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RESEARCH NOTE

DNA Barcoding of Dominant Species in the Sardine Fishery of Northern Mindanao

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DNA barcoding was done to identify the dominant species of sardines that comprised the landed catch of the sardine fishery in the Northern Mindanao Region (NMR), and juveniles or "lupoy" in the Northern Zamboanga Peninsula (NZP). Cytochrome oxidase I (COI) minibarcodes were sequenced for all samples, while 16S ribosomal subunit sequences were obtained from a subset of samples. In reference to a previously reported land catch survey, sequence data confirmed the identity of the most dominant sardines in NMR and lupoy from NZP as *Sardinella lemuru*. Meanwhile, those reported as *S. gibbosa* were identified as *Herklotsichthys quadrimaculatus*, while those reported as *S. pacifica* were identified as the recently described *S. goni*. Results highlight: 1) the limitation in identifying sardines during land catch surveys without further taxonomic verification, and 2) the utility of DNA barcoding as a tool for species identification. Moreover, genetic data revealed that *S. goni* occurs in the fisheries of NMR, thereby expanding its known habitat range. Since this study was limited only in using DNA barcodes for identification, a detailed morphological examination of the dominant sardines is recommended.

Keywords: 16S, COI, DNA barcoding, *Sardinella*

A landed catch survey was previously done on sardine fisheries in the NZP and NMR (de Guzman *et al.* 2015). Results reported that the three most dominant species were *Sardinella lemuru* [junior synonym of *S. aurita* following Stern *et al.* (2018); 93.7%], *S. pacifica* (originally reported as *S. fimbriata* but was corrected by Hata and Motomura 2019; 1.1%), and *S. gibbosa* (0.6%). The catch composition was also comprised of unidentified sardine juveniles, locally known as

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lupoy (4.7%). Since the survey was conducted for the purposes of recording species composition and land catch volumes, identification was limited only in using visual descriptions of common sardine species, following Munroe *et al*. (1999). This posed several concerns since: 1) members of the genus *Sardinella* often share similar visual diagnostic characters (Munroe *et al*. 1999; Samonte *et al*. 2009; Quilang *et al*. 2011; Stern *et al*. 2018); and 2) the diagnostic characters of juveniles are not yet fully developed, making them difficult to identify (Thomas *et al.* 2013). Species identification, therefore, requires further taxonomic verification.

Taxonomic verification of sardines can be done either through phenotypic or genetic methods. The former requires a thorough morphological examination of the fish (*e.g.* morphometrics, meristics), while the latter utilizes genetic information. DNA barcoding is a genetic method that uses a short section of DNA from genes (*e.g.* COI, 16S) for species identification (Quilang *et al*. 2011; Weigt *et al*. 2012). DNA barcoding is more suited in identifying sardines given their complex diversity and overlapping phenotypic characteristics, which led to recent taxonomic corrections (Willette and Santos 2013; Thomas *et al*. 2014; Stern *et al*. 2016, 2018; Hata and Motomura 2019). This study used DNA barcoding to identify the dominant sardine species in the sardine fisheries along the northern coast of Mindanao, as reported by de Guzman *et al.* (2015).

Sardine samples were obtained from key locations in NMR, while juveniles were collected from areas along Dapitan-Dipolog Bay in NZP (Figure 1). Samples were initially identified following de Guzman *et al.* (2015). All specimens were transported to the Marine Science Institute, University of the Philippines Diliman for genetic barcoding and phylogenetic analyses (Appendix Description I; Appendix Table I). Samples with presumptive identities are hereafter referred to as *Sl* (presumptive *S. lemuru*), *Sp* (presumptive *S. pacifica*), and *Sg* (presumptive *S. gibbosa*).

The most likely identity of the samples was based on the query results from online databases (Appendix Table II) and their respective clusters in the phylogenetic trees. COI tree (Figure 2) showed that all *Sl* and *lupoy* samples clustered with the subgenus *Sardinella* (bootstrap support ≥ 98%). This subgenus contains several sardine species characterized by nine pelvic fin rays (*S. aurita*, *S. brasiliensis*, *S. lemuru*, *S. longiceps*, *S. neglecta*; Stern et al. 2018). Meanwhile, most *Sp* clustered with *S. goni* (bootstrap support ≥ 77%), while all *Sg* and two *Sp* samples clustered with *Herklotsichthys quadrimaculatus* collected from Indonesia (bootstrap support ≥ 100%). Inferences from COI were further corroborated by the subset of samples in the 16S tree (Figure 3).

DNA barcodes confirmed that the most dominant species of sardines in northern Mindanao is *S. lemuru*, following recent taxonomic corrections (Willette and Santos 2013). Although it was recently proposed that *S. lemuru* – along with the other species under the subgenus *Sardinella* – be renamed as *S. aurita* (Stern et al. 2018), Eschmeyer's Catalog of Fishes (https://www.calacademy.org/scientists/projects/eschmeyers-catalog-of-fishes; date accessed: January 2021) still considers junior synonyms of *S. aurita* as valid scientific names. Meanwhile, the other two dominant species – *Sp* and *Sg* – were misidentified. This case of misidentification is not unusual for sardines since different species often share similar diagnostic characters.

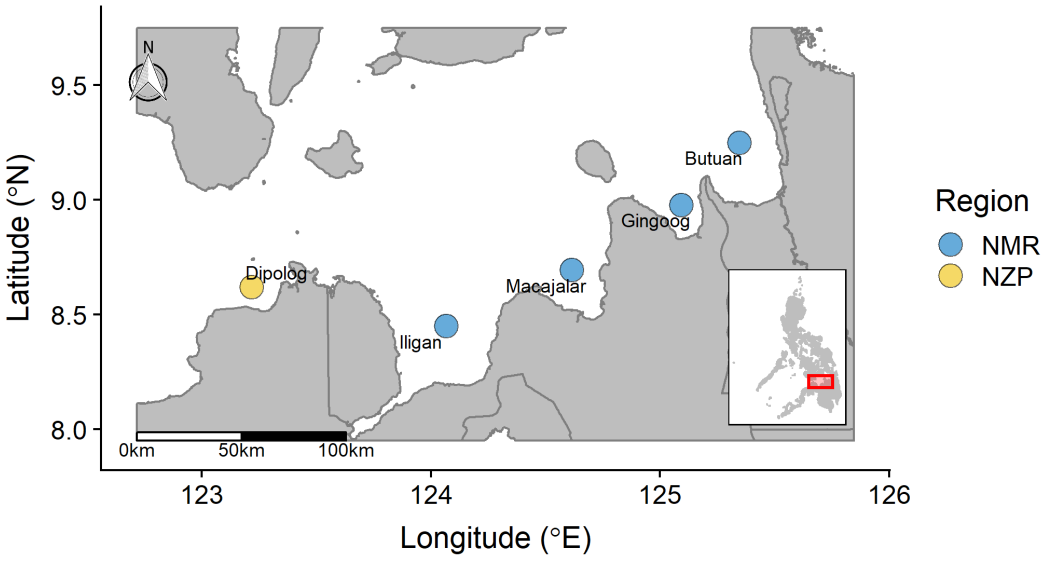

Figure 1. Sampling sites. Colors indicate the region from where the sites belong (blue – NMR; yellow – NZP). Inset shows the location relative to the Philippine archipelago.

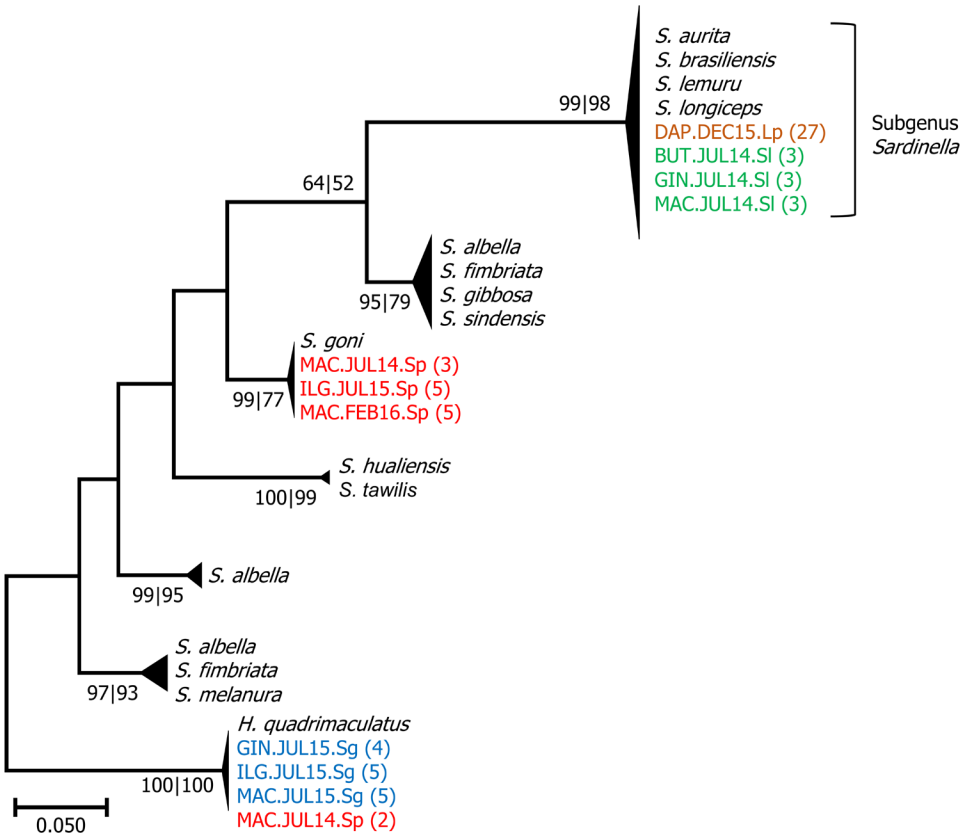

Figure 2. Maximum likelihood (ML) tree inferred from cytochrome oxidase I (COI) sequences (length = 192 bp) using K80+G model. Nodes with bootstrap support values greater than 50% are shown (NJ|ML). Labels in black indicate samples retrieved from the database, while those with color indicate sequences generated in this study. Sample notations include the site (BUT – Butuan; GIN – Gingoog; MAC – Macajalar; ILG – Iligan; DAP – Dapitan-Dipolog), the month and year of sampling, the presumptive identities (orange – "lupoy"; green – *S. lemuru*; red – *S. pacifica*; blue – *S. gibbosa*), and the number of samples (inside parenthesis). *Herklotsichthys quadrimaculatus* was used as the outgroup.

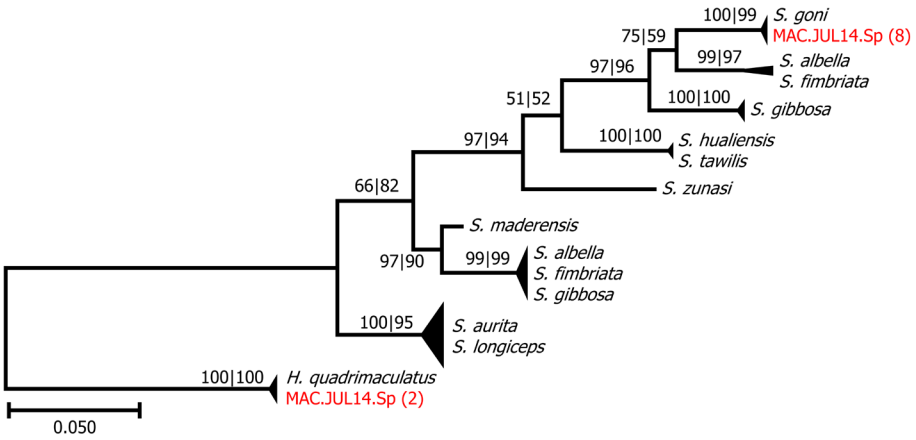

Figure 3. Maximum likelihood (ML) tree inferred from 16S ribosomal subunit sequences (length = 507 bp) using K80+G model. Nodes with bootstrap support values greater than 50% are shown (NJ|ML). Labels in black indicate samples retrieved from the database, while those in red indicate presumptive *S. pacifica* collected from Macajalar (MAC). The number of samples are shown inside the parenthesis. *Herklostichthys quadrimaculatus* was used as the outgroup.

This is particularly difficult during land catch surveys where rapid visual dentification, coupled with the loss of diagnostic characters (*e.g.* color) and physical damage to the fish, contribute to misidentification.

DNA barcodes also revealed that the second most dominant sardine species in NMR, presumed to be *S. pacifica*, was genetically identical to *S. goni*. This recently described species was first obtained from Boracay Island, Philippines (Stern *et al.* 2016). While its taxonomic description has been provided, its distribution remains to be uncovered. Results of land catch surveys (de Guzman *et al.* 2015) coupled with DNA barcoding of *Sp* samples collected from 2014–2016 suggest that the species may be common in the region.

While this paper highlighted: 1) the limitations of identifying sardines solely on morphological diagnostic characters, especially in the context of land catch surveys; and 2) the utility of DNA barcoding as a tool for species identification, there are still gaps that need to be addressed. First, this paper was unable to perform a thorough phenotypic examination of the samples that were barcoded. Morpho-meristic data are necessary to further improve the inferences from genetic data and provide more accurate identities. These will support the claim that, in addition to *S. lemuru*, the other dominant species of landed catch in the region are *H. quadrimaculatus* and *S. goni* instead of *S. pacifica* and *S. gibbosa*. Likewise, these are needed to determine whether the *S. goni* samples in NMR are morphologically similar to the ones recovered from Boracay Island. Second, while no *S. pacifica* and *S. gibbosa* were genetically identified, the possibility cannot be discounted that these two species are present in the region but were not represented in the samples that were barcoded due to sampling limitations.

Overall, correct taxonomic classification is important in managing sardine resources. Species misidentification may lead to unintended consequences, particularly in the development of management schemes that revolve around the species' biology (Willette and Santos 2013). Implications also extend to food and commercial sectors where there is a need for accurate labels of fishery products for consumption or export. Genetic barcoding is a useful tool for species identification, and it should be utilized to supplement phenotypic methods of identification. Those who are trained in molecular technologies (*e.g.* DNA barcoding) should work alongside morpho-meristics experts to prepare an integrated identification tool that will guide field researchers in sardine identification.

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NOTES ON APPENDICES

The appendix section of the study is accessible at http://www.philjournalsci.dost.gov.ph

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APPENDICES

Description I. Detailed methodology on genetic barcoding and sequence analyses.

Tissue Excision and DNA Extraction

The NFRDI's manual for tissue sampling (Agmata *et al.* 2012) was used. Tissue was excised from the dorsal muscle at the right body side of the specimen under aseptic conditions. This was placed in a pre-labelled 1.5-mL microcentrifuge tube and was preserved with 95% EtOH. A small mass of muscle tissue was used for DNA extraction using hot sodium hydroxide and Tris (HotSHOT) extraction protocol (Montero-Pau *et al*. 2008). Briefly, a small piece of tissue was placed in a 0.2-mL microtube with 50-µL alkaline lysis buffer (25 mM NaOH, 0.20 mM Na_2EDTA, pH 12.0), followed by vigorous vortexing for 5 s. The sample was then heated (95 °C, 20 min) and immediately cooled (4 °C, 20 min) using a thermal cycler (C1000 Touch™, Bio-Rad, USA). Afterwards, a 50-µL neutralizing solution (40 mM Tris-HCl, pH 5.0) was added and mixed *via* vortexing. Crude DNA extracts were stored at 4 °C until further processing.

PCR Amplification, Visualization, and Purification

Samples were initially barcoded using cytochrome oxidase I (Shokralla *et al*. 2015). Identities of a subset of samples were further verified using the 16S ribosomal subunit (Thomas *et al*. 2014). Primer sequences, components of the polymerase chain reaction (PCR) mix, and the PCR thermal regimes are provided in Appendix Table 1. Amplification success was determined through agarose gel electrophoresis (1.0% agarose gel, 100 V, 15 min) and UV-visualization. Amplicons were purified using ExoSAP-IT (Affymetrix, USA). Briefly, PCR products were mixed with ExoSAP-IT (2 µL), followed by enzyme activation (37 °C, 30 min) and enzyme deactivation (80 °C, 20 min). Amplicons were sent to 1st Base, Malaysia for sequencing.

Sequence Analysis

Sequence editing and sequence alignment were done using Geneious v8.1.5 (Kearse *et al.* 2012). MUSCLE algorithm (Edgar 2004) with manual evaluation was used for alignment. The aligned sequences were then queried to online databases: 1) Barcode of Life Database Systems (BOLD Systems; https://www.boldsystems.org), and 2) National Center for Biotechnology Information – Basic Local Alignment Search Tool (NCBI-BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). Database sequences that were almost identical (99–100% similarity) with the queried sequences were considered as the genetic identity of the samples (Appendix Table II).

In addition to the sequences generated in this study, homologous sequences of COI and 16S were retrieved from BOLD Systems and NCBI GenBank, respectively. Identical sequences for each species retrieved from the database were collapsed into a single representative haplotype using FaBox (Villesen 2007). Aligned sequences were then loaded to MEGA 7 (Kumar *et al.* 2016) for phylogenetic analyses. Model testing was carried out to select the appropriate evolutionary model

Table I. Primers used for amplification, final concentration of PCR reagents per reaction volume (25 uL), and PCR thermal regime.

	Cytochrome oxidase I	**16S ribosomal subunit**
Primer pair	FISH_miniE_F/ FISH_miniE_R (Shokralla *et al*. 2015)	16Sar/16Sbr (Palumbi 1996)
FWD sequence (5'–3')	CACGACGTTGTAAAACGACACYAAICAYAAAGAYATIGGCAC	CGCCTGTTTATCAAAAACAT
REV sequence (5'–3')	GGATAACAATTTCACACAGGCTTATRTTRTTTATICGIGGRAAIGC	CCGGTCTGAACTCAGATCACGT
Reaction concentration (final concentration in PCR master mix)		
PCR buffer (X)	1.00	0.50
MgCl (mM)	2.50	2.00
dNTPs (mM each)	0.15	0.20
BSA (X)	0.20	0.20
Primer (µM each)	0.10	0.50
Taq polymerase (U/uL)	0.02	0.03
Thermal regime		
Initial denaturation	95 °C, 5.0 min	94 °C, 10.0 min
Denaturation	94 °C, 30 s	94 °C, 30 s
Annealing	46 °C, 1.0 min	45 °C, 30 s
Extension	72 °C, 30 s	72 °C, 45 s
No. of cycles	35x	39x
Final extension	72 °C, 5.0 min	72 °C, 10 min

Table II. Presumptive and genetic barcode identities of sardine samples from the fishery of northern Mindanao. Sample ID notation: embayment (DAP – Dapitan-Dipolog; BUT – Butuan; GIN – Gingoog; MAC – Macajalar; ILG – Iligan), sampling date (month-year), and code for presumptive identities (Lp – lupoy; Sl – *S. lemuru*; Sg – *S. gibbosa*; Sp – *S. pacifica*). Barcode IDs were obtained from Barcode of Life Database (BOLD) Systems (https://www.boldsystems.org/index.php) and GenBank of the National Center for Bioinformatics Information (NCBI; https://www.ncbi.nlm.nih.gov/genbank/). Accession numbers are shown.

Sample ID	Presumptive ID*	Barcode ID (99–100% similarity)		Accession no. (miniCOI/16S)
		mini COI (192 bp)	16S (507 bp)	
DAP.DEC15.Lp01	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950773
DAP.DEC15.Lp02	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950774
DAP.DEC15.Lp05	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950775
DAP.DEC15.Lp06	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950776
DAP.DEC15.Lp07	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950777
DAP.DEC15.Lp08	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950778
DAP.DEC15.Lp09	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950799
DAP.DEC15.Lp10	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950780
DAP.DEC15.Lp11	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950781
DAP.DEC15.Lp12	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950782
DAP.DEC15.Lp13	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950783
DAP.DEC15.Lp14	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950784
DAP.DEC15.Lp15	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950785
DAP.DEC15.Lp16	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950786
DAP.DEC15.Lp17	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950787
DAP.DEC15.Lp18	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950788
DAP.DEC15.Lp19	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	NT905789
DAP.DEC15.Lp20	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950790
DAP.DEC15.Lp21	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950791
DAP.DEC15.Lp22	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950792
DAP.DEC15.Lp23	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950793
DAP.DEC15.Lp24	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950794
DAP.DEC15.Lp25	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950795
DAP.DEC15.Lp26	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950796
DAP.DEC15.Lp27	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950797
DAP.DEC15.Lp28	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950798
DAP.DEC15.Lp29	Sardine juvenile (lupoy)	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT950799
BUT.JUL14.Sl03	*Sardinella lemuru*	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT946132
BUT.JUL14.Sl05	*Sardinella lemuru*	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT946133
BUT.JUL14.Sl21	*Sardinella lemuru*	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT946137
GIN.JUL14.Sl01	*Sardinella lemuru*	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT946135
GIN.JUL14.Sl17	*Sardinella lemuru*	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT946136
GIN.JUL14.Sl21	*Sardinella lemuru*	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT946134
MAC.JUL14.Sl04	*Sardinella lemuru*	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT946157
MAC.JUL14.Sl21	*Sardinella lemuru*	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT946158
MAC.JUL14.Sl22	*Sardinella lemuru*	*S. lemuru; S. longiceps; S. brasiliensis; S. aurita*	NA	MT946159
GIN.JUL15.Sg01	*Sardinella gibbosa*	*Herklotsichthys quadrimaculatus*	NA	MT946160
GIN.JUL15.Sg02	*Sardinella gibbosa*	*Herklotsichthys quadrimaculatus*	NA	MT946161
GIN.JUL15.Sg03	*Sardinella gibbosa*	*Herklotsichthys quadrimaculatus*	NA	MT946162

Sample ID	Presumptive ID*	Barcode ID (99–100% similarity)		Accession no. (miniCOI/16S)
		mini COI (192 bp)	16S (507 bp)	
GIN.JUL15.Sg05	*Sardinella gibbosa*	*Herklotsichthys quadrimaculatus*	NA	MT946141
ILG.JUL15.Sg01	*Sardinella gibbosa*	*Herklotsichthys quadrimaculatus*	NA	MT946138
ILG.JUL15.Sg02	*Sardinella gibbosa*	*Herklotsichthys quadrimaculatus*	NA	MT946139
ILG.JUL15.Sg03	*Sardinella gibbosa*	*Herklotsichthys quadrimaculatus*	NA	MT946140
ILG.JUL15.Sg04	*Sardinella gibbosa*	*Herklotsichthys quadrimaculatus*	NA	MT946150
ILG.JUL15.Sg05	*Sardinella gibbosa*	*Herklotsichthys quadrimaculatus*	NA	MT946151
MAC.JUL15.Sg01	*Sardinella gibbosa*	*Herklotsichthys quadrimaculatus*	NA	MT946147
MAC.JUL15.Sg02	*Sardinella gibbosa*	*Herklotsichthys quadrimaculatus*	NA	MT946148
MAC.JUL15.Sg03	*Sardinella gibbosa*	*Herklotsichthys quadrimaculatus*	NA	MT946149
MAC.JUL15.Sg04	*Sardinella gibbosa*	*Herklotsichthys quadrimaculatus*	NA	MT946150
MAC.JUL15.Sg05	*Sardinella gibbosa*	*Herklotsichthys quadrimaculatus*	NA	MT946141
MAC.JUL14.Sp01	*Sardinella pacifica*	*Sardinella goni*	*Sardinella goni*	MT946165 / MT944107
MAC.JUL14.Sp02	*Sardinella pacifica*	*Sardinella goni*	*Sardinella goni*	MT946166 / MT944108
MAC.JUL14.Sp03	*Sardinella pacifica*	*Sardinella goni*	*Sardinella goni*	MT946167 / MT944109
MAC.JUL14.Sp06	*Sardinella pacifica*	*Herklotsichthys quadrimaculatus*	*Herklotsichthys quadrimaculatus*	MT946168 / MT944110
MAC.JUL14.Sp07	*Sardinella pacifica*	*Sardinella goni*	NA	MT944111
MAC.JUL14.Sp08	*Sardinella pacifica*	*Sardinella goni*	*Sardinella goni*	MT944112
MAC.JUL14.Sp09	*Sardinella pacifica*	*Sardinella goni*	*Sardinella goni*	MT944113
MAC.JUL14.Sp10	*Sardinella pacifica*	*Herklotsichthys quadrimaculatus*	*Herklotsichthys quadrimaculatus*	MT946169 / MT944114
MAC.JUL14.Sp11	*Sardinella pacifica*	*Sardinella goni*	*Sardinella goni*	MT944115
MAC.JUL14.Sp12	*Sardinella pacifica*	*Sardinella goni*	*Sardinella goni*	MT944116
ILG.JUL15.Sp01	*Sardinella pacifica*	*Sardinella goni*	NA	MT946142
ILG.JUL15.Sp02	*Sardinella pacifica*	*Sardinella goni*	NA	MT946143
ILG.JUL15.Sp03	*Sardinella pacifica*	*Sardinella goni*	NA	MT946144
ILG.JUL15.Sp04	*Sardinella pacifica*	*Sardinella goni*	NA	MT946145
ILG.JUL15.Sp05	*Sardinella pacifica*	*Sardinella goni*	NA	MT946146
MAC.FEB16.Sp01	*Sardinella pacifica*	*Sardinella goni*	NA	MT946152
MAC.FEB16.Sp02	*Sardinella pacifica*	*Sardinella goni*	NA	MT946153
MAC.FEB16.Sp03	*Sardinella pacifica*	*Sardinella goni*	NA	MT946154
MAC.FEB16.Sp04	*Sardinella pacifica*	*Sardinella goni*	NA	MT946155
MAC.FEB16.Sp05	*Sardinella pacifica*	*Sardinella goni*	NA	MT946156

*Presumptive identities were based on the report by de Guzman *et al.* (2015). *S. pacifica* was originally reported as *S. fimbriata* but was corrected following Hata and Motomura (2019). Recent taxonomic work proposed that *S. lemuru* be considered as junior synonym for *S. aurita* (Stern *et al.* 2018).

for the dataset. Phylogenetic trees were constructed using both neighbor joining (NJ) and maximum likelihood (ML) methods; parameters were based on the estimates obtained from the best model. Bootstrap replicate was set to 1000 for both runs; a cut-off value indicating strong phylogenetic support was set to ≥ 50%.

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