SHORT COMMUNICATION

First report of *Awaous ocellaris* in goby fry or "ipon" fishery in Northern Luzon, Philippines

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Goby fry found in Northern Luzon, commonly called "ipon", is an important fishery in the Philippines that is threatened by increasing demand and exploitation of the resource. The continuing decline of the "ipon" population calls for effective fisheries management and conservation. Unfortunately, effective management efforts are hindered by the unresolved identities of the goby fry species which comprise "ipon." Here, we provide the first report of *Awaous ocellaris* as one of the species included in the "ipon" fishery in Aparri, Cagayan, We used the mitochondrial gene cytochrome c oxidase subunit 1 (CO1), that is used by the International Barcode of Life (IBOL) as a standard taxonomic DNA marker for vertebrate species. The results provide important information on the identity of "ipon" which is vital to ensuring the sustainability of the resource.

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INTRODUCTION

Goby fry are greatly valued food fish in the Pacific, the Caribbean, Central America, and the Indian Ocean. In the Philippines, goby fry, which are commonly called "ipon" in Northern Luzon, are often exploited for fermentation and are known to be used as primary ingredient for the most expensive fish pastes in the country (Ruddle 1993, Bell 1999). They are mostly found in Northern Luzon (BFAR 1939), but sightings have been recorded in some places in Southern Mindanao. These species are catadromous, descending to sea for spawning, and then returning upstream to freshwater habitats during a precisely limited season (Ruddle 1993).

In the early 1930s, there were already impressive yields of "ipon" in Northern Luzon. Unfortunately, the increased demand for these post-larvae gobies through the years has encouraged the development of various capture methods to increase the yields, thereby subjecting the resource to overfishing (Bell 1999). Despite management and conservation efforts, such as the Fisheries Administrative Order No. 9 s. 1939 entitled "Regulations for the conservation of certain species of fish commonly called "Ipon" in the northern province of Luzon" (BFAR 1939), the current status of "ipon" fisheries is dire. In view of this, proper management of the "ipon" resource is

essential in order to prevent overfishing and stock depletion, which will be detrimental to the fishery as a whole (Soliman et al. 2009).

Species identification is a vital part of management efforts since species which have not been correctly identified cannot be conserved and protected effectively (Nwani et al. 2011). As a generic term for all goby fry found in Northern Luzon, "ipon" is said to include, but is not limited to, two genera of eleotrids (*Eleotris melanosoma* and *Ophiocara aporos*) and 3 genera of gobies (*Chonophorus melanocephalus/ Awaous melanocephalus, Glossogobius celebius* and *Glossogobius giurus,* and *Sicyopterus lacrymosus*) (Ruddle 1993). However, a complete inventory of the species composition of "ipon" is still nonexistent.

DNA barcoding is a useful tool in providing a clear and accurate identification of fish and fish products. Its foundation rests on the principle that species are generally well-defined by species-specific molecular tags, like those derived from the 5' region of the mitochondrial gene cytochrome c oxidase subunit 1 (CO1), which allow unambiguous identification (Hebert et al. 2003, Hubert et al. 2008). DNA barcoding not only assists in fisheries management for long term sustainability but also enables detection of mislabeled products (Ward et al. 2009). Specifically in the Philippines, DNA barcoding has been used in identifying juvenile tunas (Pedrosa-Gerasmio et al. 2012), in resolving identity issues concerning sardine species (Willette and Santos 2012, Willette et al. 2011), and in detecting mislabeled fish and fish by-products (Maralit et al. 2013).

In this study, we identified an additional species of goby *Awaous ocellaris* in the "ipon" fishery from Aparri, Cagayan using DNA barcoding. This research highlights the importance of correct species identification in fisheries management, the use of DNA-based methods in doing the identification, and raising our awareness of the need to conserve juvenile fishery resources like "ipon" in the Philippines.

METHODOLOGY

"Ipon" samples were collected by Dr. Romeo Culasing of Cagayan State University, about 1 km from the Cagayan River mouth, up to 15 km upstream, in Aparri, Cagayan. These samples were caught by local fishermen and were the ones about to be transported to the local market. Muscle tissues of about 150 mg were obtained from 10 randomly selected samples, then fixed in 95% ethanol and stored at -20 °C until DNA extraction. CTAB DNA Extraction was based on the methodology of Doyle and Doyle (1987), with modifications by Santos et al. (2010). The quality and quantity of the DNA extracts were measured using NanoPhotometer. The DNA template concentrations ranged from 141 to 147 ng/µl. For the PCR amplification, a fragment of approximately 600 bp of mitochondrial DNA covering part of the cytochrome c oxidase subunit 1 (CO1) gene was amplified by using the polymerase chain reaction (PCR) technique and fish primer cocktails C_FishF1t1/C_FishR1t1 from Ivanova et al. (2007). PCR products (2 µl) were subjected to agarose gel electrophoresis for visualization and compared with a 1 kb ladder. The result of the gel electrophoresis is shown in Figure 1. DNA sequencing was outsourced to Macrogen Corporation, Korea.

The resulting DNA sequences were edited and aligned using alignment explorer packaged in MEGA4 (Tamura et al. 2007). Species classification was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) was shown next to the branches (Felsenstein 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980). Analyses were conducted in MEGA5 (Tamura et. al. 2011). Species classification was also inferred using the Maximum-Likelihood method based on the Kimura 2-parameter model (Kimura 1980) for comparison. The percentage of trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. Analyses were also conducted in MEGA5 (Tamura et al. 2011). Voucher

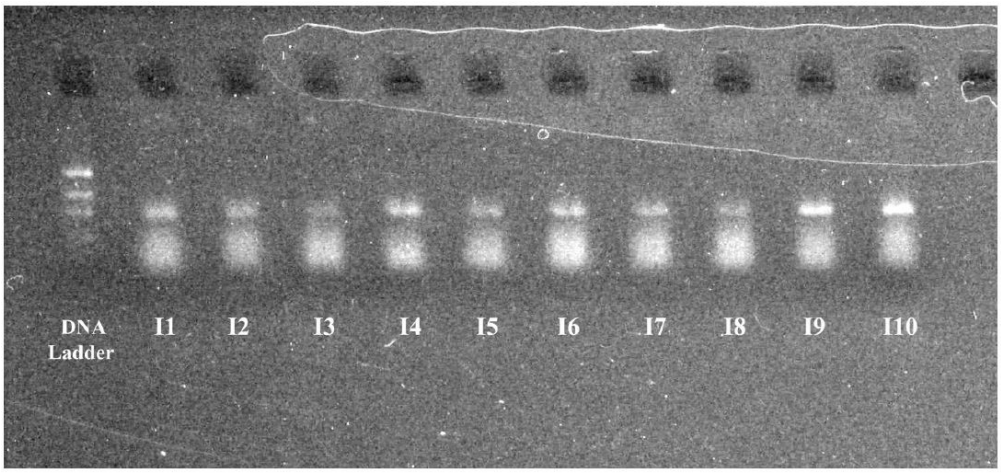

Figure1. Amplified products of "ipon" samples (lanes I1-I10) compared with a 1 kb ladder. All samples show ~700 bp amplification.

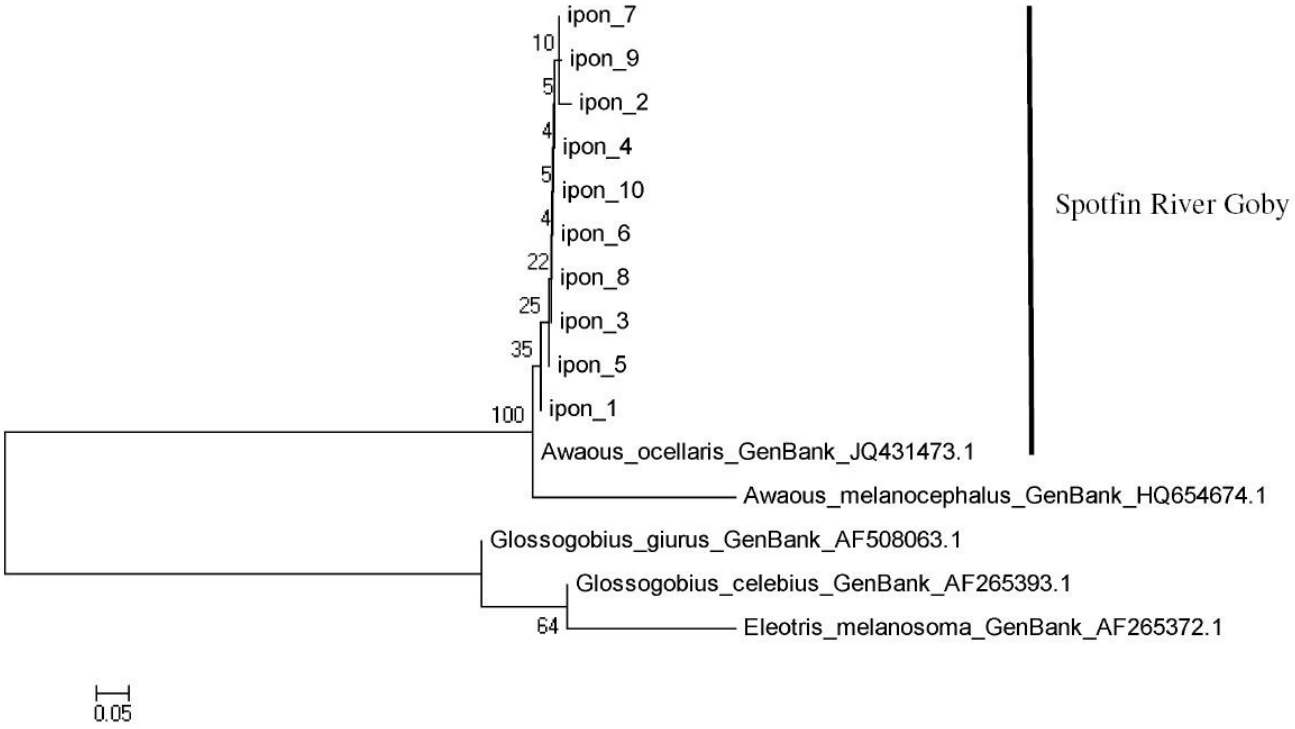

Figure 2. Neighbor-Joining Tree of "ipon" CO1 sequences and those of other closely-related species using Kimura 2-parameter model

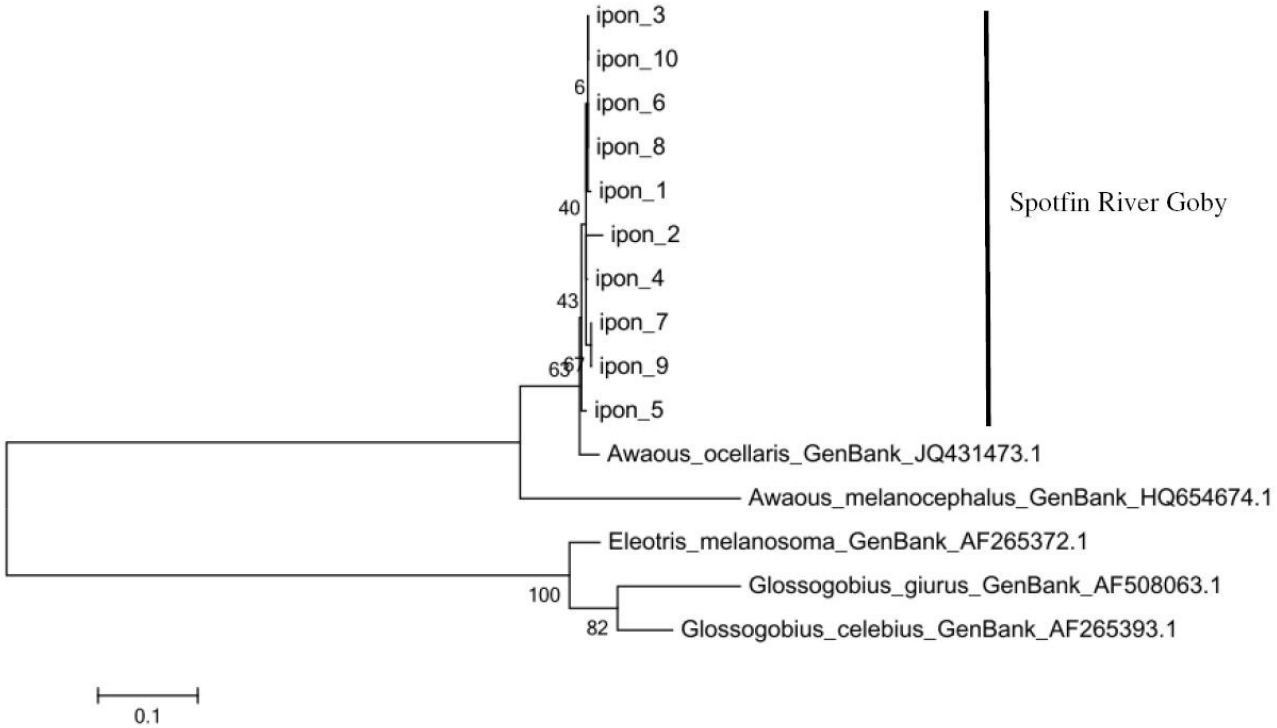

Figure 3. Maximum-Likelihood tree of "ipon" CO1 sequences and those of other closely-related species using Kimura 2-parameter model

CO1 sequences of *Awaous ocellaris* (JQ431473.1), *A. melanocephalus* (HQ654674.1), *Glossogobius giurus* (AF508063.1), *G. celebius* (AF265393.1), and *Eleotris melanosoma* (AF265372.1) were obtained from GenBank for comparison. Table 1 shows (%) similarity of the CO1 sequences of the "ipon" samples compared to the known reference barcode of its closest match, *A. ocellaris,* from BOLD and GenBank. The CO1 sequences of the "ipon" samples were submitted to BOLD with the following accession numbers: ipon_1-BFPHL077-13, ipon_2- BFPHL078-13, ipon_3- BFPHL079-13, ipon_4- BFPHL080-13, ipon_5- BFPHL081-13, ipon_6- BFPHL082-13, ipon_7- BFPHL083-13, ipon_8- BFPHL084-13, ipon_9- BFPHL085-13, and ipon_10- BFPHL086-13.

RESULTS AND DISCUSSION

A total of 10 "ipon" samples were processed for CO1 sequencing. Figure 2 shows the phylogenetic tree of CO1 sequences together with voucher sequences using the Neighbor-Joining method, Kimura 2-parameter model. The analysis involved 15 nucleotide sequences. There were a total of 321 positions in the final dataset which contained 205 parsimony informative characters. The cluster analyses were conducted in MEGA5. All of the 10 samples clustered with *A. ocellaris*, and is supported with 100% bootstrap. The Maximum-Likelihood method was also used to compare the consistency of resulting trees. Figure 3 shows the phylogenetic tree of CO1 sequences together with voucher sequences using the Maximum-Likelihood method, Kimura 2-parameter model. The analysis involved 15 nucleotide sequences. There were a total of 306 positions in the final dataset. Evolutionary analyses were also conducted in MEGA5. The results confirm consistency in the trees.

The pairwise distances were also computed and shown in Table 2. All of the samples showed relatively low pairwise genetic distance values from *A. ocellaris*. The mean interspecific genetic distance between the "ipon" samples and *A. ocellaris* was 0.028, while the interspecific distance relative to another *Awaous* species, *A. melanocephalus*, which was included in the analysis, is 0.283, thus further inferring similar identities.

Our results suggest that the "ipon" samples collected are *A. ocellaris*. This is the first definitive report of such species found in "ipon" fishery. Although *A. ocellaris* has been previously reported to be found in Northern Luzon (Herre 1953), it was not identified as being part of the "ipon" fishery. In fact, even in a more recent study made by Ruddle (1993), *A. ocellaris* was not mentioned as being part of the fishery. This may have been due to the large reliance on morphological characteristics, which in gobies are subtle and have no clear delineation (Jeon et al. 2012), or to a lack of a thorough study of the resource. Here, we demonstrated the utility of DNA barcoding in accurate species identification. Considering that a significant number of randomly collected "ipon" samples turned out to be of the genus *Awaous*, it is surprising that this species has not been previously identified as part of "ipon" fishery. In fact, our results suggest that *Awaous* is a significant part of the fishery.

Table 1. Similarity (%) of the "ipon" samples' CO1 sequences with the known reference barcode of its closest match, *A. ocellaris,* from BOLD and GenBank.

	Similarity (%) with Awaous ocellaris	
Sample species	BOLD	BLASTn
Ipon 1	99.69	97
Ipon 2	98.75	96
Ipon3	100	98
Ipon4	100	98
Ipon5	99.38	97
Ipon6	100	98
Ipon7	99.69	97
Ipon8	100	98
Ipon9	99.69	97
Ipon10	100	98

"Ipon" fishery is a very important industry in the Philippines, but very little study which concerns it has been conducted. Thus, confirmation of the presence of *A. ocellaris* as one of the species included in "ipon," paves the way in forming a more effective management plan for this fishery. Furthermore, since it is widely exploited in Northern Luzon as primary fish paste ingredient, it is imperative to identify all the species which comprise the "ipon" fishery so that it could be protected and managed well. Here the identification of *A. ocellaris* serves as an early initiative for a more thorough investigation and species identification of "ipon."

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Aquatic Resources Regional Office 2 for facilitating the collaboration between NFRDI and CSU under the Oplan Sagip Ludong (OSL).

CONFLICT OF INTEREST

None.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

The samples were collected by Dr. Romeo Culasing and Billy Joel Catacutan. Framing of the hypothesis and experimental set-up was done by Mudjekeewis D. Santos. Laboratory work, data analysis, and manuscript preparation were done by Angelli Marie Jacynth M. Asis, Altair B. Agmata, and Mudjekeewis D. Santos.

REFERENCES

Bell KNI. Overview of goby-fry fisheries. NAGA - the ICLARM quarterly 1999; 22(4):30-36.

BFAR (Bureau of Fisheries and Aquatic Resources). Fisheries Administrative Order No. 9. Regulations for the Conservation of Certain Species of Fish Commonly Called "Ipon" in the Northern Province of Luzon. 1939. BFAR, Philippines.

Doyle JJ, Doyle JL. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 1987; 19:11-15

Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. Evol 1985; 39:783-791.

Hebert PDN, Cywinska A, Ball SL, deWaard JR. Biological identifications through DNA barcodes. Proc R Soc Lond. B Biol. Sci 2003; 270:313–321.

Herre AW. Checklist of Philippine Fishes. Washington DC: United States Government Printing Office, 1953: 740.

Hubert N, Hanner R, Holm E, Mandrak NE, Taylor E, Burridge M, Watkinson D, Dumont P, Curry A, Bentzen P, Zhang J, April J, Bernatchez L. Identifying Canadian Freshwater Fishes through DNA Barcodes. PLoS ONE 2008; 3(6): e2490. doi:10.1371/journal.pone.0002490.

Table 2. Kimura- 2 parameter pairwise distances between individuals of "ipon" samples including outgroups from GenBank.

ipon_1		0.007	0.003	0.003	0.005	0.003	0.005	0.003	0.004	0.003	0.010	0.034	0.286	0.284	0.704									
ipon_2	0.016		0.006	0.006	0.008	0.006	0.007	0.006	0.007	0.006	0.011	0.034	0.382	0.386	0.891									
ipon_3	0.003	0.013		0.000	0.004	0.000	0.004	0.000	0.003	0.000	0.009	0.034	0.309	0.310	0.770									
ipon_4	0.003	0.013	0.000		0.004	0.000	0.004	0.000	0.003	0.000	0.009	0.034	0.309	0.310	0.770									
ipon_5	0.009	0.019	0.006	0.006		0.004	0.006	0.004	0.005	0.004	0.010	0.033	0.309	0.310	0.770									
ipon_6	0.003	0.013	0.000	0.000	0.006		0.004	0.000	0.003	0.000	0.009	0.034	0.309	0.310	0.770									
ipon_7	0.009	0.019	0.006	0.006	0.013	0.006		0.004	0.003	0.004	0.010	0.034	0.326	0.325	0.800									
ipon_8	0.003	0.013	0.000	0.000	0.006	0.000	0.006		0.003	0.000	0.009	0.034	0.309	0.310	0.770									
ipon_9	0.006	0.016	0.003	0.003	0.009	0.003	0.003	0.003		0.003	0.010	0.033	0.340	0.347	0.834									
ipon_10	0.003	0.013	0.000	0.000	0.006	0.000	0.006	0.000	0.003		0.009	0.034	0.309	0.310	0.770									
Awaous_ocellaris_GenBank_JQ431473.1	0.029	0.038	0.025	0.025	0.032	0.025	0.032	0.025	0.029	0.025		0.033	0.300	0.297	0.736									
Awaous_melanocephalus_GenBank_HQ654674.1	0.294	0.303	0.294	0.294	0.289	0.294	0.293	0.294	0.289	0.294	0.289		0.707	0.519	1.003									
Glossogobius_giurus_GenBank_AF508063.1	1.479	1.563	1.509	1.509	1.509	1.509	1.518	1.509	1.541	1.509	1.487	1.871		0.025	0.026									
Glossogobius_celebius_GenBank_AF265393.1	1.479	1.563	1.509	1.509	1.509	1.509	1.518	1.509	1.541	1.509	1.487	1.745	0.180		0.020									
Eleotris_melanosoma_GenBank_AF265372.1	1.845	2.049	1.908	1.908	1.908	1.908	1.935	1.908	1.979	1.908	1.869	2.318	0.196	0.125										

Ivanova NV, Zemlak TS, Hanner RH, Hebert PDN. Universal primer cocktails for fish DNA barcoding. Molecular Ecology Notes 2007; 7:544-548.

Jeon HB, Choi SH, Suk HY. Exploring the utility of partial cytochrome c oxidase subunit 1 for DNA barcoding of gobies. Anim Syst Evol Divers 2012; 28(4):269-278.

Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 1980; 16:111-120.

Maralit BA, Aguila RD, Ventolero MFH, Perez SKL, Willette DA, Santos MD. Detection of mislabeled commercial fishery by-products in the Philippines using DNA barcodes and its implications to food traceability and safety. Food Control 2013; 33(1):119-125.

Nwani CD, Becker S, Braid HE, Ude EF, Okogwu OI, Hanner R. DNA Barcoding Discriminates Freshwater Fishes from Southeastern Nigeria and Provides River System-level Phylogeographic Resolution within Some Species. Mitochondrial DNA 2011; 22(Suppl. 1):43-51.

Pedrosa-GerasmioIR, Babaran RP, Santos MD. Discrimination of juvenile yellowfin (*Thunnus albacares*) and bigeye (*T. obesus*) tunas using mitochondrial DNA control region and liver morphology. PLoS ONE. 2012; 7(4): e35604.DOI 10.1371/journal.pone.0035604.

Ruddle K. The Availability and Supply of Fish for Fermentation in Southeast Asia. In: Lee C, KH Steinkraus, PJA Reilly, eds. Fish Fermentation Technology. Japan:United Nations University Press, 1993: pp. 62-63.

Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 1987; 4:406-425.

Santos MD, Lopez GV, Barut NC. A pilot study on the genetic variation of eastern little tuna (*Euthynnus affinis*) in Southeast Asia. Phil J Sci 2010; 139(1):43-50.

Soliman VS, Bobiles RU, Yamaoka K. Overfishing of three siganid species (Family: Siganidae) in Lagonoy Gulf, Philippines. Kurushio Science 2009; 2:145-150.

Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4. Mol Biol Evol 2007; 24(8):1596–1599.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011; 28(10):2731-2739.

Ward RD, Hanner R, Herbert PDN. The campaign to DNA barcode all fishes, FISH-BOL. J Fish Biol 2009; 74:329-356.

Willette DA, Santos MD. Correcting widespread misidentifications of the highly abundant and commercially important sardine species *Sardinella lemuru*, Bleeker, 1853, in the Philippines. Journal of Applied Ichthyology. 2013; 29(4):881-885.

Willette DA, Santos MD, Aragon MA. First report of the Taiwan sardinella S*ardinella hualiensis* (Clupeiformes: Clupeidae) in the Philippines. J Fish Bi