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DNA BARCODING OF THREE SELECTED GASTROPOD SPECIES USING CYTOCHROME OXIDASE (COI) GENE

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ABSTRACT
This study focused on DNA barcoding and phylogenetic tree construction of three selected gastropod species viz., Pomacea canaliculata, Helicostyla sp. and Vivipara costata using cytochrome oxidase I (COI) gene. Genomic DNA samples of selected gastropod species were successfully isolated using modified cetyltrimethylammonium bromide (CTAB) extraction method. A set of primers, LCO1490 (5'GGTCAAATCATAAAGATATTGG 3') and HCO2198 (5' TAAACTTCAGGGTGACCAAAAAATCA 3') was used for the amplification of COI gene of mitochondria. Analysis of homology using BLASTn revealed that P. canaliculata (identified based on morphology) matched with P. canaliculata (AB728585.1), while previously identified Helicostyla sp.(identified based on morphology) was closest to Macrochlamys sp. (MF983631.1) *and V. costata (identified based on morphology) was most similar to Bellamya aeruginosa (KP150594.1) in the BLAST analysis. The morphological identification of P. canaliculata was proven correct with 100% similarity to all existing P. canaliculata sequences using BLASTn analysis. However, BLASTn showed that Helicostyla sp. and V. costata were closest to Machrochlamys sp. and B. aeruginosa with 91% and 94% similarity, respectively. The low similarity may suggest that this study is the first to submit COI sequences for Helicostyla sp. and Vivipara costata. Further, the three species had no match in the Barcode of Life Database (BOLD). The Maximum Likelihood tree revealed that the selected gastropod species were clustered together and strongly supported with a high bootstrap value of 98%. It is concluded, however, that COI gene as DNA barcode could be used to discriminate the three studied gastropod species.*
KEY WORDS: *cytochrome oxidase I, DNA barcoding, Helicostyla sp., Pomacea canaliculata, Vivipara costata*

INTRODUCTION

Taxonomy is an essential part of any biological study. In scientific studies involving a taxon, it is important to have an accurate and correct identification of that particular taxon (Fontanilla *et al*., 2014). Traditional taxonomy alone sometimes fails in identifying species (Packer *et al*., 2009) and the usefulness of traditional methods could be limited in cases of

morphologically similar species and cases when specimens are derived from juvenile life stages (Gossner & Hausmann, 2009). The molecular taxonomic methods have been extensively used to complement morphological approaches in species identification and in establishing phylogenetic relationships. Particularly, species identification through DNA barcoding has been adopted over the past decades (Hebert *et al*., 2003a; Hebert *et al*., 2003b; Ferri *et al*., 2009). DNA barcoding is a taxonomic method that uses a short genetic marker in an organisms' DNA for species identification. The sequence of mitochondrial cytochrome C oxidase subunit I (COI) serves as the standard barcode for almost all animals (Hebert *et al*., 2003b). It is one of the most widely used markers for population genetics and phylogeographic studies across the animal Kingdom (Avise, 1994).

Gastropods are single-valve, soft-bodied class of animals in the Phylum Mollusca (Galan *et al*., 2015). It is one of the most diverse taxa having 40,000 species in which 5,000 of these are freshwater snails thriving in freshwater habitats (Abbott, 1950). The golden apple snail (*Pomacea canaliculata* Lamarck, 1822) is an aquatic organism and easily found in and around rice paddy fields (Hamid *et al*., 2015). This organism is considered as pest of rice in many Asian countries because it feeds voraciously on rice seeding (Yusa, 2001). The land snail genus *Helicostyla* Ferussac, 1821 is a group of small, terrestrial, pulmonate gastropods endemic to the Philippines (Bouchet & Rocroi 2005; Flores, 2014). This genus is the most diverse with at least 128 known species among the Subfamily Helicostylinae (Faustino 1930). *Vivipara costata*, on the other hand is commonly known as ege and "kuhol" in Bisaya (Galan *et al*., (2015).

This study analyzed the molecular phylogeny of three gastropod species using COI gene. Specifically, this study isolated the partial COI gene sequences and constructed a phylogenetic tree showing the relationships of the three species. Though one recent study reported that COI gene is not suitable for phylogenetic analysis on the mollusc, *Stagnicola* (Schniebs *et al*., 2016), this study aimed to investigate if COI will be useful in these three gastropod species. The selection of the three gastropod species was in accordance to the most common collected gastropods in the province of Bukidnon in the Philippines. To the best of the authors' knowledge, this is the first report on DNA barcoding of gastropods in the province of Bukidnon, Philippines using COI gene

MATERIALS AND METHODS

Collection of Specimens. The adult snails of the three gastropod species were collected from the three identified areas in Bukidnon, Philippines namely: Central Mindanao University, Musuan, Bukidnon (*P. canaliculata*); Impalutao, Malaybalay, Bukidnon (*Helicostyla* sp.); and Catubalon, Valencia City, Bukidnon (*V. costata*). The samples were collected mostly from water reservoirs in which permits were not required. Each sample was dissected to remove its soft body parts and kept at a maintained temperature of -20°C.

Isolation of gDNA. Genomic deoxyribonucleic acid (gDNA) isolation was carried out using the cetyltrimethylammonium bromide (CTAB) extraction method with modifications. Fifty (50) mg of fresh specimens were weighed using analytical beam balance. The samples were homogenized using liquid nitrogen in a sterile mortar and pestle. Forty (40) mg of polyvinylpyropyrrolidone (PVPP) were added in the samples and ground to a uniform powder. The mixture was transferred into 2 ml microcentrifuge tubes. Eight hundred (800) µl of pre-

warmed (65°C) 2x CTAB Buffer was added. Then 1µl of β-mercaptoethanol was added to the mixture. At very low speed, the solution in the tube was vortexed and incubated at 65° C for an hour. Then after, the samples were briefly cooled and mixed with 500 µl of 24:1 Chloroform-isoamyl alcohol. Using an electronic shaker, the mixture was mixed at low speed in room temperature (37°C) for 20 minutes and centrifuged for 15 minutes at 13,000 rpm. The top aqueous layer was pipetted and transferred into a new microcentrifuge tube. A volume (600µl) of ice-cold isopropanol was added and the microcentrifuge tubes were then inverted several times to mix. The solution was incubated overnight at 4°C to precipitate DNA. The samples were then centrifuged for 15 minutes at 13,000 rpm and the pellets were formed at the bottom of the tube. Then, the supernatants were discarded into the proper disposal container. One (1) mL of 100% isopropanol was added for the final washing. The samples were centrifuged for 10 minutes at 13,000 rpm while the supernatant was discarded into the proper disposal container.

The tubes were inverted in a tissue paper and air-dried. The dried pellets were added with 100 µl 1x TE and 1µl of RNAse and were incubated at 37^{o}C for 30 minutes. One volume of ice-cold isopropanol and 0.1 vol 3M sodium acetate, pH 5.0, were added to the microtubes to precipitate DNA. After centrifugation, the supernatant was discarded and the pellet was air-dried for 10–15 minutes and resuspended in 100 µl nuclease-free water to dissolve gDNA. The gDNA samples were stored at 4 ^{0}C until use and subjected to gel electrophoresis to determine the quality of the DNA as well as concentration based on the intensity of bands.

PCR Amplification. A set of primer, LCO1490 (5'GGTCAACAAATCATAAAGATATTGG 3′) and HCO2198 (5′ TAAACTTCAGGGTGACCAAAAAATCA 3′) was used for the amplification of the COI gene of mitochondria. The gene was amplified with gDNA as a template using the Applied Biosystems Veriti 96-well Thermal Cycler. The polymerase chain reaction (PCR) components of 25 µl reaction were as follows: 1x PCR buffer – Mg Cl_2, 0.8 mM of dNTP mix, 0.4 µM forward primer, 0.4 µM reverse primer, 1.5 mM $MgCl_2$ and 0.5 U of Taq DNA polymerase. The used gDNA was first diluted to 1:40 and 1.0 µl (50–100 ng) was added per 25 µl reaction. PCR profile for the cocktail (LCO1490 and HCO2198) of COI gene consisted of initial denaturation at 95°C for 2 min, 43 cycles of 94°C for 30 sec, annealing at 45°C for 30 sec, and extension at 65°C for 5 min, with a final extension at 72°C for 5 min, followed by an indefinite hold at 4°C (Folmer *et al*., 1994; Ivanova *et al*., 2005; Ivanova *et al*., 2007).

Gel electrophoresis and Documentation. The PCR products were subjected to gel electrophoresis. One percent (1%) agarose gel was prepared using Pronadisa Agarose D-1 Low EEO and 0.25x Tris-Boric Acid-EDTA (TBE). A 0.35 g of agarose was dissolved into 35 mL of 0.25x TBE and heated in microwave for a minute. It was briefly cooled and added with 3.5 µl of Biotium GelRed. The mixture was poured into the mold and was allowed to harden with a foil cover. Once hardened, the gel was set into the electrophoresis machine. Five (5) µl of the PCR product or two (2) µl genomic DNA was added with 2 µl of 2x loading dye and loaded into the wells of the agarose gel. One (1) µl of 1 Kb plus Ladder were loaded on the first well of the gel. The gel was subjected to run in 100V for 30 minutes using Mupid-One Electrophoresis system. After, the gel was viewed using the Bio-Rad Doc EZ Imager.

Sequencing and Sequence Analyses. An account was made at the Macrogen website (http://dna.macrogen.com/eng/) and an online order was made. Three replicates of PCR products for each species of gastropods were subjected to sequencing using AB13739XL

(Macrogen, Inc., Korea). The sequences were downloaded at the Macrogen website. Nine (9) sequences of gDNA samples were aligned and edited using the BioEdit Sequence Alignment Editor. One final alignment for all 9 gDNA sequences was made using the Clustal Omega. Species identification using the final sequence was carried out using NCBI BLASTn and the Barcode of Life Database (BOLD) version 3.

Construction of Phylogenetic tree. The molecular phylogenetic analysis of the sequence data were analyzed by Maximum Likehood method based on the General Time Reversible model using the Molecular Evolutionary Genetics Analysis (MEGA) version 7.0. Several other sequences of mollusc species from GenBank were included in the analysis.

RESULTS AND DISCUSSIONS

There were three gastropod species collected from the three identified areas in Bukidnon. Each species of three gastropods have three replicates. The studied specimens were morphologically identified as *P. canaliculata*, *Helicostyla* sp. and *V. costata. P. canaliculata* or the golden apple snail (Fig. 1a) lives for 2-6 years with high fertility and its shell is tight brown and flesh is creamy white to golden pinkish or orange (Dela Cruz *et al*., 2001). Ponder *et al.* (2016) also described the shell color which varies from bright green to orange-brown with reddish spiral bands. The interior of the shell is yellowish with a suffusion of purple and marked with strong spiral bands. *P. canaliculata* has a more rounded aperture and a deep sutural channel. Umbilicus is wide and deep. Size depends on the availability of food and destructive stage is when the length of the shell is from 10 mm (about the size of a corn seed) to 40 mm (about the size of a pingpong ball) (Dela Cruz *et al*., 2001). Their size reached up to 75 mm high and the operculum is completely horny which generally does not aestivate (Ponder *et al*., 2016).

The collected *Helicostyla* species (Fig. 1b) is differentiated from other members of Helicostylinae by the presence of an ovate-globose to elongated shells with dome-like spire that lacks hydrophanous periostracum and spiral striae (Schileyko, 2004). De Chavez (2014) described this group of species having shell ovate-globose with dome-shaped spire of about three whorls. Whorl expands regularly with body whorl rounded and not keeled. Postembryonic whorls smooth. Aperture has ovate and moderately oblique with reddish-brown streak along the aperture lip that is slightly reflected on the lower half section and the umbilicus is imperforate. In addition, *V. costata* is usually harvested and cooked with coconut milk as reported by Galan *et al.* (2015) (Fig. 1c).

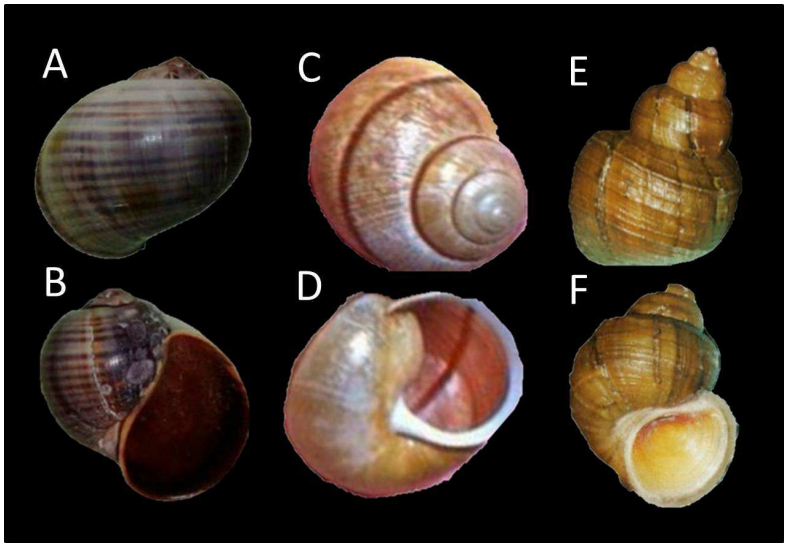

FIG. 1. Three selected gastropod species. A & B.) *P. canaliculata,* C & D.) *Helicostyla* sp. and E & F.) *V. costata.*

The 9 gDNA samples were successfully isolated using the modified CTAB method (Doyle and Doyle, 1987) and all gave PCR products (Fig. 2). The approximate size of the amplicons is almost 700 base pairs. Upon sequencing, 614 to 655 bp were obtained.

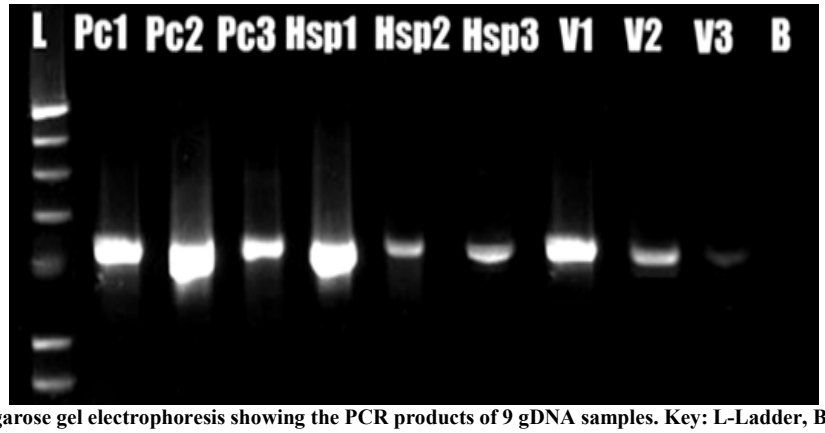

FIG. 2. Agarose gel electrophoresis showing the PCR products of 9 gDNA samples. Key: L-Ladder, B-Blank, Pc – *P. canaliculata*, Hsp – *Helicostyla* sp., V – *V. costata*. The numbers after the letters indicate the replicate individuals per species.

The data obtained from the analysis of homology using BLAST revealed that *P. canaliculata* is correctly identified with 100% similarity to the sequence of Matsukura, Wada, Yoshida and Cazzaniga which is *P. canaliculata* (AB728585.1). The following few studies on the same species was found to utilize other genes than COI. The study of Bian *et al*. (2013)

showed that the partial sequences of mitochondrial (mt) adenosine triphosphate subunit 6 (p*atp*6) genes of two apple snails species from eight provinces in China revealed that *P. canaliculata* and *P. insularum* were grouped in different clades, but the genetic trees could not reveal geographically genetic relationships of *P. canaliculata* isolates from different origins. Additionally, phylogenetic analysis of Li *et al*. (2013) based on combined sequences of mitochondrial (mt) 12S and 16S of 9 *P. canaliculata* isolates from 5 southern provinces in China revealed complex genetic structure. Two phylogenetic groups of *P. canaliculata* were indicated in China with one group sistered to *P. canaliculata* isolates from USA, and two groups were even found in the same province.

Helicostyla sp. revealed 91% similarity to *Macrochlamys* sp. (MF983631.1) by Slapcinsky and Mulcahy (2017). Summary results are shown in Table 1. They however differ in their morphological characteristics. On the other hand, the analysis of homology for *V. costata* revealed 94% similarity to the sequence of Gu *et al.* (2012) which is *Bellamya aeruginosa* (KP150594.1), a widely distributed species in China in habitats of lakes, rivers, ditches, and ponds and has great importance both for human consumption and for crab culture (Chen *et al*., 2005; Liu *et al*., 1993). This species feed on detritus or algae on lake sediment or other substrata (Zhang *et al.,* 2007) and is also known to exhibit gonochorism and ovoviviparity (Gu *et al*., 2012). They however differ in morphology. The low similarity of 90-94% indicates that there is no match. Further, BOLD identification system revealed no match for the three collected gastropods. This means that the sequences are still not available in the said database. This study will be the first to provide the COI sequence for these species of *Helicostyla* and *Vivipara*.

Phylogenetic analysis (Fig. 3) showed that the three species share a common ancestor as they cluster together with a high bootstrap support of 95%. It is however seen that the sequence of *P. canaliculata* from GenBank group together with the sequence of this study. This shows that the morphology and COI sequence jived for *P. canaliculata*. This is contrary to the incongruence between morphology and COI sequence analysis reported by Schniebs *et al*. (2016) in the genus *Stagnicola*.

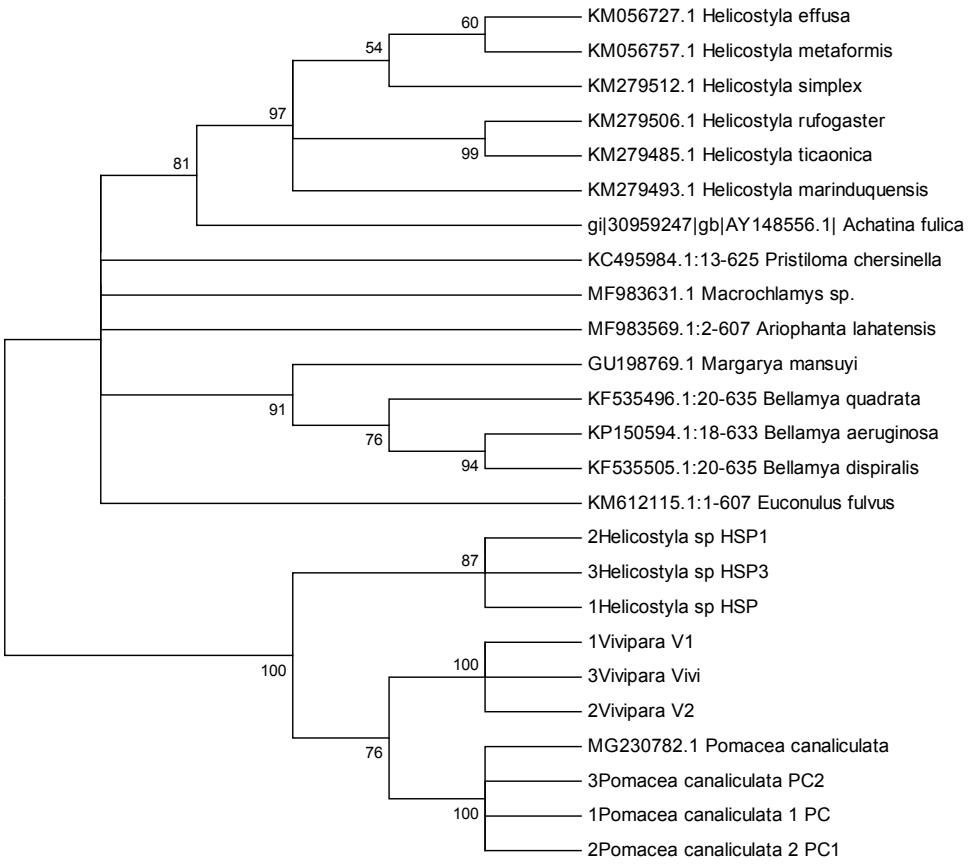

FIG 3. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model. The tree with the highest log likelihood (-4864.8484) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.3991)). The analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 495 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

TABLE 1. Summary of top nucleotide BLAST Hits of COI gene sequences of the three gastropod species.

Species Code	Morphological identification	BLAST search match	BLAST % Identification	BLAST Query cover	E-value	Accession number
Pc1	*Pomacea canaliculata* 1	*Pomacea canaliculata*	100%	100%	0.0	AB728585.1
Pc2	*Pomacea canaliculata* 2	*Pomacea canaliculata*	100%	100%	0.0	AB728585.1
Pc3	*Pomacea canaliculata* 3	*Pomacea canaliculata*	100%	100%	0.0	AB728585.1
Hsp1	*Helicostyla* sp. 1	*Macrochlamys* sp.	91%	98%	0.0	MF983631.1
Hsp2	*Helicostyla* sp. 2	*Macrochlamys* sp.	91%	98%	0.0	MF983631.1
Hsp3	*Helicostyla* sp. 3	*Macrochlamys* sp.	91%	98%	0.0	MF983631.1
V1	*Vivipara costata* 1	*Bellamya aeruginosa*	94%	99%	0.0	KP150594.1
V2	*Vivipara costata* 2	*Bellamya aeruginosa*	94%	99%	0.0	KP150594.1
V3	*Vivipara costata* 3	*Bellamya aeruginosa*	94%	99%	0.0	KP150594.1

Key: Pc – *P. canaliculata*, Hsp – *Helicostyla* sp., V – *V. costata*. The numbers after the letters indicate the individuals per species.

CONCLUSIONS

It is concluded that the cytochrome oxidase I gene is useful in identifying *Pomacea canaliculata* and was able to discriminate it from the two other collected species, *Helicostyla* sp. and *Vivipara costata*. However, the last two species were not identified using their COI sequences. This is so because the COI in publicly available databases are not yet complete for species identification to be successful.

It is recommended that further investigations be done to determine which gene or gene combinations should be used for accurate phylogenetic analysis in molluscs. Mollusc species not yet barcoded should be studied.

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