RESEARCH ARTICLE

Updates on Identification and Distribution of the River Mullet "Ludong" in Northern Philippines

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ABSTRACT

"Ludong", also known as the president's fish, is a river mullet known to be found in Cagayan River, Northern Philippines. Unfortunately, this species has been heavily exploited over the years. As a conservation measure, the Department of Agriculture - Bureau of Fisheries and Aquatic Resources (DA-BFAR) issued the BFAR Administrative Circular (BAC) 247 of 2013, declaring a closed fishing season for "ludong" during its spawning from October 1 to November 15. Despite these efforts, the identity and distribution of "ludong" remain unclear. Here, the species identification and habitat range of "ludong" have been determined and updated by DNA barcoding using mitochondrial cytochrome c oxidase subunit 1 (CO1) as a marker. Twenty-five (25) "ludong" samples were collected from Cagayan River and Abra River traversing Regions 1, 2, and the Cordillera Administrative Region. BLAST, phylogenetic and pairwise analyses showed that all 25 specimens were *Cestraeus goldiei*. Consequently, the known distribution of *C. goldiei* has been extended from Cagayan River to Abra River, from Region 2 to Region 1 and CAR. Knowledge of the definitive species identification and distribution of "ludong" will contribute to further understanding of its biology and fisheries and boost its conservation.

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1. INTRODUCTION

Cestraeus spp. belong to the family Mugilidae and is catadromous in nature, living mostly in freshwater areas and then migrating to marine habitats to spawn. There are three recognized species under *Cestraeus* (Fricke et al. 2022): *C. plicatilis* (Valenciennes 1836), *C. oxyrhynchus* (Valenciennes 1836), and *C. goldiei* (Macleay 1883). These species are found throughout the Melanesia region, including New Caledonia, Fiji Islands, New Hebrides, and Solomon Islands, with varying status (Naikatini et al. 2017; Keith et al. 2014). In Choiseul Island in the Solomon Islands, *C. plicatilis* population has declined due to the overharvesting of spawners and juveniles and sedimentation of streams and rivers (Boseto et al. 2007). The same species was listed as critically endangered in the Red List of Japanese freshwater fishes released in August 2007 by the Japanese government (Hosoya 2008).

In the Philippines, *Cestraeus* spp., also known as "ludong" or the "President's fish," is said to be the most expensive freshwater fish in the country, with a price reaching about PHP 5,000/kilo (Javier et al. 2016; Dela Cruz 2008). Due to its popularity and rarity, demand for this fish has resulted in overfishing, coupled with dredging and mining activities, the "ludong" population in Cagayan River has declined over the years (Agasen et al. 2007; Dela Cruz 2008; Ventolero et al. 2014). To sustain the "ludong" population in the river, the Department of Agriculture – Bureau of Fisheries and Aquatic Resources (DA-

BFAR) promulgated BFAR Administrative Circular (BAC) 247 of 2013, declaring a closed fishing season for "ludong" from October 1 to November 15, during its spawning period.

However, despite the attention afforded to "ludong," its identity remains unclear. Thomson (1997) first reported the occurrence of *C. plicatilis* in the Philippines. The same species has been cited by Agasen et al. (2007) and Javier et al. (2016) to be the "ludong" in Cagayan River. In contrast, Harrison and Senou (1999) mentioned in their book chapter on Mugiliformes included in the "FAO Species Identification Guide for Fishery Purposes" that the "ludong" in the Philippines were *C. oxyrhynchus* and *C. goldiei*. They reported that *C. oxyrhynchus* was differentiated from *C. goldiei* and *C. plicatilis* based on the extension of the medial lobes on the lower jaw. *Cestraeus* with medial lobes not reaching the corner of the mouth is identified as *C. oxyrhynchus*, while those with medial lobes reaching the corner of the mouth are identified as *C. goldiei* or *C. plicatilis*. *C. goldiei* and *C. plicatilis* were further differentiated by the length of the pectoral fins compared to the head and the difference in tooth patches on vomer and dentary symphysis. Ventolero et al. (2014), using the species key by Harrison and Senou (1999), morphologically identified their specimens from Cagayan River as *C. oxyrhynchus* and *C. goldiei*. However, Harrison and Senou (1999) and Ventolero et al. (2014) also posited that straightforward diagnoses of "ludong" are challenging as many species under the Mugilidae family share morphological or meristic features. Using DNA analysis, Durand et al. (2012) identified *C. goldiei* in Cagayan River and *C. oxyrhynchus* in New Caledonia.

In terms of its range distribution, "ludong" is so far known to occur in the waters of Cagayan River and its tributaries (Agasen et al. 2007; Durand et al. 2012; Ventolero et al. 2014). It was also reported to thrive in Bantay-Abra of the Abra River System, traversing the provinces of Ilocos Sur and Abra (Rosario 2004 as cited by Javier et al. 2016). However, this report did not show any published scientific evidence to support such a claim.

DNA barcoding is a rapid and accurate species identification technique that is reliable, sensitive, economically viable, and environment friendly (Bandyopadhyaya et al. 2013; Bingpeng et al. 2018). It utilizes the highly polymorphic characteristic of mtDNA as genetic tags used in aquaculture and fisheries biology (Frankham 2005; Van Dyke 2008; Coker 2017). Specifically, the mitochondrial Cytochrome C Oxidase subunit 1 (CO1) is used as the standard barcode marker for many invertebrate and vertebrate species (Kress and Erickson 2008; Barcaccia et al. 2016). In the Philippines, numerous CO1 sequences have been reported for class Actinopterygii or ray-finned bony fishes (Fontanilla et al. 2014). In particular, CO1 was used to identify mullets (Guino-o et al. 2017) and freshwater fishes from Northern Luzon, such as *Mesopristes cancellatus* or "Pigek" (Maralit et al. 2012) and *Awaous ocellaris* or "Ipon" (Asis et al. 2013)

This study investigated the species identity as well as the range distribution of "ludong" in the Northern Philippines using the CO1 marker. The results obtained has important implications for understanding the biology and ecology of the species as well as its sustainable management.

2. MATERIALS AND METHODS

2.1 Sampling sites and procedure

"Ludong" samples were collected from Northern Philippines by personnel from the BFAR Regions 1, 2, and Cordillera Administrative Region (CAR) (Fig. 1, Table 1). A Gratuitous Permit (No. 0163-18) was secured by BFAR Region 2 from the Department of Agriculture (DA) to collect "ludong"

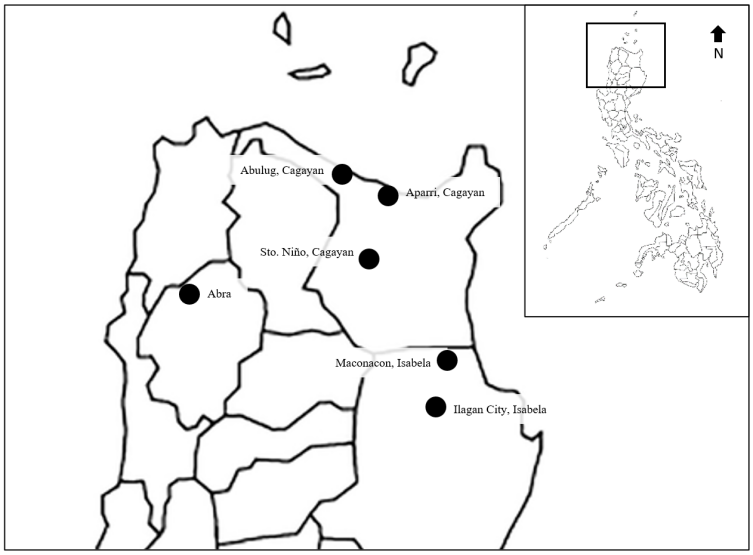
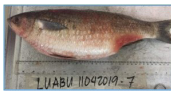


Figure 1. Areas around Northern Philippines where "ludong" samples were collected

Table 1. BLAST analysis of all 25 "ludong" samples collected from Northern Philippines

Number	Sampling Code	Sampling Location	Sampling Date	Fish sample	GenBank Accession Number	% Identity with *Cestraeus goldiei* (Accession No. JQ060406)
1	LUD4	Cab 10, Ilagan City, Isabela, Region 2	June 19, 2019		ON406954	99.81%
2	LUD5	Cab 10, Ilagan City, Isabela, Region 2	June 19, 2019		ON406955	99.82%
3	LUD7	Talungan, Aparri, Cagayan, Region 2	November 3, 2018		ON406953	100.00%
4	LUD9	Talungan, Aparri, Cagayan, Region 2	November 3, 2018		ON406951	99.83%
5	LUD10	Blue Rm, Maconacon, Isabela. Region 2	June 3, 2018		ON406950	99.80%
6	LUD11	Cab 10, Ilagan City, Isabela, Region 2	April 29, 2018		ON406949	99.82%
7	LUD32	Abulug, Cagayan, Region 2	November 4, 2019		ON406948	99.45%
8	LUD34	Abulug, Cagayan, Region 2	November 4, 2019		ON406947	99.80%
9	LUD35	Abulug, Cagayan, Region 2	November 4, 2019		ON406946	99.81%
10	LUD36	Abulug, Cagayan, Region 2	November 4, 2019		ON406945	99.81%
11	LUD37	Abulug, Cagayan, Region 2	November 4, 2019		ON406944	99.78%
12	LUD38	Abulug, Cagayan, Region 2	November 4, 2019		ON406943	99.66%
13	LUD39	Abulug, Cagayan, Region 2	November 4, 2019		ON406942	99.80%
14	LUD41	Abulug, Cagayan, Region 2	November 4, 2019		ON406940	99.83%
15	LUD42	Abulug, Cagayan, Region 2	November 4, 2019		ON406939	99.77%

Number	Sampling Code	Sampling Location	Sampling Date	Fish sample	GenBank Accession Number	% Identity with *Cestraeus goldiei* (Accession No. JQ060406)
16	LUD51	Sto. Nino, Cagayan, Region 2	November 6, 2019		ON406938	99.82%
17	LUD52	Sto. Nino, Cagayan, Region 2	November 6, 2019		ON406937	99.60%
18	LUD128	San Juan, Abra, Cordillera Administrative Region	December 3, 2020		ON406936	99.82%
19	L2 Cgol	Aparri, Cagayan, Region 2			ON40635	99.82%
20	L60 Coxy	Aparri, Cagayan, Region 2			ON40634	99.63%
21	LE2 Cgol	Aparri, Cagayan. Region 2		From previous publication	ON40633	99.63%
22	LE3 Cgol	Aparri, Cagayan, Region 2		From previous publication	ON40632	99.63%
23	LE6 Coxy	Aparri, Cagayan, Region 2		From previous publication	ON40631	99.63%
24	LE9 Cgol	Aparri, Cagayan, Region 2		From previous publication	ON40630	99.63%
25	LE10 Cgol	Aparri, Cagayan, Region 2		From previous publication	ON40629	99.63%

samples for scientific research purposes.

A total of 20 fish specimens labeled as "ludong" were sent to the National Fisheries Research and Development Institute - Genetic Fingerprinting Laboratory (NFRDI-GFL), Quezon City. Muscle tissue samples from each fish or fin clip's dorsal portion were obtained, preserved in 95% ethanol, and placed in 1.5 mL Eppendorf tubes for extraction.

2.2 DNA extraction, amplification, and sequencing

Genomic DNA was extracted using the Cetyl trimethyl ammonium bromide (CTAB) extraction method of Santos et al. (2010). An alternative method used was Chelex DNA extraction (Walsh et al. 1991). Partial fragments of the CO1 gene were amplified using 24µl of PCR mix, which was a mixture of water, 10X PCR Buffer, 10mM DNTP, 2.0µl each of 10µM forward primer and reverse primer (Ward et al. 2005; Ivanova et al. 2007), 50mM MgCl, 0.2-unit Taq polymerase, and 2µl DNA template. The most effective primer pair for the samples was that of Ward et al. (2005) with PCR conditions set as follows: initial denaturation of 94°C for 5 min, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C and extension for 1 min at 72°C, and a final extension of 5 min at 72°C. Amplicons were electrophoresed through a 1% agarose gel stained with ethidium bromide and submerged in 1X TAE Buffer. Standard PCR purification and DNA sequencing were outsourced to 1st Base Sequencing Company.

2.3 DNA sequences from archive

CO1 sequences from seven “ludong” samples obtained during the study of Ventolero et al. in 2014 and archived in the NFRDI-GFL Database were

included in the analysis (Table 1).

2.4 Data analysis

The three *Cestraeus* CO1 sequences available in GenBank and BOLD Systems databases were used as reference: 1 for *Cestraeus goldiei* with GenBank Accession Number JQ060406 and 2 for *C. oxyrhynchus* with GenBank Accession Numbers JQ060407 and JF911704. CO1 sequences for *C. plicatilis* are not available in both databases.

Consensus sequences were created, aligned, and edited using Geneious 6.1.8 (https://www.geneious.com). Phylogenetic trees with 500 bootstrap values were generated using MEGA 6: Molecular Evolutionary Genetics Analysis (Kumar et al. 2018). A pairwise comparison was made using the Kimura 2-parameter (K2P) model (Kimura 1980).

3. RESULTS

All 20 samples were putatively identified as "ludong" by the different BFAR personnel from Regions 1, 2, and CAR, who collected and submitted them to NFRDI-GFL. No initial morphological identification was conducted during the laboratory analysis.

Twenty CO1 sequences from each of the 20 "ludong" samples were generated by this study. Another seven were taken from the NFRDI-GFL archives for a total of 27 sequences initially used for analysis (Fig. S1). Only 25 sequences, which had a minimum of 441 base pairs, were finally considered (Table 1). All 25 sequences were identified using BLAST as *Cestraeus goldiei*. As compared to the reference sequence from Durand et al. (2012), the percentage identity ranged from 99.45% to 100%. This identification was further supported by the Neighbor-Joining tree, where all 25 sequences clustered with *C. goldiei* CO1 sequence published by Durand et al. (2012) at 100% bootstrap value (Figure 2). Lastly, the pairwise comparison showed 0.002 – 0.005 distance values between samples and *C. goldiei* reference sequence in contrast to the 0.060 – 0.062 distance values between samples and *C. oxyrhynchus* (Table 2).

Twenty-four (24) out of the 25 samples were taken from the Cagayan River and its tributaries. Specifically, 20 of the samples (82%) were from Cagayan Province, primarily collected from the municipality of Abulug and Aparri, while 4 out of 25 (15%) came from the Province of Isabela, specifically from the municipalities of Ilagan City and Maconacon. In addition, 1 out of 25 samples (4%) was collected from Abra River in the Province of Abra, Cordillera Administrative Region.

4. DISCUSSION

All 25 samples were identified as *C. goldiei* based on the BLAST, Neighbor-Joining tree, and pairwise comparison analysis. While this is in accordance with the report of Durand et al. (2012), the identification of all samples as *C. goldiei* was surprising since, based on the morphological identification of Harrison and Senou (1999) and Ventolero et al. (2014), there were at least 2 *Cestraeus* spp. present in Cagayan River, *C. goldiei* and *C. oxyrhynchus*. Such findings strongly suggest that only *C. goldiei* is present in Northern Philippines, not *C. oxyrhynchus* or *C. plicatilis*.

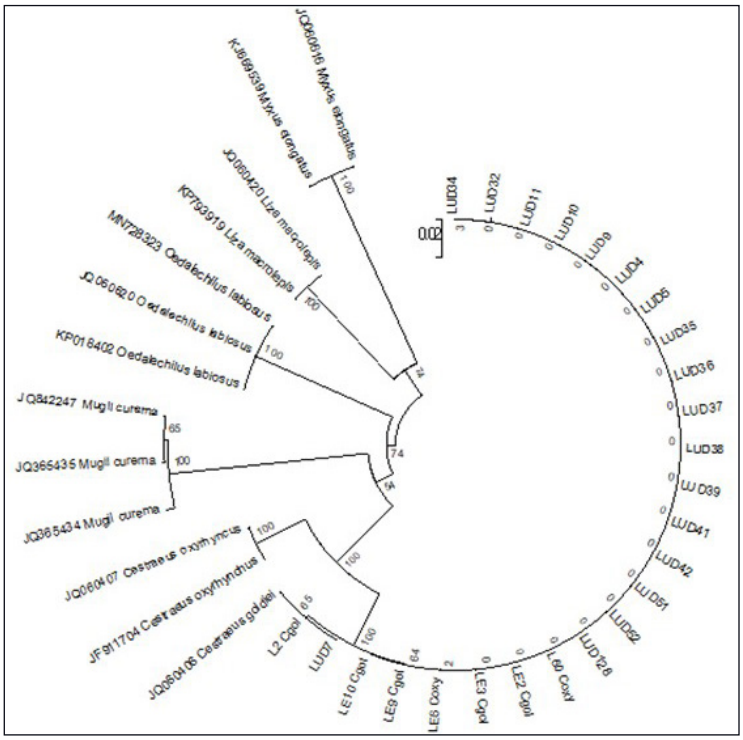


Figure 2. Neighbor-Joining tree (wheel type) of the CO1 sequences generated from all 25 "ludong" samples and other mullets, including *Cestraeus goldiei* and *C. oxyrhynchus* as reference sequences. The tree is based on 441 nucleotides using the K2P model of DNA substitution. Numbers on nodes represent percentage bootstrap support out of 500 samples; values less than 50% are not shown.

Table 2. Pairwise comparison of all 25 "ludong" samples with other mullets, including *Cestraus goldiei* and *C. oxyrhynchus*

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	LUD7 & L2_Cgol																	
2	LUD All (except #1 & #3)	0.002																
3	LUD32	0.005	0.002															
4	*Cestraeus goldiei* JQ060406	0.000	0.002	0.005														
5	*Cestraeus oxyrhynchus* JF911704	0.062	0.060	0.062	0.062													
6	*Cestraeus oxyrhyncus* JQ060407	0.062	0.060	0.062	0.062	0.000												
7	*Oedalechilus labiosus* KP018402	0.159	0.162	0.165	0.159	0.154	0.154											
8	*Oedalechilus labiosus* JQ060620	0.159	0.162	0.165	0.159	0.154	0.154	0.000										
9	*Oedalechilus labiosus* MN728323	0.159	0.162	0.165	0.159	0.154	0.154	0.000	0.000									
10	*Liza macrolepis* KP793919	0.167	0.170	0.173	0.167	0.185	0.185	0.157	0.157	0.157								
11	*Liza macrolepis* JQ060420	0.167	0.170	0.173	0.167	0.185	0.185	0.157	0.157	0.157	0.000							
12	*Mugil curema* JQ365435	0.184	0.181	0.183	0.184	0.187	0.187	0.216	0.216	0.216	0.207	0.207	0.210					
13	*Mugil curema* JQ842247	0.184	0.181	0.183	0.184	0.187	0.187	0.216	0.216	0.216	0.207	0.207	0.210	0.002				
14	*Mugil curema* JQ365434	0.187	0.184	0.187	0.187	0.183	0.183	0.209	0.209	0.209	0.210	0.210	0.214	0.009	0.007			
15	*Myxus elongatus* KJ669539	0.213	0.216	0.216	0.213	0.190	0.190	0.218	0.218	0.218	0.176	0.176	0.179	0.218	0.218	0.221		
16	*Myxus elongatus* JQ060616	0.213	0.216	0.216	0.213	0.190	0.190	0.218	0.218	0.218	0.176	0.176	0.179	0.218	0.218	0.221	0.000	

There is an apparent disagreement between the identification of Harrison and Senou (1999) and Ventolero et al. (2014) in this study. It is possible that *C. goldiei* and *C. oxyrhynchus* are synonymous, and the distinguishing characteristic, the length of the medial lobes, is variable. The same phenomenon was observed by Decru et al. (2016), wherein morphology-based species identification of freshwater fishes was compared with DNA barcoding results using a COI marker, and four cases of possible synonymy were detected. The clustering of *Marcusenius monteiri* and *M. stanleyanus* was one of the cases detected. The only distinguishing characteristic between the two species is tooth morphology: *M. monteiri* has notched teeth, while *M. stanleyanus* has conical or truncated teeth. However, it was mentioned that both tooth shapes were observed in the same specimen, which could mean that this characteristic is variable or a result of hybridization. Another possible explanation is intraspecific hybridization. Several studies have observed natural hybridization on freshwater species (Scribner et al. 2000) such as tilapia (Bradbeer et al. 2019), carps (Dwivedi 2019; Pallipuram 2020), bitterlings (Hata et al. 2019), and lernaea (Hua et al. 2019). Numerous factors lead to hybridization, including external fertilization, competition for limited spawning habitat, predation, and habitat alteration.

Direct evidence to show the occurrence of *C. goldiei* in Abra River, a different system from Cagayan River, is also an important contribution of this study as it extends the known range of the species in the Northern Philippines. This is similar to the findings of Maralit et al. (2012) on another rare, high-value, freshwater therapontid species, *Mesopristes cancellatus*, locally known as "pigek" where its known range distribution was extended from Mindanao to Northern Luzon. Knowledge of habitat range, especially of threatened species, is vital for its management as more protected habitats can ensure the population's sustainability. In addition, best practices in managing "ludong" in Cagayan River could already be adopted and jumpstart management in Abra River. For example, communities in Cagayan River have been reported to have positive attitudes toward the current conservation program for "ludong" especially if the program has lower negative impacts on their income and livelihood (Javier et al. 2016).

CONCLUSION

Accurate species identification helps in assessing and understanding the species, which assists in the formulation of conservation strategies such as monitoring of population trends, evaluation of management plans and health assessments of ecosystems, and extinction analysis (Austen et al. 2016; Kürzel et al. 2022). Based on the results, having only *C. goldiei* in Northern Philippines would narrow the conservation programs and policies intended for this species. In addition, provinces of Northern Philippines that are traversed by the Cagayan and Abra River systems can work concurrently for robust and efficient management of "ludong." Further morphological and molecular studies on identifying "ludong" with more samples from other locations are recommended. Also, there is a need to study the genetic diversity and population structure of "ludong" to support the formulation of joint conservation and management strategies.

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SUPPLEMENTARY MATERIAL

Below is the link to the electronic supplementary material.
Supplementary file

AUTHOR CONTRIBUTIONS

Escaño VMC: Investigation, Formal Analysis, Data Curation, Writing – original draft. **Rey JTS:** Investigation, Resources. **Ame EC:** Conceptualization, Project Administration, Resources. **Gaerlan RS:** Conceptualization, Project Administration, Resources**. Tauli M:** Conceptualization, Project Administration, Resources. **Encarnacion A:** Project Administration, Writing – review and editing. **Santos MD:** Conceptualization, Supervision, Project Administration, Writing – review and editing.

CONFLICTS OF INTEREST

To the best of our knowledge, no conflict of interest exists.

ETHICS STATEMENT

The authors followed all institutional and national guidelines for the care and use of laboratory animals. Moreover, the authors obtained a Gratuitous Permit (No. 0163-18) from the Bureau of Fisheries and Aquatic Resources (BFAR) Region II of the Department of Agriculture (DA) to collect "ludong" samples.

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