mL of TAE Buffer) containing 4 µL of Ethidium Bromide as a staining agent to visualize the PCR products. A mixture of 3 µL of the PCR products and 1 µL of loading dye was transferred into the gel wells before performing electrophoresis at 220 V and 400 mA for 25 minutes. The PCR products were purified using a Qiagen purification kit in correspondence with the instructions of the manufacturer, then the purified samples were then sent to First Base in Malaysia for sequencing.

Data analysis

DNA barcode

This research obtained partial sequences of the COI gene based on 618 bp from four individuals of *Pila* snails. Initially, each sequence was translated into an amino acid sequence to eliminate any pseudogenes, following protocols stated by Song et al. (2008) and Šlechtová et al. (2021). Subsequently, chromatogram analysis was performed using Finch TV software and the translated amino acid sequences were then verified through the ExPASy website (https://web.expasy.org/translate/) (Duvaud et al. 2021). All sequences were compared with close relatives of the samples through the Basic Local Alignment Search Tool (BLAST) (Boratyn et al. 2013) and the Barcode of Life Data Systems (BOLD) System (Baena-Bejarano et al. 2023). A total of 18 accessions from GenBank (NCBI) were selected as ingroup and outgroup for phylogenetic tree construction. Multiple sequence alignment was performed using Clustal X (Ferrari and Patrizio 2021), followed by manual verification with BioEdit software. The partial COI gene sequences from *Pila* in this study were submitted to GenBank with corresponding accession numbers, as presented in Table 1.

Phylogenetic reconstruction

Phylogenetic reconstruction based on partial sequences of the COI gene included a total of 12 sequences, both ingroup and outgroup accessions, which were obtained from GenBank (Table 1). The analysis in this procedure aimed to construct the grouping of different species with related species. Phylogenetic tree reconstruction was conducted using MEGA X version 10.2.6, with the Neighbor-Joining (NJ) and Maximum-Likelihood (ML) methods. For both NJ and ML tree reconstructions, the Kimura 2-Parameter (K2P) substitution model was used (Nishimaki and Sato 2019). In addition, the rate variation among sites was modeled with a Gamma distribution, and a bootstrap consensus tree was inferred from 1000 replicates (De Moraes Russo and Selvatti 2018).

Species delimitation using ABGD

The Automatic Barcode Gap Discovery (ABGD) method, as stated by Puillandre et al. in (2012), was implemented to differentiate *Pila* species using the COI gene. Spart Explorer https://spartexplorer.mnhn.fr/ was used to strengthen the ABGD analysis. This algorithm identified potential species by detecting significant gaps in the distribution between intra- and interspecific distances, which is then used to delimit species hypotheses. ABGD was performed by repeatedly dividing the dataset into potential species groups to achieve the most refined categorization. The process started with the development of a pairwise uncorrected p-distance matrix using MEGA software, excluding any ambiguous positions in the sequence pairs. The ABGD analysis was conducted using an online tool accessible at http://wwwabi.snv.jussieu.fr/public/abgd/. This analysis incorporated 2 different models, namely Jukes-Cantor and K2P. These models were used to calculate genetic distances while considering different assumptions about nucleotide substitution rates.

Table 1. DNA sequences from NCBI GenBank used as comparisons

Species	Location	Haplotype	ACC number
Pila virescens T1	Tlambah Reservoir, Sampang, Indonesia	T1	PQ896824
Pila virescens T2	Tlambah Reservoir, Sampang, Indonesia	T2	PQ896825
Pila virescens T3	Tlambah Reservoir, Sampang, Indonesia	T3	PQ896843
Pila virescens T4	Tlambah Reservoir, Sampang, Indonesia	T4	PQ896844
Pila virescens isolate THAMP044_18	Singapore	THAMP044_18	MN104580.1
Pila virescens isolate THAMP042_18	Singapore	THAMP042_18	MN104578.1
Pila celebensis isolate THAMP057-19	Singapore	THAMP057-19	MN207238.1
Pila celebensis isolate THAMP056-19	Singapore	THAMP056-19	MN207234.1
Pila gracilis isolate THAMP023_18	Singapore	THAMP023_18	MN104559.1
Pila gracilis isolate THAMP015_18	Singapore	THAMP015_18	MN104551.1
Pila sp. HI_141	USA	HI_141	EF514947
Pila sp. KH_140	Cambodia	KH_140	EF514946
Pila virescens	Vietnam	BM250710	EU528475
Pila virescens	Vietnam	VN21	FJ710304
Pila virescens	Thailand	THAMP031_18	MN104567
Pila virescens	Thailand	THAMP032_18	MN104568
Pila virescens	Thailand	THAMP033_18	MN104569
Pila virescens	Thailand	THAMP034_18	MN104570
Pila virescens	Laos	THAMP035_18	MN104571
Pila virescens	Thailand	THAMP036_18	MN104572
Pila virescens	Thailand	THAMP037_18	MN104573
Pila virescens	Thailand	THAMP038_18	MN104574
Pila virescens	Thailand	THAMP039_18	MN104575
Pila virescens	Thailand	THAMP040_18	MN104576
Pila virescens	Thailand	THAMP041_18	MN104577
Pila virescens	Thailand	THAMP043_18	MN104579
Filopaludina javanica voucher ZMB 11275	Germany	ZMB 112753	MN997939.1
Filopaludina javanica voucher ZMB 127082	Germany	MN997940	MN997940.1

RESULTS AND DISCUSSION

Descriptions of *Pila virescens* (Deshayes, 1824)

Synonym

Ampullaria brohardi (Granger, 1892); *Ampullaria callistoma* (Morelet, 1866); *Ampullaria pagoda* (Morelet, 1865); *Ampullaria polita* (Deshayes, 1830); *Ampullaria polita* var. *compressa* (G. Nevill, 1885); *Ampullaria polita* var. *major* (Dautzenberg & H. Fischer, 1906); *Ampullaria virescens* (Deshayes, 1824); *Pachylabra polita* (Deshayes, 1830); *Pila polita* (Deshayes, 1830)

Morphology

Shell. Large (shell height about 23.8-67 mm, shell width 19.4-52.8 mm - see Table 2), subglobose, thick, and solid. Yellowish green to greenish brown. The shell coils in a dextral direction with about 6-6.5 whorls. The surface is plain and smooth with a soft luster. Striated with transverse lines, spiral band absent. Shell spire elevated. Suture flat without any channel. Umbilicus narrow or closed. The Aperture oval to elongate lunar or oblique, apertural lips is somewhat thickened.

Operculum. Solid and calcified, oval to elongate lunar. Multispiral with subcentral nucleus. Slightly concave on its external surface (Figure 3).

Habitat

Prefers to live in lentic environments such as ponds, swamps, or lakes.

Table 2. Morphometry of *Pila virescens* from Madura (n = 23)

Parameters (mm)	*Pila virescens*
Shell Height (SH)	23.8-67
Spire (S)	2-14.7
Body Whorl Height (BWH)	20.8-53.1
Shell Width (SW)	19.4-52.8
Aperture Width (AW)	11-31.4
Aperture Height (AH)	15.9-40.5

Figure 3. Shell of *Pila virescens* (scale bar: 10 mm)

The discovery of *P. virescens* in the Tlambah Reservoir was the first rediscovered record on Madura Island, about 83 years after it was first reported. Furthermore, the distribution of this snail in Indonesia has only been reported from Java Island. The size of the snail found in Madura is slightly smaller than that reported from Java, namely a shell size of 56.77-71.13 mm high and a width of 38.22-50.32 mm (Marwoto et al. 2020a).

Sequence composition and genetic diversity

The successful amplification of the COI gene was confirmed by a distinct DNA band appearing at approximately 618 bp, without any smearing (Figure 4), signifying that the amplification process effectively captured functional mitochondrial COI sequences. The absence of smearing in the gel electrophoresis results further supported the quality of the amplification. Moreover, the lack of stop codons in the amplified sequences is a clear indication that nuclear DNA sequences originating from mitochondrial DNA (NUMTs) appearing shorter than 618 bp (Inoue et al. 2020) were excluded from the sequencing process. The COI gene is widely recognized for its reliability in species identification since pseudogenes are eliminated during protein translation. Numerous investigations found COI sequences to be effective for differentiating species across various taxa. For example, recent biodiversity research generated new COI sequences for a significant number of species (Venera-Pontón et al. 2020).

The sequence validation results obtained from the online facilities of BLAST and BOLD showed that the sequence samples matched the available accessions in the database with a query cover ranging from 99% to 99.8% (Table 3). Furthermore, the high query cover percentages suggest a strong alignment between the sequenced samples and the references in the database, confirming the identity of the samples as *P. virescens*. This level of similarity is essential for ensuring the accuracy of species identification (Cossu et al. 2014). High query cover percentages signify the reliability of the sequence data. Hence, a query cover of 99% or greater in the context of molecular barcoding suggests that the sequences are highly conserved and that the species has been well-characterized in the database. This is particularly important in biodiversity research, where accurate species identification is crucial for ecological assessments and conservation strategies.

DNA barcoding was highly effective in distinguishing *Pila* genus across various regions. Recent studies have demonstrated the high efficacy of DNA barcoding in distinguishing *Pila* species across various regions. For instance, Kulsantiwong et al. (2013) highlighted the utility of DNA barcoding in identifying freshwater snails, emphasizing its role in detecting cryptic species that might not be distinguishable through morphological characteristics only. This is particularly relevant for *Pila*, where species may exhibit minimal morphological variation yet possess significant genetic divergence. The successful application of DNA barcoding in identifying *Pila* species is further supported by the results of Mitchell (Mitchell 2015), stating that even decades-old dried museum specimens could yield reliable DNA sequences for barcoding purposes. This signifies that DNA barcoding can effectively use historical samples, enhancing its applicability in biodiversity assessments and conservation efforts.

The *Pila* genus, comprising various freshwater snails, has significant genetic diversity that is crucial for understanding evolutionary relationships and species differentiation. Metrics such as haplotype diversity (Hd) and nucleotide diversity (π) provide insights into the genetic variability in populations. This research reports a Hd of 0.09091 and π of 0.166, representing a wide range of genetic variations among the studied *Pila* populations (Table 4). The observed Hd of 0.09091 suggests a rich genetic structure within the *Pila* populations, reflecting the presence of multiple haplotypes across different geographical regions. High haplotype diversity is often associated with larger effective population sizes and historical demographic events that promote genetic variation (Estupiñán et al. 2016). The nucleotide diversity (π) of 0.166 further emphasizes the genetic variability in these populations, implying that the COI gene sequences show considerable differences among individuals.

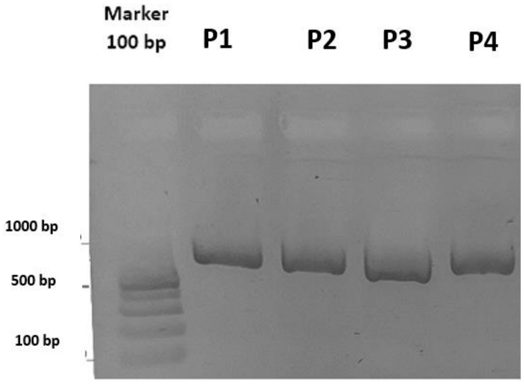

Figure 4. Visualization of DNA specimens of *Pila virescens* from Sampang, Madura Island, Indonesia

Table 3. Validation of partial sequence of COI gene of *Pila* with ingroup through the BLAST (GenBank/NCBI) and the BOLD System online facilities

Species	Highest BOLD identification	GenBank NCBI			Bold systems	
		Query cover (%)	E-value	% Similarity	Similarities (%)	Status
*Pila virescens*_T1	*Pila virescens*	99.2	0	99.6	99.24	Published
*Pila virescens*_T2	*Pila virescens*	99.3	0	99.7	99.35	Published
*Pila virescens*_T3	*Pila virescens*	99.6	0	99.8	99.6	Published
*Pila virescens*_T4	*Pila virescens*	99.5	0	99	99.7	Published

Table 4. Characteristics of partial COI gene sequence used for phylogenetic trees reconstruction and genetic distance analysis, including sequences from the research samples and the GenBank/BOLD system (ingroup and outgroup

Parameters	**Position at codon**			
	1st	**2nd**	**3rd**	**Total**
Thyrosine frequency	26.73%	38.4%	24.9%	618 bp
Cytosine frequency	30.21%	30.2%	33.1%	618 bp
Adenine frequency	24.65%	14.5%	27.3%	618 bp
Guanine frequency	22.41%	16.9%	15.6%	618 bp
Frequency of invariable sites	58.35%			
Frequency of parsimony informative sites	26.48%			
Nucleotide diversity (Pi)	0.17676			
Haplotype diversity	0.09091			
Number of haplotypes	15			
Polymorphic sites	18			
Variance of Haplotype diversity	0.00197			
ts/tv ratio (R)	2.4			
Gamma discrete distribution	0.68			
Standard Deviation of Haplotype Diversity	0.031			
Mean of evolutionary rate	0.00, 0.04, 0.05, 0.08, 0.22, 0.24, 0.26 and 2.2 substitutions per site			

Note: The COI gene sequence characteristics were based on the 618 bp sequence length

Low haplotype diversity indicates that the population has limited genetic variation at the haplotype level, which can lead to reduced adaptability to environmental changes and increased vulnerability to diseases (Camacho et al. 2017). Conversely, the relatively higher nucleotide diversity suggests that, although the population may have a limited number of haplotypes, the genetic variations within those haplotypes are more pronounced. From a conservation perspective, the low haplotype diversity indicates a pressing need for management strategies that enhance genetic diversity. This could involve habitat protection to prevent further genetic erosion and the implementation of breeding programs that introduce genetic variability (Wongsa et al. 2017).

For instance, conservation efforts could focus on creating wildlife corridors to facilitate gene flow between isolated populations, thereby increasing the overall genetic diversity and resilience of the population. Factors influencing population genetic structure include environmental variability, habitat fragmentation, and anthropogenic impacts. For example, high haplotype diversity coupled with low nucleotide diversity has been linked to genetic bottlenecks caused by habitat loss or overexploitation, which can severely limit gene flow and reduce the effective population size (Ukenye et al. 2020).

The frequency of parsimony informative sites at 25.48% suggested the presence of informative genetic variations that could facilitate species differentiation. The 10 polymorphic sites and the overall ts/tv ratio of 2.82 further showed the genetic variability and evolutionary dynamics in the explored *Pila*. The mean evolutionary rate ranging from 0.00 to 2.2 substitutions per site represented the rate of genetic changes over time in the COI gene sequences. The assessment of genetic distance between species was crucial for understanding the relationships and similarities among different *Pila*. The genetic distance between *Pila* and other ingroup species is 3.2%. At the same time, the overall transition/transversion (ts/tv) ratio of 2.82 signifies a predominance of transitions over transversions, a common pattern observed in mitochondrial DNA sequences. This familiar pattern of transitions over transversions is significant due to reflecting the evolutionary dynamics and mutation processes affecting the COI gene. The mean evolutionary rate of COI is generally considered to be approximately 0.0140 substitutions per million years, suggesting that the COI gene evolves at a relatively rapid pace compared to other mitochondrial genes (Estupiñán et al. 2016; Guy-Haim et al. 2018).

Understanding the genetic distance between *Pila* species is crucial for elucidating the relationships and similarities. The genetic distance of 3.2% between *Pila* and other ingroup species represents a moderate level of divergence, which is essential for assessing phylogenetic relationships and potential speciation events in the genus. The genetic analysis results showed the importance of using molecular methods, such as DNA barcoding, for species differentiation in the *Pila* genus. The high levels of genetic diversity and the presence of informative sites suggest that these genetic markers can be effectively used for identifying species and understanding evolutionary relationships (Yu et al. 2021).

Identification using BOLD Systems

The highest accuracy in identifying *Pila* from Madura Island, using the Barcode of Life Data (BOLD) Systems online platform, ranged from 99.58% to 100%. This significant accuracy shows the effectiveness of DNA barcoding for species identification, specifically considering the complexity and diversity of *Pila* species, which often display subtle morphological variations that can be difficult to detect through traditional taxonomy only. This research confirmed the BOLD system as a superior tool for identifying species when compared to other databases and achieving high identification accuracy, corresponding with the results of Modeel et al. (2023). The results showed no differences in BOLD identification between the variants of *Pila* genus (Table 3). A genetic distance greater than 2% represented distinct species in the group. In comparison, a genetic distance of less than 3% suggested that the individuals belonged to the same species or closely related species (Wong et al. 2009). This genetic distance also correlated with GenBank data for each sample and related species. Variations in genetic distance were attributed to genetic diversity in the groups where a genetic distance value below 2% signified no closely related species and was classified as very low. However, high genetic distances could signify identification at the family or genus level (Martin et al. 2020).

Phylogenetics reconstruction

Phylogenetic reconstruction analysis led to the construction of NJ and ML trees shown in Figures 5 and 6, respectively. Each species was associated with a distinct DNA barcode cluster, facilitating the clear delineation of existing phylogenetic relationships. Both NJ and ML trees exhibited had 4 divergent clusters, implying robust support for the inferred relationships. The phylogenetic trees showed an unambiguous branching pattern for *Pila* genus with different clusters. The proximity of these 2 species at the same node suggested a close evolutionary relationship, consistent with the calculated genetic distance of 4.11%, which represented the furthest genetic separation. In general, the NJ, ML, and genetic distance analyses supported the genetic distinctiveness of *Pila,* by signifying the divergence and evolutionary relationships in the genus for sustainability and conservation. The phylogenetic reconstruction analysis also led to the construction of NJ and ML trees, as shown in Figures 5 and 6, respectively. Each species in the *Pila* genus was associated with a distinct DNA barcode cluster, facilitating a clear delineation of phylogenetic relationships. The NJ and ML trees exhibited two divergent clusters, implying robust support for the inferred relationships among the species. The unambiguous branching pattern observed in the phylogenetic trees for the *Pila* genus showed the genetic distinctiveness of the species. The presence of distinct clusters suggested that the species passed through significant evolutionary divergence, which was essential for understanding the ecological roles and evolutionary history.

The method used for constructing phylogenetic trees at a substitution rate of 0.02 per site typically involves maximum likelihood and Neighbor-joining estimation, with node support assessed through bootstrap analysis. The scale of the tree is influenced by both the number of taxa and the evolutionary divergence, necessitating careful consideration of these factors to ensure accurate phylogenetic inference (Xue et al. 2020). In the *Pila* genus, the nodes in the phylogenetic tree were likely supported by high bootstrap values, indicating robust phylogenetic relationships among the haplotypes and haplogroups identified. The results contributed to the understanding of *Pila* phylogeny and genetic diversity, which was essential for their conservation efforts. As these *Pila* faced increasing threats from habitat loss and invasive snails, the genetic information became crucial for developing targeted conservation measures (Liu et al. 2023). The result showed that *Pila* were resolved sister taxa with robust bootstrap support, and the genetic diversity observed between these clusters was very low, with values greater than 2%. Established criteria stated that a genetic distance value exceeding 2% signifies species differentiation, while less than 3% suggested intra-specific or conspecific groupings (Chakraborty and Ghost 2014; Bektas et al. 2018). Consequently, the genetic distance analysis supported the distinction between species and provided insight into the genetic diversity between the species in Indonesia, particularly Madura Island.

Species delimitation using ABGD

The ABGD analysis identified six groups for *Pila*, including both ingroup and outgroup samples (Figure 7). The initial partitioning was conducted with a prior maximal distance (P) of 0.059948, and the barcode gap threshold was calculated at 0.100, while the K80 Kimura distance was determined to be 1.68. The analysis showed a barcode gap centered around 1.45% divergence between the available COI sequences, representing a significant genetic distinction among the groups. The prior interspecific genetic divergence values of 4.11%. This divergence value was considered more plausible than the presence of three or more species that had intraspecific divergence values below 0.28%. However, a single species designation was associated with intraspecific divergence values exceeding 3.2%. This corresponded with the results of the ABGD grouping, which divided the species into three distinct groups. The calculated divergence values provided a framework for understanding the evolutionary relationships among *Pila* species. The interspecific divergence of 4.11% signified a clear genetic separation, while the intraspecific divergence values showed the potential for cryptic diversity within species. These insights are crucial for taxonomic classification and can help to resolve ambiguities in species identification.

Haplotype network of *Pila*

The MJ network analysis provided a comprehensive description of the genetic variation in *Pila* genus, presenting 15 haplotypes categorized into 4 distinct haplogroups. The network showed that *Pila* species formed an ingroup based on their unique haplotypes, where each *Pila* possessed a distinct, non-homologous haplotype corresponding with the geographical location. This genetic clustering suggested a close relationship of *Pila* with the origin locations as presented in Figure 8. The results provided valuable insights into the genetic diversity and relationships in *Pila*, emphasizing the importance of further research and conservation efforts. The results of the MJ network analysis revealed that the *Pila* species formed a clear ingroup, suggesting a close genetic relationship among the haplotypes within the genus. Each *Pila* species possessed unique haplotypes, which were not homologous to those of other species, indicating a high level of genetic differentiation. This genetic clustering is significant as it suggests that the *Pila* species have adapted to their respective environments, leading to the development of distinct haplotypes that reflect their geographical origins (Zhang et al. 2017). The identification of nine haplotypes categorized into four distinct haplogroups suggests a structured genetic variation that is closely linked to geographical distribution. Each *Pila* species exhibiting unique haplotypes corresponding to their geographical locations indicates that local adaptation and historical biogeographical processes have played crucial roles in shaping the genetic landscape of this genus (Thomassen et al. 2011; Duo et al. 2015).

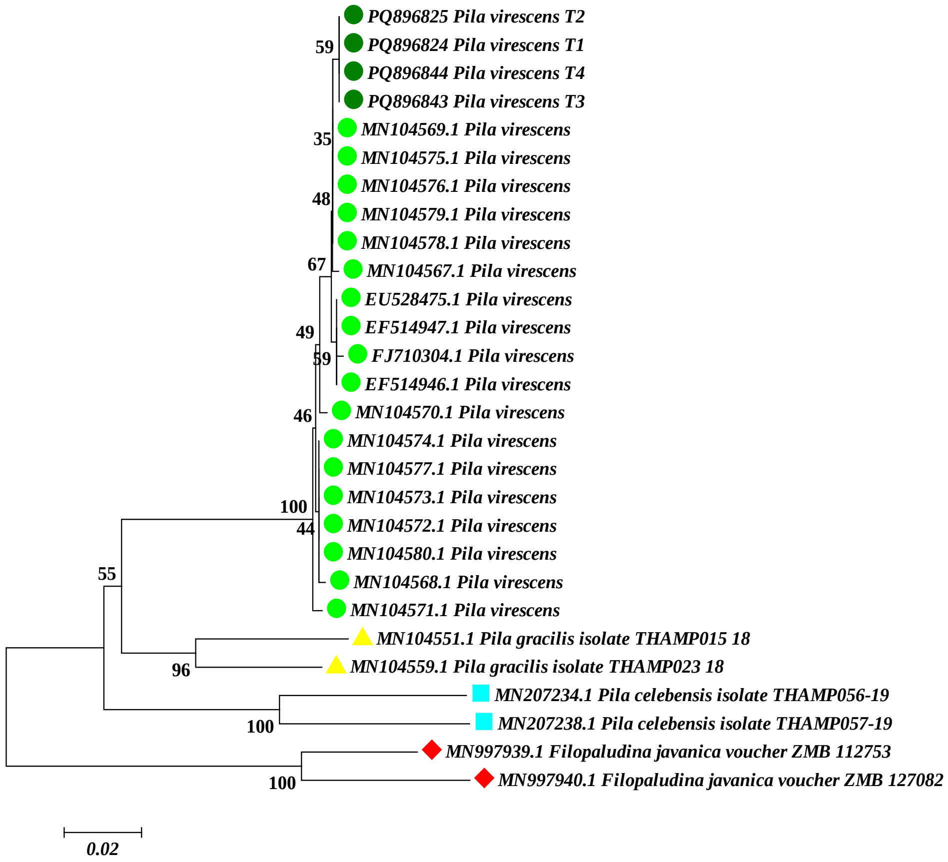

Figure 5. Neighbour Joining (NJ) phylogenetic tree of *Pila*

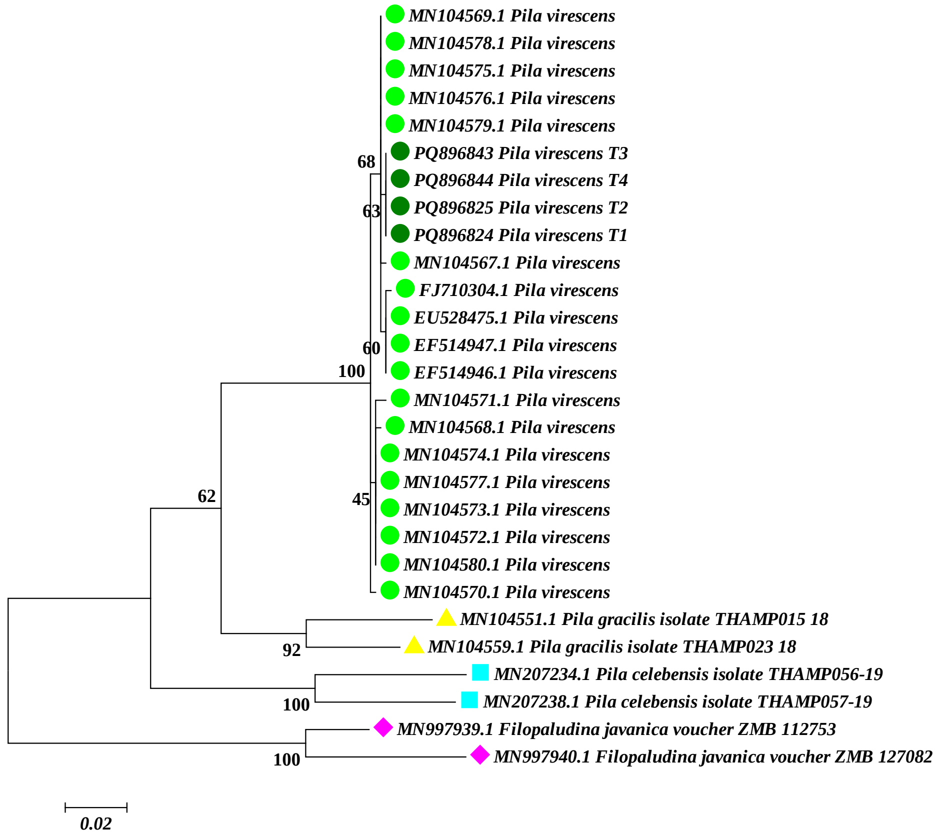

Figure 6. Maximum Likelihood (ML) phylogenetic tree of *Pila*

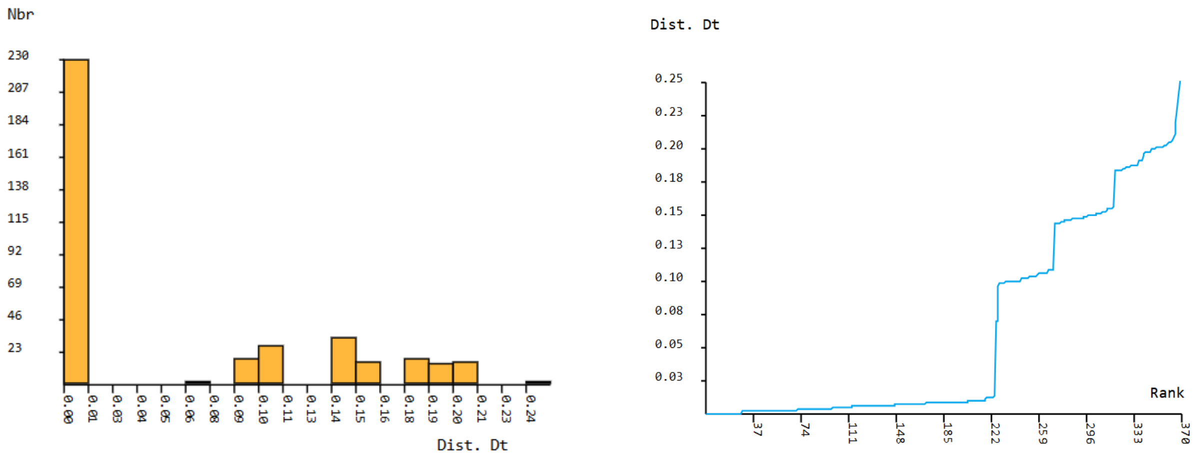

Figure 7. Analysis of gap barcodes of *Pila* species generated by ABGD (Puillandre et al. 2012). Distribution of K2P distances between each pair of samples for the COI gene: A. Distance histogram; B. Rank distance intraspecific divergence

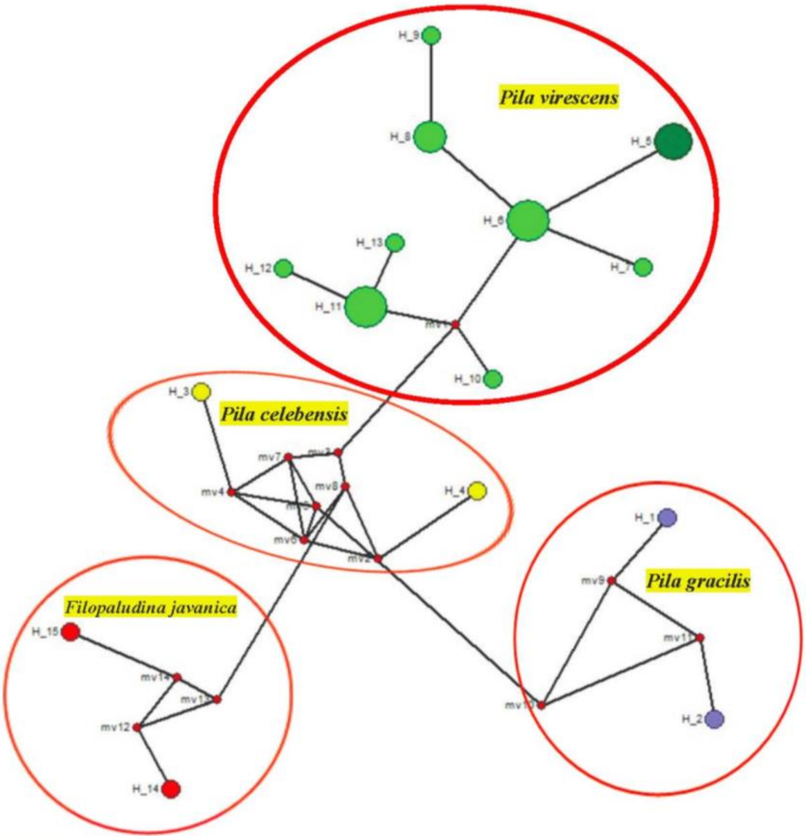

Figure 8. Haplotype network of *Pila*

Each species identified with MJ network analysis has a specific, non-homologous haplotype correlating with the geographical location. This pattern of genetic differentiation suggests that environmental factors and geographical isolation may play significant roles in shaping the genetic structure of *Pila* populations. The identification of distinct haplotypes and haplogroups in the *Pila* genus provides essential insights into the genetic diversity and evolutionary relationships among species. The presence of unique haplotypes associated with specific locations shows the importance of conserving these populations because of the potential to harbor unique genetic traits critical for the resilience and adaptability of the species.

In conclusion, this research confirmed the existence of *Pila virescens* in Madura Island, Indonesia, and provided the species' molecular characteristics. Additionally, the results would significantly enhance the understanding of genetic diversity in the *Pila* genus needed for future conservation programs.

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