DNA barcoding of freshwater gastropods found in the upstream of Jalaur River in Barangay Garangan, Calinog, Central Philippines

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Abstract

Multiple species of gastropods can be found in the Jalaur River which encompasses almost the entire province of Iloilo. However, the identification of these species can be challenging with their complex morphology. DNA barcoding using the cytochrome oxidase I (COI) gene was performed to accurately identify and classify the organisms of Class Gastropoda. Through phylogenetic analysis, one sample was identified to be *Pomacea canaliculata* while the remaining four samples remained unidentified due to unrepresented taxa in the GenBank. This shows a lack of available information about the organisms found in the Jalaur River as nucleotide sequences of these species have not been provided to public databases. With this, further research must be done to know more about the species found in Jalaur River and their conservation status. It is recommended to maintain the storage condition of samples to prevent DNA degradation. PCR conditions should also be adjusted to achieve optimal results.

Introduction. - Systematics and taxonomies are the basis of all biology as it ensures the quality of life of future generations [1]. However, there are still a vast number of unknown species that have not yet been identified. Studies suggest that the Earth is home to about 8.7 million species [2]; meanwhile, only 14% had been identified as of 2011 [3]. The Philippines is home to about 22,000 mollusks species [4]. Gastropods, in particular, have a wide range of habitats and are prone to evolve as they are sensitive to slight changes in the environment [5]. In addition to this, gastropods have one of the highest numbers of documented extinctions among the major taxonomic groups in the world making its identification necessary [6].

Moreover, gastropods are also ecologically important as some of their species can be used as indicators to assess the condition of the aquatic habitat along with the quality of any water impoundments [7]. Gastropods may also serve as pests to agriculture with the potential to invade and alter the ecosystem [8]. Additionally, some gastropods species may be intermediate hosts of infections despite being a source of food for fishes, birds, and humans [7,9]. Despite this, the understanding of its systematics is still incomplete [10] and the phylogenetics among its family is largely unresolved [11].

Studies had been done assessing freshwater gastropods in the Philippines which was identified as *P. canaliculata*, *V. costata*, *L. natalensis*, *M. tuberculata*, *M. turricula*, *T. granifera*, *G. ladacensis*, *L. accuminata*, *L. caperata*, and Planorbis sp. [7, 12];. Alcala et al. [13] were also able to taxonomically identify *N. polita, T. granifera, P. porcellana, T. scabra, P. canaliculata, L. scabra, C. cucullate, C. manillensis, C. plicata,* and Ostrea sp. in Jalaur River. Despite their findings, morphological identification may not always be accurate due to the existence of cryptic species having similar morphology but different DNA sequences [14, 15].

To address this, past research recommends the use of DNA barcoding as an effective tool to authenticate and accurately identify organisms [14, 15]. DNA barcoding is a process that involves sequencing a short fragment of the COI gene taken from the unidentified organisms and comparing their DNA barcodes to existing sequences [16]. It can reveal possible misidentified organisms, discover overlooked species, and identify new or evolved species promptly and accurately as complete data can be collected from a single specimen irrespective of its morphological features or its stage in life [1, 17]. The DNA barcodes can be used to generate a phylogenetic tree for the evaluation of each organism, along with the phylogeny, diversity, and relationship among the organisms. With the data,

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actions can be taken for an organism's conservation and management [18]. The significance of DNA barcoding makes it a prominent research topic as it can authenticate the organisms to assess their safety, conserve genetics, and detect possible invasive alien species [14].

The Jalaur River encompasses almost the entire Iloilo with its upstream located at Barangay Garangan, Calinog, Iloilo. Previous studies mentioned that freshwater gastropods, specifically stream snails, are known to exhibit upstream migration in temperate and tropical regions [19,20]. Additionally, this area is also home to the Panay Bukidnon indigenous people thus with the identity of the freshwater gastropods, awareness will be given to the locals regarding the availability of certain gastropods in their area. Monitoring and assessment of the species could also be done along with its conservation and management. Additionally, the result of this research can be used as a baseline for further taxonomic research.

This study aimed to identify selected freshwater gastropods in the Jalaur River located in Barangay Garangan, Calinog, Iloilo, Philippines by analyzing gene sequences to establish their relationship and conservation status. Specifically, this study aimed to:

(i) identify selected species of freshwater gastropods collected from Jalaur River in Barangay Garangan, Calinog, Iloilo using phylogenetic analysis;

(ii) determine the relationship among the collected gastropod species by interpreting the phylogenetic tree; and

(iii) query the conservation status of each identified gastropod species on the International Union for Conservation of Nature (IUCN) Red List Index.

Methods. - This research is a descriptive study on the phylogenetics of freshwater gastropods. The methodology includes sample collection, DNA extraction, DNA amplification, agarose gel electrophoresis, DNA sequencing and alignment, species identification, and phylogenetic analysis.

Sample Collection and Preparation. To collect samples, a permit was requested from the National Commission on Indigenous Peoples (NCIP) since the sampling site is part of the Panay-Bukidnon's ancestral domain. Freshwater gastropods in shallow waters were collected through handpicking [7]. A total of 11 samples were collected from upstream of the Jalaur River in Barangay Garangan, Calinog, Iloilo, Philippines with the geographic coordinate 11°11'29" N 122°27'13" E at 367.0 meters of elevation using opportunistic sampling with the help of the indigenous people.

The collected samples were then stored in separate airtight bags labeled according to their vernacular names that were provided by the indigenous people and were placed in a cooler for transportation. The samples' foot muscle was then extracted and cut into small pieces then submerged in 70% ethanol to prevent the degradation of DNA [20].

DNA Extraction. DNA extraction was performed following the standard protocol for animal tissue according to the NucleoSpin Tissue Genomic DNA Purification User Manual. It was then tested in the Thermo Scientific™ Multiskan™ GO to check the quality of the extracted DNA.

DNA Amplification. DNA amplification was done through polymerase chain reaction (PCR). The universal primers for the amplification of the (COI) gene, 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′) were used [21]. The PCR was performed following the thermal regime: 3 min at 94 ℃, then 25 cycles of 20 sec at 94 ℃, 30 sec at 50 ℃, and 1 min at 72 ℃, followed by extension for *5* min at 72 ℃ set for seashells at the West Visayas State University laboratory.

Agarose Gel Electrophoresis. The amplified DNA was subjected to gel electrophoresis using 1% agarose gel and stained with Invitrogen 10X BlueJuice to check the integrity of the samples. The result was then viewed under the UV transilluminator.

DNA Sequencing and Alignment. The PCR products were sent to Macrogen Inc., Seoul, South Korea for sequencing. The sequences were then assembled using their forward and reverse sequences with DNABaser.

Species Identification. Each assembled DNA was queried using Basic Local Alignment Search Tool (BLAST) to compare the sequences to the available sequences in GenBank. The species with the lowest E-value and the highest bit score was determined for each sample. From the family, sequences of multiple species were downloaded to be included in the phylogenetic tree.

Phylogenetic Analysis. Using MEGA X, the sequences were aligned using ClustalW. This software also generated the best model for DNA substitution to be the Tamura 3-parameter model. Then a Neighbor-Joining tree from 1000 replicates was constructed using the maximum likelihood statistical method. Clades with bootstrap values higher than 70 were considered well-supported [22]. These were used as the basis to verify the identity of the sample. After verifying each sample's species, it was queried in the IUCN Red List Index for the conservation status.

Safety Procedure. Proper protective equipment was utilized at all times. Lab gowns and nitrile gloves were used during DNA extraction, DNA amplification, and gel electrophoresis to avoid exposure to hazardous chemicals. After the collection of DNA, the waste materials and the samples were autoclaved and disposed of properly as medical/biohazard waste.

Results and Discussion. - Freshwater gastropods in the upstream of Jalaur River were identified through DNA barcoding. The process includes sample collection, DNA extraction, DNA amplification, agarose gel electrophoresis, DNA sequencing and alignment, species identification, and phylogenetic analysis. From the 11 samples that were collected, only *5* were successfully barcoded due to possible DNA degradation and non-optimal conditions set for the

PCR amplification. Furthermore, only one of the five was identified to its species-level namely *Pomacea canaliculata* while the other 4 barcoded samples remained unidentified due to unrepresented taxa.

The initial identification was done based on their vernacular names. After obtaining the assembled DNA, initial species identification was performed using BLAST to classify them into specific species. The program presents the e-value and bit score of the top matches for each sequence. The e-value shows the statistical significance of a match while the bit score gives an indication of the quality of the alignment [22]. A low E-value and bit score higher than 950 are suggested for assigning species identity; thus, the sequence with the lowest E-value and the highest bit score was chosen as the BLAST identification [23]. These identifications are summarized in Table 1.

Table 1. The barcoded samples are presented with their corresponding vernacular name and BLAST Identification with the lowest E-value and the highest bit score.

Sample	Vernacular Name	BLAST Identification	E-value	Bit Score
A1	Awis	*Stenomelania* sp.	0	1158
A2	Awis	*Stenomelania* sp.	0	1122
K2	Kuol	*Pomacea canaliculata*	0	1179
TG	Tamburuko (gurob-gurob)	*Tarebia granifera*	0	1210
TM1	Tamburuko (mugot)	*Stenomelania denisoniensis*	0	1031

While BLAST was able to identify the samples with E-value = 0 and bit score > 1000, the study conducted by Ross et al. [24] raises major concerns as many taxa are unrepresented. In addition to this, it cannot give accurate identification of species because top hits are often not the closest phylogenetic relatives of the organisms [24, 25]. In a study by Hillis and Bull [26], clades having bootstrap values ≥70 correspond to a ≥95% probability of it being real. Thus, to verify the initial identity of each sample, phylogenetic analysis was performed where clades with bootstrap values of seventy (70) or higher were considered well-supported to be of the same species after undergoing one thousand (1000) replicates. Figure 1 shows the bootstrap consensus tree of the five (5) samples along with representative sequences from the family of their BLAST identities with the outgroup *Paludomus siamensis*.

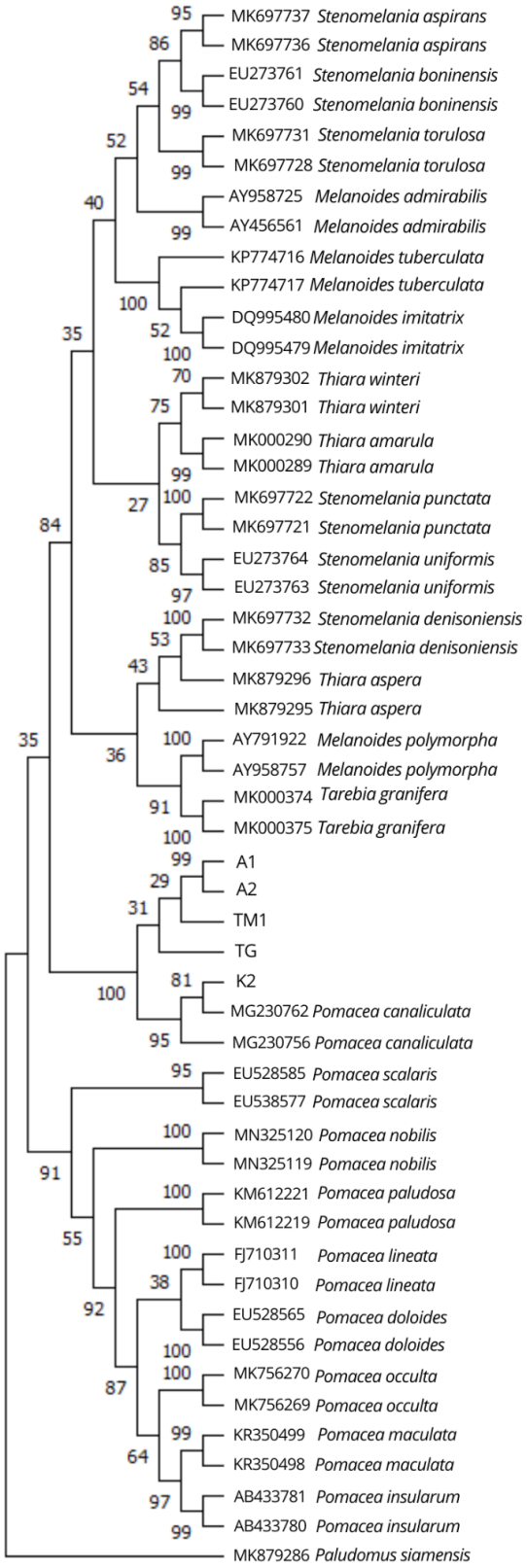

Figure 1. Bootstrap consensus tree of the samples is shown along with representative sequences from the family of their BLAST identification with *Paludomus siamensis* as the outgroup.

The phylogenetic tree shows that K2 can be identified to be *Pomacea canaliculata* with a bootstrap value of 81. This is the same species of gastropods that was identified by Alcala et al. [13] in the same river in

2010. Aside from this, it was also found in other parts of the Philippines such as Bukidnon and Agusan del Sur [7,12].

The identified species for K2 was queried in the IUCN Red List of Threatened Species for the identification of their conservation status. *Pomacea canaliculata* is categorized as Least Concern. Though it is not a focus of species conservation, its management is still necessary as it can serve as pests to rice crops [27].

Additionally, *Pomacea canaliculat*a was found to be an invasive species in the Global Invasive Species Database (GISD) [28]. With its ability to adapt to harsh environmental conditions along with its high reproductive rate, it can colonize and invade natural habitats [27, 29] which results in alterations in the ecosystem [8].

The remaining four samples belong to the same clade. It can be interpreted that A1 and A2 that have the same vernacular name, "Awis", are of the same species based on the phylogenetic analysis having a bootstrap value of 99. Collectively, the samples were more closely related to each other than their BLAST identification. This may be due to more common ancestors shared by the samples. The inaccuracy of the BLAST identification can be attributed to unrepresented taxa in the GenBank [24]. This shows a lack of available information about the organisms found in the Jalaur River as nucleotide sequences of these species have not been provided to public databases.

Limitations. The study barcoded less than the actual number of samples collected due to possible degradation of DNA and non-optimal conditions during the DNA amplification. Moreover, some samples were unrepresented species of gastropods in the GenBank, thus out of the five barcoded samples, only one organism was identified.

Conclusion. - In conclusion, "Kuol", identified to be *Pomacea canaliculata* or Golden Apple Snail, can be found in the Jalaur River along with four other unidentified gastropods. The identified species was not found to be a focus of species conservation since it is under the Least Concern categorization; however, its management is necessary as it is an invasive species and pests to rice crops. Aside from the Jalaur River in Iloilo, *Pomacea canaliculata* was also found in other areas in the Philippines. The four other organisms were not identified due to the limited sequences recorded in GenBank or the possibility of evolution.

Recommendations. - Further research must be done to identify the gastropods found in Jalaur River for their proper management. It is also recommended to perform all procedures in one laboratory with complete equipment for DNA barcoding to maintain a constant storage temperature of -20 ℃ and prevent DNA degradation [30]. More time should also be dedicated in the study to allow adjustments in PCR conditions to achieve optimal results which may vary depending on the organism being amplified.

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