# DNA barcoding of molluscs (Bivalvia) in selected sites in Capiz, Philippines

ANDREA PEARL D. AREÑO[1], LAURA ELYSE J. CAMBEL[1], MARIENELLE S. HILAPAD[1], ANDREA LUCYLE M. BELA-ONG[1], RAYMOND VINCENT F. CASTILLO[2], and MARCOS B. VALDEZ JR.[3]

[1]*Philippine Science High School - Western Visayas Campus, Brgy. Bito-on, Jaro, Iloilo City 5000, Department of Science and Technology – Science Education Institute, Philippines*

[2]*Department of Biology, College of Science, De La Salle University, Malate, Manila 1004, Philippines*

[3]*Department of Biological Sciences, Institute of Arts and Sciences, Far Eastern University, Sampaloc, Manila 11000, Philippines*

**Abstract**

Molluscs exhibit complex morphological characteristics and have the same vernacular for the same species which makes species identification difficult. Using morphology as the lone identifier of species populations was found to be problematic. DNA barcoding using the CO1 gene is found to be a useful tool for species identification when traditional taxonomy is ineffective. This study aimed to contribute to the current DNA barcoding data of molluscs in the Philippines and provide identification of commercially available bivalves in Roxas City, Capiz for conservation and diversity assessment. A discordance between morphological and molecular identification was found and nine putative new species were discovered. DNA barcoding can be used for species identification, food safety, conservation management, market surveillance and discovery of putative new species of bivalves in markets in Roxas City, Capiz. In addition, combining morphological and DNA taxonomic analysis can help in conserving and monitoring of commercially-available marine species.

***Keywords:*** *DNA barcoding, bivalves, putative, mislabeling, CO1 gene*

**Introduction.** As an archipelagic country, the Philippines is rich in aquatic resources. Notably, it hosts to about 10% (22, 000) of the conservative mollusk species richness worldwide [1]. Mollusks comprised 28% of the inland fisheries production [2].

In Western Visayas, Roxas City in Capiz is known as the "Seafood Capital of the Philippines" with a total fisheries production of 30,053.663 metric tons. Oyster and mussel production equate to 150 metric tons (3000 sacks) and 292.5 metric tons (5850 sacks), respectively, for food purposes every year. The proliferation of aquatic products continues worldwide as its daily consumption increases. Along with this, is the rising diversity in the aquatic species and products available in the market [3]. However, though molluscs are significant to the society and economy, comprehensive knowledge about the bivalve species of Roxas City, Capiz in the Philippines is limited. Throughout the years, there was a decline in biodiversity and a distinct increase in the number of endangered species observed for marine molluscs caused by climate change, coastal environment deterioration and anthropogenic activities [4]. Thus, the need for proper and accurate species identification for economic and conservation purposes. DNA barcoding is a method used to verify the species in common economic aquatic products to secure correct consumer information, effectively supervise the aquatic market trade and promote species conservation [5].

DNA barcoding represents a tool for biodiversity assessment, quickly sorting collections into species-like units [4]. It is currently being used to identify invasive species and improving biosecurity [6]. In the past, morphology was used as the lone identifier of species populations. However, it was found to be ineffective [7]. There are cases where morphological characteristics are missing or misleading, making it difficult to classify organisms. Complex morphological approaches of species in the phylum Mollusca hinder its proper conservation and management [4]. In the present, molecular identification of species allows accurate authentication of aquatic products. DNA barcoding can be useful for species identification and more reliable to assign species when traditional taxonomy is problematic [8]. Most DNA-based methods utilize a specific conserved gene region which has moderate variability. Mitochondrial DNA (mtDNA) is maternally inherited and therefore the succeeding generation would only have the maternal DNA. Due to this, mtDNA sequences can be used to differentiate species. Hebert et al. (2003) proposed the use of mtDNA gene cytochrome oxidase 81 subunit I (COI) as a global identification system for animals. Previous studies have proven that the COI gene can correctly identify species of marine aquatic organisms [10,11,12,13,14].

The current study proposed to barcode economically important bivalves in Roxas City, Capiz using the CO1 gene as biomarker due to the lack of DNA barcoding data of mollusc species in the Philippines. In addition, morphological and molecular identification were done to assess market label authenticity in relation to conservation of species and mislabeling. This study aimed to provide DNA barcoding data of economically important bivalve species in Roxas City, Capiz. It specifically aimed to:

(i) identify bivalve species collected using morphological identification

(ii) identify bivalve species collected using mitochondrial cytochrome c oxidase subunit (CO1) gene

(iii) analyze diversity and relationships between bivalve species using different software packages

(iv) identify possible mislabeled products basing on their vernacular names and local names given in published sources

(v) compare DNA sequences to sequences available in GenBank using BLAST and BOLD search

(vi) compare identified bivalve species identified using morphological and molecular methods

**Methods.** This is a descriptive study which includes methods on the collection and preservation of samples, extraction of DNA, PCR amplification, gel electrophoresis, gene sequencing and analysis of DNA samples using softwares and programs. The sample collection was done in Bagong Lipunan Market and Ivisan Market in Roxas City, Capiz, Philippines. The study was conducted at Far Eastern University - Manila Molecular Laboratory.

*Sample Collection and Morphological Identification.* A total of 118 samples were collected from Bagong Lipunan Market and Ivisan Market in Capiz. The muscle tissues from the adductor muscle of bivalves were preserved and subsequently stored in 95% alcohol. Basing on vernacular names, 22 representative samples were selected for DNA extraction. Shells were used for morphological species-level identification.

*DNA Extraction and PCR Amplification.* DNA extraction was performed using Macherey-Nagel NucleoSpin Tissue kit. Extracted DNA was subjected to PCR amplification using CO1 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'). Amplification was performed with a 25 µL master mix consisting of 16.4 µL PCR grade water, 2.5 µL 10X PCR Buffer with 1.5 mM MgCl2, 2.5 µL 10mM DNTP, 0.5 µL Primer A, 0.5 µL Primer B, 0.10 µL Taq polymerase, 0.75 µL DMSO, 0.75 µL 3X BSA, and 1 µL DNA Template. PCR was carried out using the following thermal regime: 5 min at 95°C, then 35 cycles of 1 min at 94°C, 1 min at 43°C and 1:30 min at 72°C, followed by extension for 7 min at 72°C.

*Gel Electrophoresis.* Successfully amplified PCR products were subjected to gel electrophoresis to check the presence of DNA. The size and quality of PCR products were assessed in 1.5% agarose gel and stained with ethidium bromide.

*DNA Sequencing.* Amplified PCR products were sent to University of California, Berkeley for DNA sequencing.

*Sequence Assembly and Alignment.* All sequences were assembled in Geneious R11 and aligned using MUSCLE in MEGA7.

*BLAST and BOLD Identification.* Each sequence was queried in BLAST for comparison of DNA sequences available in GenBank. Along with BLAST, BOLD was used to minimize the risk of using contaminated sequences. All identified species under BLAST search was checked on IUCN red list of threatened species to identify endangered species.

*Phylogenetic Analysis.* For analysis of the base composition and visualization of the relationships among bivalve species included in this study, the software package MEGA7 was used. Phylogenetic analysis using the Neighbor-Joining (NJ) tree model was conducted. Pairwise distances were also calculated along with the intraspecific and interspecific genetic divergences of the samples.

*Safety Procedure.* Unused tissue samples from the bivalves were disposed following biosafety laboratory procedures. Lab gowns and nitrile gloves were used during maceration, DNA extraction, PCR amplification, and gel electrophoresis to avoid direct contact with hazardous chemicals.

**Results and Discussion.** Comparison of results in both methods of identification were used to assess market supervision data. Moreover, sequence identification in BLAST and BOLD search were used to evaluate current conservation status of the species and genetic relationship between samples.

After collection, shells were separated for morphological identification. Basing on vernacular names, there were 11 species collected among 22 samples, namely: Bagaycay (n=6), Balinday (n=2), Litob (n=3), Tikab (n=2), Halaan (n=1), Bilao (n=1), Tuway (n=2), Lampirong (n=1), Tahong (n=2), Talaba (n=1) and Punaw (n=1). Species-level morphological identification was performed and then verified by Dr. Laureen Manalo (University of the Philippines Research Center) with basis from Laureta's Compendium of the Economically Important Seashells in Panay, Philippines which resulted to the identification of thirteen species: *Mactra achatina* (n=2), *Katelysia sp.* (n=2), *Scapharca inaequivalvis* (n=2), *Perna viridis* (n=2), *Chlamys senatoria* (n=1), *Gari togata* (n=1), *Polymesoda erosa* (n=2), *Anadara granosa* (n=1), *Amusium pleuronectes* (n=1), *Crassostrea iredalei* (n=1), *Azorinus acutidens* (n=1), *Katelysia hiantina* (n=4), and *Gafrarium sp.* (n=1).

Comparing vernacular names given by market vendors and based on the reference material, a discordance between the two sets of information was detected (see Table 1). Among 22 samples, there were 11 species identified by vernacular names while there were 13 species by species-level morphological identification. However, PNML7 called "Bilao" has a similar name "Bila-og" given in the reference for morphological identification. Although 12 samples had the same local name for both sets, remaining eleven differed greatly. Bagaycay, Balinday, Butigis, Punaw, and Halaan were the mostly interchanged samples. It can also be noted that 'Bagaycay' is not considered as a local name among bivalves. However, samples under this name were listed as butigis (PNML1), punaw (PNML11, PNML19) or malinday (PNML18, PNML20) based on the published article

used as basis for species-level morphological identification.

**Table 1.** Comparison of vernacular name to local name on given to samples from Laureta's Compendium of the Economically Important Seashells in Panay, Philippines.

| Specimen Code | Vernacular Name (given by vendors) | Local name on published reference |
|---|---|---|
| PNML1 | Bagaycay | Butigis |
| PNML2 | Balinday | Punaw |
| PNML3 | Litob | Litob, Litog |
| PNML4 | Tahong | Tahong |
| PNML5 | Tikab | Tikab |
| PNML6 | Halaan | Punaw |
| PNML7 | Bilao | Bayuyan, Bila-og |
| PNML8 | Tuway | Tuway |
| PNML9 | Balinday | Butigis |
| PNML10 | Litob | Bakalan, Litob, Litog |
| PNML11 | Bagaycay | Punaw |
| PNML12 | Lampirong | Escalop, Kapis |
| PNML13 | Tahong | Tahong |
| PNML14 | Talaba | Talaba |
| PNML15 | Tuway | Tuway |
| PNML16 | Tikab | Tikhan, Tudlo-tudlo |
| PNML17 | Bagaycay | Punaw, Malinday |
| PNML18 | Punaw | Punaw, Malinday |
| PNML19 | Bagaycay | Punaw |
| PNML20 | Punaw | Punaw, Malinday |
| PNML21 | Litob | Litob, Litog |
| PNML22 | Punaw | Punaw, Malinday |

For molecular identification, obtained DNA sequences from bivalve samples were compared to publicly available sequences in GenBank and BOLD Systems. Closest species match generated by BLAST was used to compare with morphological species identification. Of the 15 amplified samples, 60% (nine samples) were identified to match in both BLAST and BOLD searches while six (6) samples differed in the closest species match: PNML7 differed in order-level identification, PNML16, PNML20, and PNML22 differed in genus-level identification, and PNML1 and PNML14 differed in species-level identification.

Maximum species identities of 98-100% were obtained from five (5) generated COI sequences (PNML3, PNML4, PNML8, PNML14, and PNML15) in GenBank and/or BOLD. However, using BLAST search, 10 sequences returned matches of less than 97% (range 75-89%) maximum identity. Likewise, these 10 sequences also returned matches of less than 97% (range 78-91%) in BOLD. In GenBank, the number of COI sequences per species varied between 1 and 320 with a mean of 69. Due to this, these species did not have high match values due to the limited number of COI sequences available, which resulted to less information for sequence comparison [15].

Nine (9) different species were identified using BLAST search, namely: *Meretrix lyrata* (n=5; PNML1, PNML2, PNML6, PNML9, PNML11), *Scapharca cornea* (n=1; PNML3), *Perna viridis* (n=1; PNML4), *Hiatella arctica* (n=1; PNML7), *Geloina expansa* (n=2; PNML8, PNML15), *Crassostrea iredalei* (n=1; PNML14), *Novaculina gangetica* (n=1; PNML16), *Merisca capsoides* (n=1; PNML18), and *Macridiscus melanaegis* (n=2; PNML20, PNML22). On the other hand, 10 species were identified using BOLD search: *Meretrix meretrix* (n=1; PNML1), *Meretrix lyrata* (n=4; PNML2, PNML6, PNML9, PNML11), *Scapharca cornea* (n=1; PNML3), *Perna viridis* (n=1; PNML4), *Glauconome rugosa* (n=1; PNML7), *Geloina expansa* (n=2; PNML8, PNML15), *Crassostrea sp.* KL-200 (n=1; PNML14), *Sinonovacula constricta* (n=1; PNML16), *Serratina capsoides* (n=1; PNML18), and *Paphia dura* (n=2; PNML20, PNML22).

Comparison of the molecular identity in BLAST and the morphological identity based on Laureta's (2008) published article, a discordance was detected (73%) between the 15 successfully barcoded samples. Only four (4) samples matched its morphological and BLAST identity: PNML4 (*Perna viridis*), PNML14 (*Geloina expansa*), PNML8 (*Crassostrea iredalei*), and PNML15 (*Geloina expansa*); while PNML3 (*Scapharca cornea*) only showed identical genus-level identification (see Table 2).

Mislabeling was revealed in six (6) common economic aquatic products. *M. lyrata* is commonly interchanged with *M. achatina* and *Katelysia sp.* with substitution rates of 40% and 60%, respectively. *S. cornea*, *H. arctica*, *N. gangetica*, *M. capsoides*, *M. melanaegis* are substituted as *S. inaequivalvis*, *G. togata*, *A. acutidens*, *K. hiantina*, respectively.

Overall, the rate of mislabeling in bivalve species has been found to be high in this study (73%), in comparison with other published market substitution reports in other countries. This suggests the need for an updated re-evaluation of commercially sold bivalves in Panay. It can also be noted that there were no online references available that can be used for basis of identification by other researchers. Therefore, online references for morphological identification of bivalve species should be available for the public emphasizing several implications that can be deduced from mislabeling, including consumer fraud [16] and wrong information on the real stock status of the product [17].

A huge difference between species identification of the two methods can be noted since a low concordance for both sets is evident. These cases are common when traditional taxonomy is compared to molecular identification.

**Table 2.** Comparison of morphological and molecular species identity of 15 bivalve samples species using BLAST search and based from the Compendium of the Economically Important Seashells in Panay, Philippines.

| Specimen Code | Morphological Identification | BLAST Identification |
|---|---|---|
| PNML1 | *Mactra achatina* | *Meretrix lyrata* |
| PNML2 | *Katelysia sp.* | *Meretrix lyrata* |
| PNML3 | *Scapharca inaequivalvis* accepted as *Anadara inaequivalvis* | *Scapharca cornea* accepted as *Anadara cornea* |
| PNML4 | *Perna viridis* | *Perna viridis* |
| PNML6 | *Katelysia sp.* | *Meretrix lyrata* |
| PNML7 | *Gari togata* | *Hiatella arctica* |
| PNML8 | *Polymesoda erosa* accepted as *Geloina expansa* | *Geloina expansa* |
| PNML9 | *Mactra achatina* | *Meretrix lyrata* |
| PNML11 | *Katelysia sp.* | *Meretrix lyrata* |
| PNML14 | *Crassostrea iredalei* accepted as *Crassostrea bilineata* | *Crassostrea iredalei* accepted as *Crassostrea bilineata* |
| PNML15 | *Polymesoda erosa* accepted as *Geloina expansa* | *Geloina expansa* |
| PNML16 | *Azorinus acutidens* | *Novaculina gangetica* |
| PNML18 | *Katelysia hiantina* accepted as *Marcia hiantina* | *Merisca capsoides* accepted as *Serratina capsoides* |
| PNML20 | *Katelysia hiantina* accepted as *Marcia hiantina* | *Macridiscus melanaegis* accepted as *Macridiscus aequilatera* |
| PNML22 | *Katelysia hiantina* accepted as *Marcia hiantina* | *Macridiscus melanaegis* accepted as *Macridiscus aequilatera* |

*Note: Accepted names and classification are based from the World Register of Marine Species (WoRMS).

Previous studies [3,4,7] have shown that traditional morphological identification is not always suitable. Due to bivalves' complex morphology, species identification is difficult and fraudulent product mislabeling usually happens which could result in health issues, economic fraud, and illegal trade of protected species [3]. As a result, the Global Trade Operations requires strict certifications on fish labels and related aspects. In the Philippines, all products must be properly labeled accurately base on its nature, quality, and quantity in accordance to Republic Act no. 7394 or Consumer Act of the Philippines. However, it is difficult to comply with this, therefore, monitoring agencies look for safe technologies for market supervision and species identification and the use of molecular methods to identify species is being employed. Previous studies [3, 4, 7] have shown that traditional morphological identification is not always suitable.

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Discrepancy results suggest that identification based on physical characteristics of species is not reliable enough to confirm and verify the identity of marine bivalve species as misidentification of these species can cause species substitutions in markets. Furthermore, the study supports the claims that molecular identification of marine bivalves is more accurate compared to morphological identification.

Neighbor-Joining tree and bootstrapping were used to support the identity of successfully identified species using the BLAST search. Accession numbers were also acquired in both GenBank and BOLD system. Highest possible identity percentage in GenBank (BLAST) was chosen for each specimen to identify matches for species identification. A Neighbor-Joining tree of 39 CO1 sequences of experimental DNA sequences with *Aurelia aurita* as the outgroup and GenBank sequences using the Kimura-2-parameter model (K2P) was generated (see Figure 1). The K2P model corrects for multiple hits, accounting transitional and transversional substitution rates, while assuming that the four nucleotide frequencies are the same and that substitution rates do not vary among sites (MEGA v7). There was a total of 121 positions in the final data set. High bootstrap values (>90) indicate well-supported clades in a 1000-replicate phylogenetic analysis.

Intraspecific divergences ranged from 0.0% to 32.7% while interspecific divergences ranged from 19.3% to 76.0% (see Table 3). No barcoding gap is present due to the overlap between intraspecific and interspecific divergences. *H. arctica* and *G. expansa* species share the same interspecific divergence (19.3%) which suggests that they are the closest species groups among the bivalves in the study. On the other hand, *M. lyrata* (28.7%) and *M. melanaegis* (32.7%) had intraspecific divergences that overlapped with the overall lowest (19.3%) interspecific divergence. The overlap between intra- and interspecific divergences suggests that a universal cut-off value or threshold

cannot be defined for the species delineation of these species.

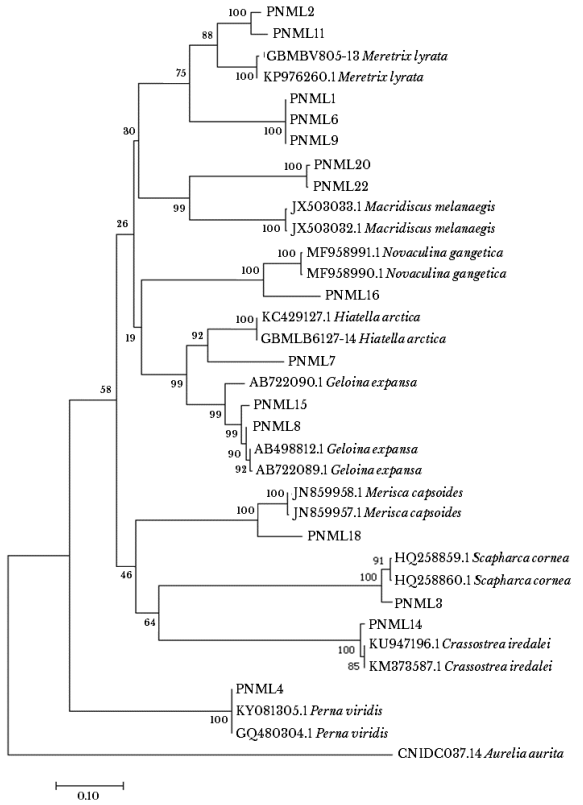


**Figure 1.** Neighbor-joining tree of 39 sequences from 9 bivalve species and *Aurelia aurita* as the outgroup based on cytochrome c oxidase subunit I (COI) gene.

PNML1, PNML6, PNML9 belonging to *M. lyrata* species group, and PNML20 and PNML22 belonging to *M. melanaegis* species group had overlaps between their highest intraspecific divergences and the overall lowest interspecific divergence which suggest that they are possible putative new species. PNML3 (*S. cornea*), PNML7 (*H. arctica*), PNML16 (*N. gangetica*), and PNML18 (*M. capsoides*) are also possible putative new species because they represent the basal clade of the species group as supported by their bootstrap values (see Figure 1) and they return low species identity matches (except for *S. cornea*) in GenBank. Although PNML3 (*S. cornea*) returned a high species identity match in GenBank, it represented a basal clade for its group. PNML4 is surely considered as *P. viridis* because it returned a high species identity match and showed no branching from the reference sequences in the phylogenetic tree in Figure 1.

Samples were also queried in International Union for Conservation of Nature (IUCN) database to check the inclusion of the species in the red list of threatened species. Only one (PNML16; *N. gangetica* of the 15 specimens was listed as known and classified as Least Concern among others that are not yet assessed. The lack of available information for bivalves in the IUCN Red List database suggests insufficient knowledge on species habitat, location, geographic range, threats, and proper conservation actions. More studies should be conducted to provide more information regarding these bivalve species.

**Table 3.** Intraspecific and interspecific Kimura-2-Parameter divergences

| Species Name | Intraspecific Divergence | | | Interspecific Divergence | | |
|---|---|---|---|---|---|---|
| | Min (%) | Mean (%) | Max (%) | Min (%) | Mean (%) | Max (%) |
| *Meretrix lyrata* | 0.00 | 17.0 | 28.7 | 32.6 | 50.4 | 71.6 |
| *Scapharca cornea* | 0.00 | 2.00 | 3.00 | 56.2 | 64.6 | 76.0 |
| *Perna viridis* | 0.00 | 0.00 | 0.00 | 51.8 | 59.7 | 68.8 |
| *Hiatella arctica* | 0.00 | 12.6 | 18.9 | 19.3 | 46.1 | 62.3 |
| Geloina expansa | 0.00 | 3.47 | 6.87 | 19.3 | 44.3 | 63.5 |
| *Crassostrea iredalei* | 0.00 | 0.66 | 0.99 | 53.6 | 55.6 | 68.8 |
| *Novaculina gangetica* | 0.00 | 9.57 | 14.4 | 38.6 | 50.9 | 63.6 |
| *Merisca capsoides* | 0.00 | 7.58 | 11.4 | 45.9 | 53.5 | 63.6 |
| *Macridiscus melanaegis* | 0.00 | 21.6 | 32.7 | 37.4 | 53.3 | 76.0 |

*Error Analysis.* Upon dissection, tissue samples may be subjected to contamination in the laboratory. Extracted DNA may also be contaminated which led to the unsuccessful sequencing of the seven samples sent to the University of California, Berkeley.

**Conclusion.** In conclusion, DNA barcoding is an invaluable tool for identification of mollusks species. It can be used for species identification, food safety, conservation management and market surveillance. It also allows the discovery of putative new species bivalves that can be found in markets in Roxas City, Capiz. However, a weakness of DNA barcoding is the lack of voucher specimens for many reference sequences found in databases as confirmed in our study. This study illustrates how combining morphological and DNA taxonomic analysis can help in conservation and monitoring of commercially available marine species. It highlights the usefulness of combining the two methods when phenotypic plasticity of samples, or reference sequences in the public datasets (BOLD and BLAST) is lacking.

**Recommendations.** Further studies in this field should consider adding more sampling sites to widen the scope of the study. In addition to this, it is also recommended to increase the sample size to have more accurate results and to have a clearer picture of the relationship of species available in the sampling areas. Future researches are also encouraged to use other methods of DNA extraction to have a higher yield of DNA. Aside from Neighbor-Joining tree, studies should also employ phylogenetic analysis using Maximum Likelihood Tree and Bayesian Tree. The current study also proposes to use other statistical models aside from Kimura-2-parameter model to

examine species' diversity in other dimensions of analysis.

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