Philippine Journal of Science
149 (2): 335-346, June 2020
ISSN 0031 - 7683
Date Received: 14 June 2019

DNA Barcoding and Diversity Analysis of 19 Economically Important Philippine Sea Cucumbers (Holothuroidea)

Ma. Neda Alcudia-Catalma[1§], **Maria Genaleen Q. Diaz**[2], **Roberta N. Garcia**[1], **Pablo P. Ocampo**[2], **Antonio C. Laurena**[1], and **Evelyn Mae Tecson-Mendoza**[1*]

[1]Institute of Plant Breeding, College of Agriculture and Food Science
[2]Institute of Biological Sciences, College of Arts and Sciences
University of the Philippines Los Baños, College 4031 Laguna, Philippines
[§]Silk/Silkworm Genomics Project, Research and Development Division
Philippine Textile Research Institute, Department of Science and Technology
Bicutan 1631 Taguig City, Philippines

This study established the DNA barcodes of 19 economically important Philippine sea cucumbers belonging to Class Holothuroidea under Phylum Echinodermata using the *cytochrome c oxidase I* (*COI*) gene. These include sea cucumbers from the Family Holothuriidae under Order Aspidochirotida: *Bohadschia marmorata, B. koellikeri*, *B. vitiensis, B. argus, Bohadschia* sp. *1*, *Actinopyga echinites, A. lecanora, Holothuria scabra, H. fuscogilva, H. atra*, *H. impatiens*, and *H. albiventer*; from the Family Stichopodiidae under Order Aspidochirotida: *Stichopus horrens*, *S. monotuberculatus, S. vastus, S. hermanni, S. chloronotus*, and *Thelenota ananas*; and from the Family Phyllophoriidae under Order Dendochirotida was *Neocucumis proteus*. Based on Kimura-2 pairwise (K2P) distances, low genetic variation within species of 0.005–0.018 was observed except for several species such as *S. chloronotus, H. albiventer, A. echinites*, and *H. scabra* – which had 0.057, 0.181, 0.207, and 0.215 within-species genetic variations, respectively. On the other hand, variation between species within a genus was 0.123 for *Bohadschia*, 0.18 for *Actinopyga*, 0.19 for *Holothuria*, and 0.071 for *Stichopus*. Phylogenetic tree using neighbor-joining analysis showed monophyletic clades for the genera *Bohadschia, Actinopyga, Stichopus, Thelenota*, and *Neocucumis* while paraphyletic clade was formed for the genus *Holothuria*. *COI* DNA sequences and barcodes were established for the first time for *Neocucumis proteus* and *Holothuria albiventer*.

Keywords: *cytochrome oxidase I* (*COI*), DNA barcodes, Holothuroidea, Philippine sea cucumbers

INTRODUCTION

The sea cucumber is an aquatic marine invertebrate that belongs to Class Holothuroidea, Phylum Echinodermata. It is globally traded because its dried product – commonly known as "balat," "bat," "gamat," or "trepang" in Asia and "bêche-de-mer" in France – is used as an exotic Asian cuisine and source of natural products. The dried sea cucumbers command high price in the international market with retail price ranging from USD 15–385/kg and are exported to Hong Kong, China, Japan, South Korea, Singapore, and the US (Purcell 2014). The sea cucumber is also explored for biomedical research, and for medicinal and therapeutic properties due to the presence of high valued bioactive compounds (Bordbar *et al*. 2011).

*Corresponding Author: etmendoza@up.edu.ph

Sea cucumbers of very diverse species used to abound in Philippine coastal waters. Due to heavy exploitation, sea cucumber production in the Philippines declined from 4,000 metric tons in the 1980s to less than 1,000 metric tons. In 2015, production of dried sea cucumbers was reported at 164 metric tons worth USD 3,900,000 (BFAR 2015) and 408 metric tons worth USD 6,841,000 in 2016 (BFAR 2017).

The Philippines is located in the Indo-Pacific region, which is a center of Holothurian species diversity (Hamel *et al*. 2001; Choo 2008). In 2005, the Indo-Malay-Philippine Archipelago was confirmed as the center of marine biodiversity, with the Philippines having the highest diversity (Carpenter and Springer 2005). Sea cucumbers in Philippine seas are estimated to consist of 200 species, which include 40 high-value species (Juinio-Menez and Samonte 2016). Earlier reports include survey and description of Philippine sea cucumbers started by Prof. Carl Semper who documented 41 species (Semper 1868; Samyn *et al.* 2013), then Seale with 64 species (1911) and Domantay with 99 species (1957). More recent surveys aimed to aid management measures. The existence of 44 species under the family Caudinidae (1), Holothuridiidae (26), Stichopodidae (16), and Synaptidae (1) was reported by Jontila *et al.* (2014) in 16 sites in the province of Palawan. Twenty-three of these were newly reported in the province, while 36 are usually commercially traded and locally consumed. A total of 18 sea cucumbers species were reported by Dolorosa (2015) at the Tubbataha Reefs Natural Park, Philippines. Twelve of these were new records and three are listed in the International Union for Conservation of Nature (IUCN) Red List. Dolorosa *et al.* (2017) studied sea cucumber fishing in Rasa Island Wildlife Sanctuary in Narra, Palawan where they identified 24 species (69% of the 35 reported number of important sea cucumber species in Palawan). Among the 24, three species (*Actinopyga echinites*, *Holothuria scabra*, and *Stichopus herrmanni*) are listed as threatened by the IUCN.

Difficulty with the taxonomy of many species of sea cucumber remains a challenging problem for conservation management, biodiversity studies, species identification, correct pricing, and trade considering that many of these species are endangered (Uthicke *et al*. 2010). In the Philippines, sea cucumbers are called by various local names; thus, a single species can have more than one local name or a local name can be shared by more than one species (Gamboa *et al*. 2004; Choo 2008).

The use of molecular tools such as DNA barcoding has contributed to further the knowledge on species identification, genetic relationships, and lineage of these organisms (Curole and Kocher 1999; Fan *et al*. 2011). Among the protein-coding genes, *COI* is used as standard for DNA barcoding of animal species, which is done by analysis of the 650 base pair fragment of *COI* among closely related species and across diverse phyla in the animal kingdom (Hebert *et al*. 2003).

DNA barcoding has been used to detect food safety, traceability, and authenticity such as detecting adulteration in marine and aquatic products (Galimberti *et al*. 2013) and authenticating commercial seafood products such as molluscs, crustaceans, and fishes (Nicole *et al*. 2012).

The *COI* gene has been used in studying the molecular phylogeny of sea cucumbers from the Eastern Pacific region (Arndt *et al*. 1996) and for DNA barcoding and diversity analysis of 47 species of sea cucumbers belonging to Class Holothuroidea under Phylum Echinodermata obtained from several locations in the Atlantic, Pacific, and Indian Oceans (Uthicke *et al*. 2010). On the other hand, Byrne *et al*. (2010) used the *COI* gene for determination of molecular taxonomy, phylogeny, and evolution of the sea cucumbers from the family Stichopodidae. In 2011, Borrero-Perez *et al*. found the *COI* gene to be more effective than mitochondrial *16S* in determining population structure of sea cucumbers of *Holothuria mammata* found in the Atlantic-Mediterranean basins.

The *COI* gene has also been used as a genetic marker to resolve the confusion in the morphology of the species from the genus *Bohadschia* (Kim *et al*. 2013), and in the revision of the genus *Phyrella* with the addition of new species from Guam (Michonneau and Paulay 2014). Using allozyme and mtDNA sequences, Uthicke *et al.* (2005) further showed that natural hybridization did not dissolve species boundaries of commercially available sea cucumbers. The two sympatric holuthurians, *Holothuria scabra* and *H. s*. var. *versicolor*, were found to be distinct but young biological and phylogenetic species using allozyme and mtDNA sequence analyses. However, individuals of intermediate phenotype – their hybrids – were intermediate in terms of allozyme alleles although similar in mtDNA sequences to those of either species.

Among the many studies of DNA barcoding for sea cucumbers worldwide, Uthicke *et al*. (2010) included only two species from the Philippines: *Pearsonothuria graeffei* and *Stichopus horrens*. Thus, it is very important that the molecular identities of the many strains and species of sea cucumbers in Philippine seas, starting with the commercially important ones, be established. This molecular tool will also allow the determination of the diversity among individuals within a species and the relationships between some Philippine sea cucumbers.

In this study, we established the *COI* sequences and DNA barcodes for 19 economically important sea cucumbers from the Philippine seas and studied their genetic

variability and relationships. These include the first report for *Neocucumis proteus* and *Holothuria albiventer.*

MATERIALS AND METHODS

Sea Cucumber Samples

The sea cucumbers were collected from the coastal areas of Luzon (Ilocos Norte, Ilocos Sur, Pangasinan, Cagayan, La Union, Aurora, and Palawan), Visayas (Leyte, Cebu, Bohol, Negros Oriental, and Iloilo), and Mindanao (Davao del Norte, Misamis Oriental, and Tawi-Tawi) (Figure 1). Majority of the samples were provided and identified by collaborators under the joint project of PCAARRD (Philippine Council for Agriculture, Aquatic, and Natural Resources Research and Development) and CHED (Commission on Higher Education) on "Inventory and Stock Assessment of the Sea Cucumbers in the Philippines" led by the University of the Philippines (UP) Diliman Marine Science Institute. The sea cucumbers were morphologically characterized by the project staff and small pieces of tissues (longitudinal muscles, body wall, or tentacles) were preserved in 95–100% ethanol in cryogenic tubes and were sent through courier to the Biochemistry Laboratory, Institute of Plant Breeding, College of Agriculture and Food Science, UP Los Baños and kept at –20 °C until needed. Appendix Table I presents the list of economically important sea cucumbers in the Philippines, which were collected and identified by collaborators who are listed in this table. These collaborators were previously trained by the UP Marine Science Institute staff in their station in Bolinao, Pangasinan. The species were photographed, identified based on morphological examination and examination of fixed ossicles under the electron microscope, and preserved in 70% ethanol in their institutions.

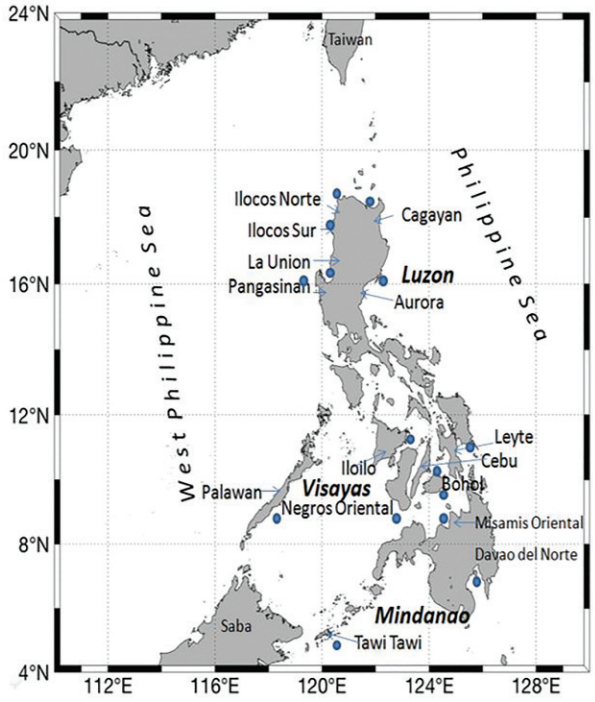

Figure 1. Collection sites of sea cucumber specimens from coastal waters (●) of provinces in the Philippines, as indicated.

DNA Extraction, PCR Amplification, and Sequencing

Genomic DNA was isolated from ethanol preserved tissues using the CTAB+Chelex® method for sea cucumbers adopted from Doyle and Doyle (1990) and Yue and Orban (2005) with modifications such as slicing of animal tissues, preferably longitudinal muscles, into very fine pieces and grinding them in CTAB (cetyltrimethylammonium bromide) buffer and thereafter incubating at 56 °C for 1.5 h. This method was found to work well with samples from genera *Bohadschia* and *Holothuria*. For samples from genera *Neocucumis* and *Stichopus*, the protocol of Yue and Orban (2005) – which uses Chelex® and proteinase K modified by CTAB-NaCl purification – was adopted. Tissues which were difficult to amplify were again extracted with DNeasy® kit (Qiagen) following the manufacturer's protocol. Each PCR (polymerase chain reaction) mixture had a final concentration of 1x PCR buffer, 1.0 µM of each primers, 2.5 mM $MgCl_2$, 200 µM dNTPs (deoxynucleoside triphosphates), 2.5 U *Taq* polymerase (Vivantis®), 1x bovine serum albumin, and 30–60 ng DNA template. The primers for amplification were the published echinoderm universal primers CO1-e F, 5′-ATAATGATAGGAGGRTTTGG-3′ CO1-e R, 5′-GCTCGTGTRTCTACRTCCAT-3′ (Uthicke *et al*. 2010; Borrero-Perez *et al*. 2010; Arndt *et al*. 1996). PCR profile for amplification included initial denaturation at 95°C for 60 s – followed by 40 cycles of denaturation at 95 °C for 30 s; annealing at 50 °C for 30 s, extension at 72 °C for 80 s, and final extension of 72 °C for 10 min. The amplified PCR products were quality-checked and sent to the Macrogen DNA sequencing facility, Seoul, Korea and AIT Sequencing Laboratory in Singapore for bidirectional DNA sequencing.

Analysis of Sequenced Data

Prior to analysis of forward and reverse DNA sequences, the reverse sequences were submitted to reverse complement (www.bioinformatics.org/sms/rev_comp.html), the sequence of which – together with the forward sequence – was aligned using Clustal Omega (Sievers and Higgins 2014; www.ebi.ac.uk/Tools/msa/clustalo/). The overlapping sequences were considered as the final sequence. The sequences were registered with the GenBank and the BOLD Systems under the project Cuke

DNA barcoding with the accession numbers/code ID, as stated in Appendix Table I.

The *COI* DNA sequences of about 650 bp were submitted for BLAST analysis (www.blast.ncbi.nlm.nih.gov/) to search their best matched species in the GenBank (Altschul *et al*. 1990). Sequence analysis was carried out by aligning the *COI* DNA sequences using the multiple sequence alignment Clustal Omega (Sievers and Higgins 2014; www.ebi.ac.uk/Tools/msa/clustalo).

The consensus sequence of each species of economically important Philippine sea cucumbers was generated by multiple sequence alignment of the *COI* DNA sequences of 4–12 individuals within the species conducted in MEGA 6.06 (Molecular Evolutionary Genetic Analysis) (Tamura *et al*. 2013). The *COI* DNA sequences were trimmed at both ends to obtain uniform length (449 bp) and saved in FASTA format. Displaced sequence refers to *COI* DNA sequence of a species, which is quite far from the consensus sequence as shown by phylogenetic analysis and which supports the high genetic variation within this species.

Sequence Divergence and Molecular Phylogenetic Tree

Data on sequence divergence and analyses of genetic distance within the species, genus, family, order, and class were computed using the software MEGA 6.0 (Tamura *et al*. 2013). DNA sequences of individuals belonging to the same species were aligned and computed, then all species belonging to the same genus, all genera belonging to same family, and all members of the family belonging to the same class. The study concentrated on the genetic variability within the species (conspecific variation) and genetic variability within the genus (congeneric variation).

Pairwise distances were calculated using the K2P parameter model of transition+ transversion base substitution (Kimura 1980).

For the phylogenetic analysis, the consensus sequences for each species were obtained and molecular analyses was also conducted in MEGA 6.0. Phylogenetic trees were constructed to present the graphical representation of distance matrix using the neighbor-joining method of Saitou and Nei (1987). Reference sequences were obtained from GenBank.

Establishment of DNA Barcodes

For the establishment of DNA barcodes, at least three DNA sequences of sea cucumbers of the same species were aligned by multiple sequence alignment through MEGA software and trimmed at both ends, resulting in 449 bp. The resulting output data of consensus sequence was run in SpIDER (Species Identity Evolution in R statistical package) to establish the barcodes in which each base was represented as a colored vertical line corresponding to its nucleotide [Brown *et al*. 2012; R-Forge (http://spider.r-forge.r-project.org/].

RESULTS AND DISCUSSION

Determination of *COI* Sequences of Philippine Sea Cucumbers

A total of 56 *COI* DNA sequences from 19 different species of sea cucumbers belonging to six genera were sequenced and their identity determined or confirmed through comparison with reference sequences in the GenBank (Appendix Table I). DNA sequences were also submitted to BOLD Systems and images were uploaded and their code numbers provided.

The identity of 17 out of 19 species was confirmed by > 98% identity with the reference sequences using BLAST. However, two species were found to have no *COI* reference sequences in the GenBank. The sequence of one species, identified morphologically as *Neocucumis proteus*, did not have a match with any sea cucumber in the GenBank, the highest being with *Phyllophorus brocki* at 90% identity. This indicates that the *COI* sequences for *Neocucumis proteus* reported here would be the first to be registered in the GenBank (GenBank acc. nos. MH834563–MH834574) (Appendix Table I). *Neocucumis proteus* (Lampert 1885). locally known as "Bola-bola." is an endemic sea cucumber found in the Visayan seas. It is considered as newly emerging high value sea cucumber but less documented and was not yet included in the catalog by Purcell *et al*. (2012).

Another species morphologically identified as *Holothuria albiventer* (Semper 1868) has no available *COI* DNA sequence in the GenBank. The four *Holothuria albiventer COI* DNA sequences (GenBank acc. nos. MH834586 and MH834587, two were not registered) generated from this study were only 84% identical to *Holothuria cinerascens*, 82% identical to *Holothuria erinacea*, 94% identical to *Holothuria impatiens*, and 84% identical to *Holothuria notabilis* (Appendix Table I; Table 1).

The generated *COI* DNA barcodes from 19 species of sea cucumbers collected from the Philippine seas are presented in Figure 2. The consensus sequence from individuals of the species was the assigned barcode for the particular species. The DNA barcodes generated in this study can be used as baseline information to identify cryptic, unidentified, and unique species; serve as a genetic tool to track the nearest relatives for description of new species; determine sequence divergence in the

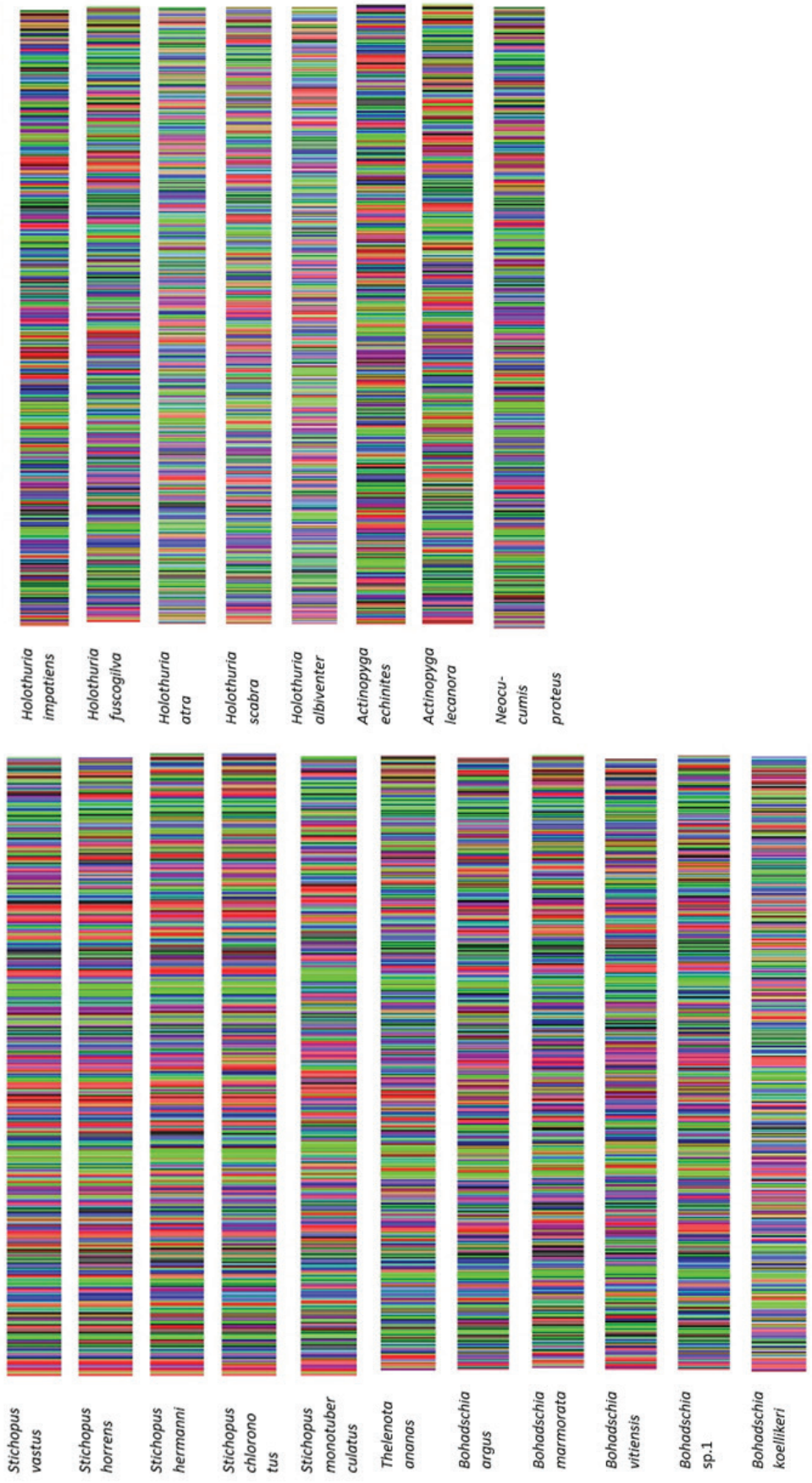

Figure 2. DNA barcodes established for economically important Philippine sea cucumbers based on consensus *COI* DNA.

morphospecies lineage; and detect correct species of commercial sea cucumber products.

Genetic Analyses

Genetic variations within and between species across Class Holothuroidea are summarized in Table 1.

Variation within species. Results show that the following had low (≤ 0.02) variations within the species: *Bohadschia marmorata*, *Bohadschia* sp. 1, *B. vitiensis*, *Holothuria fuscogilva*, *H. atra*, *Stichopus monotuberculatus, S. vastus*, and *Thelenota ananas*.

The following species had sequence variation or difference of > 0.02 to < 0.05 within the species: *Bohadschia koellikeri*, *B. argus*, *Actinopyga lecanora*, *Stichopus horrens*, and *S. hermanni.* The following species had > 0.05 to < 0.1 sequence variation within the species: *Stichopus chloronotus* and *Neocucumis proteus*. High > 0.1 sequence differences were obtained for the following: *Actinopyga echinites*, *Holothuria impatiens*, *H. scabra*, and *H. albiventer*. *A. echinites* and *H. scabra* had very high sequence variation of 0.207–0.215, respectively. These high sequence variations confirmed the high level of diversity of the family Holothuriidae, which have been the subject of debate since the early 20th century (Samyn *et al.* 2005).

Generally, DNA sequences of individuals belonging to the same species have sequence difference level of < 0.02 (Ward *et al*. 2008; Uthicke *et al*. 2010). Hoareau and Boissin (2010) reported level of divergence that exceeded 0.03 within the species level among the echinoderms, which they called morphospecies in *Actinopyga lecanora* with 0.041 level of divergence. However, according to Uthicke *et al*. (2010), species that have sequence differences of 0.02–0.05 fall under the gray area and requires further investigation.

Table 1. Genetic variation within species and genus of economically important Philippine sea cucumbers using the K2P distances.

Species name	N	Genetic variability within species	Genetic variability within genus
Order: Aspidochirotida			
Family: Holothuriidae			
Bohadschia marmorata	7	0.005 ± 0.00	0.123 ± 0.06
Bohadschia koellikeri	5	0.040 ± 0.02	
Bohadschia argus	5	0.040 ± 0.02	
Bohadschia sp 1	4	0.007 ± 0.00	
Bohadschia vitiensis	5	0.014 ± 0.006	
Actinopyga lecanora	5	0.026 ± 0.03	0.18 ± 0.14
Actinopyga echinites	5	0.207 ± 0.18	
Holothuria impatiens	10	0.153 ± 0.09	0.19 ± 0.08
Holothuria fuscogilva	7	0.018 ± 0.01	
Holothuria atra	8	0.015 ± 0.01	
Holothuria scabra	5	0.215 ± 0.08	
Holothuria albiventer	4	0.181 ± 0.01	
Family: Stichopodiidae			
Stichopus horrens	11	0.025 ± 0.02	0.071 ± 0.05
Stichopus hermanni	6	0.023 ± 0.03	
Stichopus chloronotus	5	0.057 ± 0.07	
Stichopus monotuberculatus	5	0.020 ± 0.01	
Stichopus vastus	6	0.015 ± 0.01	
Thelenota ananas	6	0.013 ± 0.01	
Order: Dendochirotida			
Family: Phyllophoriidae			
Neocucumis proteus	12		0.079 ± 0.08

Variation between species. Table 1 further shows the average sequence divergence between species within the genus computed *via* distance matrix using the K2P distances. The variation between species within a genus was 0.123 for *Bohadschia*, 0.18 for *Actinopyga*, 0.19 for *Holothuria*, and 0.071 for *Stichopus*. The value obtained for *Bohadschia* was comparable with the values obtained by Kim *et al.* (2013) of 0.111 ± 0.028 and by Uthicke *et al.* (2010) of 0.1205.

For the genus *Stichopus*, the sequence divergence between species within the genus was 0.071, lower than the 0.1002 from the previous study of Uthicke *et al*. (2010) and 0.093 from Byrne *et al*. (2010). The genus *Actinopyga* had higher sequence divergence of 0.18 in this study compared to 0.15 in the study of Uthicke *et al*. (2010). The genus *Holothuria* had the highest sequence variation of 0.19, which is similar to 0.169 obtained by Uthicke *et al*. (2010).

The species of the genus *Neocucumis* has not yet been studied and documented and/or barcoded. Similarly, the genus *Thelenota* had not been well-studied, perhaps because members of this species are rare and critically endangered. Sea cucumbers of the genus *Thelenota* are fished but are considered threatened species and included in the red list of the IUCN. Recent *COI* DNA sequences of this genus in the Genbank includes 10 sequences of *Thelenota ananas*, six sequences of *Thelenota anax*, and one sequence of *Thelenota rubralineata*.

The genetic distance of 0.071–0.123 within the genus level reported in this study is within the range of 0.036–0.211 (average, 0.154) for all species across echinoderms (Ward *et al*. 2008), 0.102–0.186 (average, 0.169) within Holothuroidea (Uthicke *et al*. 2010), and 0.158–0.248 (average, 0.206) among echinoderms in the study of Hoareau and Boissin (2010).

The problem of cryptic species was confirmed by high sequence divergence within the species of *Actinopyga echinites*, *Holothuria impatiens*, *H. scabra*, *H. albiventer*, *Stichopus chloronotus*, and *Neocucumis proteus* (Table 1). Michonneau (2015) reported a variation of 0.153 for the cryptic and not-so-cryptic species in the complex *Holothuria* (*Thymiosycia*) *impatiens*. Uthicke *et al.* (2010) also reported that cryptic species may occur in *H. atra*, as shown by high average genetic distance and high variance of the mean (average: 1.8%, SD: 1.1%; N = 77) and three clades in neighbor-joining analysis of *COI* sequences.

High genetic difference between and among individuals was reported by Uthicke and Benzie (2003) for *Holothuria nobilis* with sequence divergence of > 0.07, compared with 0.045 for *Holothuria atra* (Uthicke *et al*. 2010).

Genetic distances. Genetic distances between genera of sea cucumbers computed using the K2P distance are presented in Table 2 and ranged from 0.187–0.250. The lowest genetic distance of 0.187 was the K2P between the genera *Bohadschia* and *Neocucumis* while the highest genetic distance of 0.250 was between the genera *Neocucumis* and *Stichopus.*

The results of genetic distance of species across the Class Holothuroidea are presented in Appendix Table II, as computed using the K2P parameter. In this study, the variation for the genus *Stichopus* ranged from 0.030–0.154 with the lowest sequence divergence between *Stichopus vastus* and *S. hermanni* and the highest divergence between *S. hermanni* and *S. chloronotus*, similar to the results of Byrne *et al*. (2010). In the genus *Bohadschia,* the lowest sequence divergence of 0.090 between *Bohadschia argus* and *Bohadschia* sp. 1 was observed, similar to the results of Kim *et al*. (2013); the highest divergence of 0.162 was both observed between *B. argus* and *B. marmorata*, and between *Bohadschia* sp. 1 and *B. marmorata.* The lowest distance was 0.007 between *Holothuria atra* and *H. scabra* and the highest distance was 0.283 between *Stichopus chloronotus* and *Bohadschia vitiensis*, as also shown in the phylogenetic tree (Figure 3).

Phylogenetic Analysis

Neighbor-joining tree of different species. The phylogenetic tree of the different species of economically important sea cucumbers in the Philippines and reference sequences from GenBank constructed by the neighbor-joining method in MEGA 6.0 is presented in Figure 3, with sea urchin *Strongylocentrotus purpuratus* as the outgroup.

Table 2. Genetic distance of *COI* DNA sequences of economically important Philippine sea cucumbers compared per genera based on K2P distances.

Group	*Neocucumis*	*Stichopus*	*Bohadschia*	*Holothuria*	*Actinopyga*	*Thelenota*
Neocucumis						
Stichopus	0.25					
Bohadschia	0.187	0.220				
Holothuria	0.213	0.243	0.217			
Actinopyga	0.199	0.239	0.206	0.213		
Thelenota	0.234	0.240	0.229	0.219	0.22	

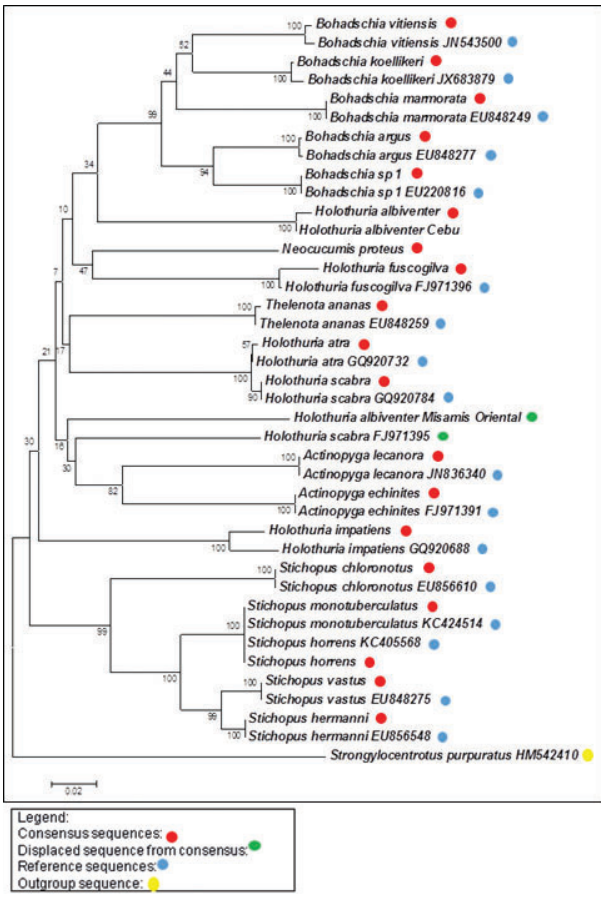

Figure 3. Phylogenetic tree of Philippine sea cucumbers generated in MEGA 6.0 using the neighbor-joining method and inferred using 1,000 bootstraps. The sea urchin *Strongylocentrotus purpuratus* was used as outgroup. Numbers on the clades indicate bootstrap support.

In general, species belonging to the same genera grouped together; these are *Bohadschia*, *Actinopyga*, *Neocucumis*, *Thelenota*, and *Stichopus*. On the other hand, the genus *Holothuria* formed paraphyletic clades. *Holuthuria albiventer*, *H. scabra*, *H. fuscogilva*, *H. atra*, and *H. impatiens* were in different clades. Even the two *H. scabra* reference sequences were found in different clades. *H. albiventer* (consensus and *H. albiventer* Cebu) interestingly formed a clade with the genus *Bohadschia*. However, *H. albiventer* Misamis Oriental (representing a displaced sequence) and *H. scabra FJ971395* (a reference sequence and displaced sequence) were in a separate clade with *Actinopyga* (Figure 3). These indicate and support the high genetic variation within and the crypticity of the species of *H. albiventer* and *H. scabra* shown in Table 1 and discussed above.

The genus *Holothuria* is known to be a morphologically diverse member of family Holothuridae with around 150 species and has a complex history in nomenclature (Samyn *et al*. 2005). Morphologically, this genus is considered complex with the presence of different types of ossicles or exoskeletons (Massin *et al*. 2000) and scientists have subdivided it into 18 subgenera for classifications (Samyn *et al*. 2005; Kerr *et al.* 2005).

Neocucumis proteus, whose *COI* sequence was first reported in this paper, formed a clade with *Holuthuria fuscogilva* but separate from other *Holuthuria*.

With the genus *Stichopus*, *Stichopus chloronotus* deviated from the others – similar to the observation of Uthicke *et al.* (2010) – since, based on its reproductive biology, it can reproduce both sexually and asexually. On the other hand, those that clustered together –*Stichopus horrens* and *S. monotuberculatus*, and *S. vastus* and *S. hermanni* – reproduce asexually by fission and sexually, respectively (Purcell *et al*. 2012).

Hoareau and Boissin (2010) observed that *Holothuria scabra* was found to be within the clade of *Holothuria fuscopunctata* while in this study, species belonging to genus *Holothuria* produced paraphyly as it clustered with other genera. This study also corroborates with the paraphyletic clades of the genus *Holothuria* from the phylogenetic tree of Samyn *et al*. (2005) using morphological characters, Kerr *et al*. (2005) based on *16S rRNA* gene, and Borrero-Perez *et al*. (2010) using *COI* or *16S*.

Neighbor-joining tree of individual specimens of species with high conspecific genetic variation. Expectedly, the specimens of species found to have high conspecific genetic variation, *Holothuria albiventer*, *H. impatiens*, *H.*

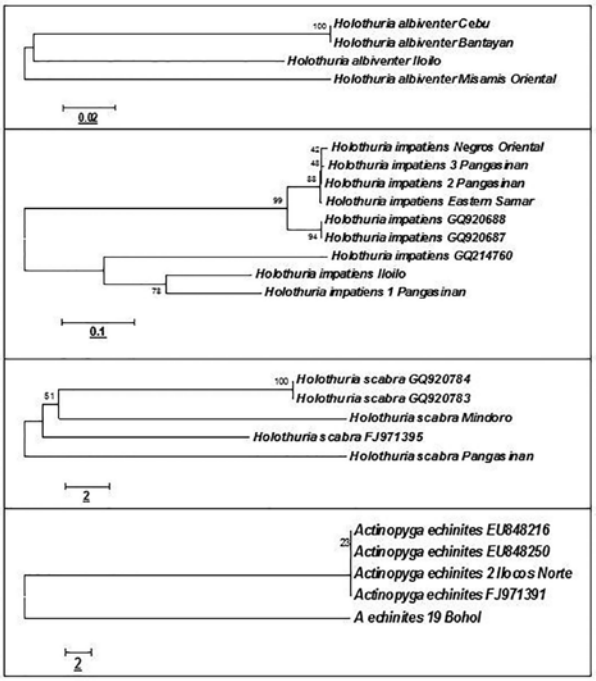

Figure 4. Neighbor-joining tree of *Holothuria albiventer*, *Holothuria impatiens*, *Holothuria scabra*, and *Actinopyga echinites*. Numbers on the clades indicate bootstrap support.

scabra, and *Actinopyga echinites* (0.153–0.215, Table 1) – separated in two or even three clades (Figure 4). These further confirm *Holothuria*'s complex species variations.

The *COI* sequence of *Actinopyga echinites* collected from Ilocos Norte clustered with the three reference sequences, implying that these four sequences are highly similar. On the other hand, *A. echinites* collected from Bohol separated from the cluster, suggesting that its *COI* sequence show considerable difference to those of the other four sequences.

The high genetic variation between the *A. echinites* from Bohol and from Ilocos Norte might be due to the geophysical isolation of the two populations (Figure 1). In New Caledonia, Uthicke and Purcell (2004) observed that *Holothuria scabra* populations at two sites with limited water exchange in the southern location were significantly different at three other locations on the west coast. Furthermore, they observed that Roger's genetic distance values between populations were significantly related to geographic distances. Similarly, Lizano (2017) observed that populations of *Stichopus cf. horrens* located in the center of the Philippine archipelago are characterized by higher levels of genetic diversity and connectivity relative to peripheral populations from the South China Sea, Sulu Sea, Celebes Sea, and Philippine Sea sites, which constituted genetically distinct groups.

Uthicke *et al.* (2010) observed that *Holothuria atra* had three clades existing within this species, which had an average of 0.0185 conspecific variation and highest of 0.045, indicating that cryptic species may occur in *H. atra*.

Nucleotide Frequency in the *COI* Gene

Detailed analysis of base composition of each species of economically important Philippine sea cucumbers was undertaken to assess the pattern of genetic variation used in construction of the phylogenetic tree. The average base frequencies for the entire 449 data set of *COI* gene generated from the 19 Philippine sea cucumbers were: A, 28.0%; C, 25.3%; T, 28.4%; G, 18.3%; and GC, 43.6% (Appendix Table III). In this study, the summary of the GC content per genera is as follows: *Stichopus*, 42.76%; *Thelenota*, 45.8%; *Holothuria*, 44.58%; *Bohadschia*, 44.38%; *Actinopyga*, 39.95%; and *Neocucumis*, 44.1%. In the study of Arndt *et al*. (1996), the GC content of the *COI* gene of Eastern Pacific sea cucumbers averaged 42% and ranged from 34.7% in *Pseudostichopus mollis* to 43.5% in *Cucumaria miniata.*

Majority of variable sites was found in the third codon position with bias against guanine (G) because of the codon degeneracy. The proportion of G, as shown in Appendix Table III, is low – as was also reported for numerous organisms, including a number of other echinoderms (Arndt *et al*. 1996) – with a corresponding increase in adenine (A) and thymine (T). It is of interest that the echinoderm mtDNA genetic code utilizes only ATG for methionine and AAG for lysine. The effect of the bias against G was further reflected in the codon usage in the *COI* sequence of sea cucumbers pioneered by Arndt *et al*. (1996).

Base composition and percent GC content in the *COI* gene of sea cucumbers from this study will contribute to data previously analyzed by Arndt *et al*. (1996) on sea cucumbers from the Eastern Pacific Ocean, which were mostly from Order Dendochirotida. The sea cucumbers from the Philippines in this study were from the western edge of the Pacific Ocean (Figure 1) and were mostly from the Order Aspidochirotida.

CONCLUSION

The *COI* DNA barcodes for 19 species of economically important sea cucumbers collected from 16 different geographical locations along the Philippine archipelago were established. Of the 19 species of sea cucumbers, eight species had 0.005–0.02 sequence differences, five species had > 0.02 to < 0.05, two species had > 0.05 to < 0.1, and four had very high sequence differences > 0.1. The sea cucumbers with high sequence differences were mostly from the family Holothuriidae in the genera *Actinopyga* and *Holothuria*, some of which are considered as species complex when examined morphologically and supported by molecular data in this study. This pioneering study was able to generate two new sea cucumber COI DNA sequences and barcodes for the Philippine endemic *Neocucumis proteus* and *Holothuria albiventer*.

ACKNOWLEDGMENTS

The authors acknowledge the scholarship under the Department of Science and Technology – Science Education Institute's Accelerated Science and Technology Human Resource Development Program through PCAARRD; the Junior Research Fellowship Grant (DA Biotech F1118) from the Department of Agriculture's Biotechnology Program; and a grant from the UP Los Baños Graduate School Graduate Mentoring and Apprenticeship Program to the first author (MNAC) – as well as the support of the Biochemistry Laboratory, Institute of Plant Breeding, College of Agriculture and Food Science, UP Los Baños. The assistance of the following researchers who helped in identifying and providing the specimens used in this study is very much appreciated: Christine Edullantes and Faith Paran; Victor Sanidad; Vicky Malaya; Veronica Grande; Helen Bangui;

Rudolph Balisco and Jeanbeth Jontilla; Frances Nievales; Marius Panahon; Corazon Batoy; Romy Billones and Ruby Tizon; Serapion Tanduyan; Venus Bantoto; and Mariefe Quiñones. We also thank sincerely the UP – Marine Science Institute for the workshop, training, and support for this study.

AUTHORS' CONTRIBUTIONS

MNAC and EMTM conceptualized and designed the study. MNAC performed the experiments under the supervision of RNG. All authors participated in the analysis and discussion of the results. MGQD, RNG, PPO, and ACL provided technical advice on various aspects of the study. MNAC and EMTM wrote the paper. MNAC, EMTM, MGQD, and RNG actively contributed to the revision of the paper. All authors approved the paper for publication.

STATEMENT ON CONFLICT OF INTEREST

The authors declare no conflict of interest with any financial, personal, or other relationships with people or organizations related to the material discussed in the manuscript.

NOTE ON APPENDICES

The complete appendices section of the study is accessible at http://philjournsci.dost.gov.ph

REFERENCES

ALTSCHUL SF, GISH W, MILLER W, MYERS EW, LIPMAN DJ. 1990. Basic Local Alignment Search Tool. J Mol Biol 215: 403–410.

ARNDT A, MARQUEZ C, LAMBERT P, SMITH MJ. 1996. Molecular phylogeny of Eastern Pacific sea cucumbers (Echinodermata: Holothuroidea) based on mitochondrial DNA sequences. Molecular Phylogenetics and Evolution 6: 425–437.

[BFAR] Bureau of Fisheries and Aquatic Resources. 2015. Philippine Fisheries Profile 2015. Department of Agriculture, Quezon City, Philippines.

[BFAR] Bureau of Fisheries and Aquatic Resources. 2017. Philippine Fisheries Profile 2017. Department of Agriculture, Quezon City, Philippines.

BORDBAR S, ANWAR F, SAARI N. 2011. High value components and bioactives from sea cucumber for functional foods: a review. Marine Drugs 9: 1761–1805.

BORRERO-PEREZ GH, GONZALEZ-WANGUEMERT M, MARCOS C, PEREZ-RUZAFA A. 2011. Phylogeography of the Atlanto-Mediterranean sea cucumber *Holothuria* (*Holothuria*) *mammata*: the combined effects of historical processes and current oceanographical pattern. Molecular Ecology 20 (9): 1964–1975.

BORRERO-PEREZ GH, GOMEZ-ZURITA J, GONZALEZ-WANGUEBERT M, MARCOS C, PEREZ-RUZAFA A. 2010. Molecular systematics of the genus *Holothuria* in the Mediterranean and Northeastern Atlantic and a molecular clock for the diversification of the Holothuriidae (Echinodermata: Holothuroidea). Molecular Phylogenetics and Evolution 57: 899–906.

BROWN SDJ, COLLINS RA, BOYER S, LEFORT MC, MALUMBRES-OLARTE J, VINK C, CRUICKSHANK RH. 2012. SPIDER: An R package for the analysis of species identity and evolution, with particular reference to DNA barcoding. Molecular Ecology Resources 12: 562–565.

BYRNE M, ROWE F, UTHICKE S. 2010. Molecular taxonomy, phylogeny and evolution in the family Stichopodidae (Aspidochirotida: Holothuroidea) based on COI and 16S mitochondrial DNA. Molecular Phylogenetics and Evolution 56: 1068–1081.

CARPENTER KE, SPRINGER VG. 2005. The center of the center of marine shore fish biodiversity: the Philippine Islands. Environmental Biology of Fishes 72: 467–480.

CHOO PS. 2008. The Philippines: a hotspot of sea cucumber fishery in Asia. In: Sea cucumbers: a global review of fisheries and trade. Toral-Granda V, Lovatelli A, Vasconcellos M eds. FAO Fisheries and Aquaculture Technical Paper No 516. p. 119–140.

CUROLE JP, KOCHER TD. 1999. Mitogenomics: digging deeper with complete mitochondrial genomes. Trends Ecology & Evolution 14: 394–398.

DOLOROSA RG. 2015. The sea cucumbers of Tubbataha Reefs Natural Park, Philippines. SPC Beche-de-Mer Information Bulletin 35: 10–17.

DOLOROSA RG, SALAZAR CB, DELFIN MTV, PADUGA JR, BALISCO RAT. 2017. Sea cucumber fisheries in Rasa Island Wildlife Sanctuary, Narra, Palawan, Philippines. SPC Beche-de-mer Information Bulletin 37: 9–20.

DOMANTAY JS. 1957. The Philippine Commercial Holothurians. The Philippine Journal of Commerce 10: 5–7.

DOYLE JJ, DOYLE JL. 1990. Isolation of plant DNA from fresh tissue. Focus 12: 13–15.

FAN SG, HA CQ, WEN J, ZHANG LP. 2011. Characterization of mitochondrial genome of sea cucumber Stichopus horrens: a novel gene rearrangement in Holothuroidea. Sci China Life Sci 54: 434–441.

GALIMBERTI A, DE MATTIA F, LOSA A, BRUNI I, FEDERICI S, CASIRAGHI M, MARTELLOS S, LABRA M. 2013. DNA barcoding as a new tool for food traceability. Food Research International 50: 55–63.

GAMBOA R, GOMEZ A, NIEVALES MF. 2004. The status of sea cucumber fishery and mariculture in the Philippines. In: The sea cucumber *Holothuria scabra* (Holothuroidea: Echinodermata): its biology and exploitation as bêche-de-mer. Lovatelli A, Conand C, Hamel J, Conand C, Pawson Dl, Mercier A eds. Adv Mar Biol 41: 130–223.

HAMEL J-F, CONAND C, PAWSON D, MERCIER A. 2001. The sea cucumber *Holothuria scabra* (Holothuroidea: Echinodermata). Advances in Marine Biology 41: 129–223. 10.1016/S0065-2881(01)41003-0.

HEBERT PDN, CYWINSKA A, BALL SL, DE WAARD JR. 2003. Biological identification through DNA barcodes. Proceedings of the Royal Society of London Series B 270: 313–322.

HOAREAU TB, BOISSIN E. 2010. Design of phylum-specific hybrid primers for DNA barcoding: addressing the need for efficient COI amplification in Echinodermata. Molecular Ecology Resources 10: 960–967.

JONTILA JBS, BALISCO RAT, MATILLANO JB. 2014. The sea cucumbers (Holothuroidea) of Palawan, Philippines, Aquaculture, Aquarium, Conservation and Legislation International Journal of the Bioflux Society 7: 194–206.

JUINIO-MENEZ MA, SAMONTE PC. 2016. Harnessing S&T for a sustainable and competitive sea cucumber industry. Retrieved on 12 Jan 2019 from http://www.pcaarrd.dost.gov.ph

KERR AM, JANIES DA, CLOUSE RM, SAMYN Y, KUSZAK J, KIM J. 2005. Molecular phylogeny of coral-reef sea cucumbers (Holothuriidae: Aspidochirotida) based on 16S mitochondrial ribosomal DNA sequence. Marine Biotechnology 7: 53–60.

KIM SW, KERR AM, PAULAY G. 2013. Colour, confusion and crossing of species problems in *Bohadschia* (Echinodermata: Holothuroidea). Zoological Journal of the Linnean Society 168: 81–97.

KIMURA M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16: 111–120.

LAMPERT K. 1885. Die Seewalzen; Holothurioidea; Eine systematische Monographie. In: Reisen im Archipel der Philippinen, Teil 2. Semper C ed. Wissenschaftliche Resultat 4: 1–310.

LIZANO AM. 2017. Genetic diversity and population genetic structure among Phillipine populations of *Stichopus* cf. Horrens [M.S. Thesis]. University of the Philippines Diliman, Quezon City.

MASSIN C, MERCIER A, HAMEL JF. 2000. Ossicle change in *Holothuria scabra* with a discussion of ossicle evolution within the Holothuriidae (Echinodermata). Acta Zoologica (Stockholm) 81: 77–89.

MICHONNEAU F. 2015. Cryptic and not-so-cryptic species in the complex *Holothuria* (*Thymiosycia*) *impatiens* (Forsskal, 1775) (Echinodermata: Holothuroidea). bioRxiv (The preprint server for biology). http://dx.doi.org/10.1101/014225.

MICHONNEAU F, PAULAY G. 2014. Revision of the genus *Phyrella* (Holothurodiea: Dendrochirotida) with the description of a new species from Guam. Zootaxa 3760: 101–140.

NICOLE S, NEGRISOLO E, ECCHER G, MANTOVANTI R, PATANELLO T, ERICKSON DL, KRESS WJ, BARCACCIA G. 2012. DNA barcoding as a reliable method for the authentication of commercial seafood products. Food Technology and Biotechnology 50: 387–398.

PURCELL SW, SAMYN Y, CONAND C. 2012. Commercially important sea cucumbers in the world. FAO Species Catalogue for Fishery Purposes No. 6. Rome: FAO. 180p.

PURCELL SW. 2014. Value, market preferences, and trade of Bêche-de-Mer from Pacific Island sea cucumbers. PLOS ONE 9(4): e95075.

SAITOU N, NEI M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406–425.

SAMYN Y, APPELTANS W, KERR AM. 2005. Phylogeny of Labidodemas and the Holothuriidae (Holothuroidea: Aspidochirotida) as inferred from morphology. Zoological Journal of the Linnean Society 144: 103–120.

SAMYN Y, SMIRNOV A, MASSIN C. 2013. Carl Gottfried Semper (1832–1893) and the location of his type specimens of sea cucumbers. Archives of Natural History 40: 324–339.

SEALE A. 1911. The Fishery Resources of the Philippine Islands, Part IV. Miscellaneous Marine Products. The

Philippine Journal of Science 8(6): 384–320.

SEMPER C. 1868. Reisen im Archipel der Philippinen. Holothurien. 2. Wissenschaftliche Resultate i–x, 1–288, pls. 1–40.

SIEVERS F, HIGGINS DG. 2014. Clustal Omega, accurate alignment of very large numbers of sequences. Methods Mol Biol 1079: 105–116. Doi:10.1007/978-1-62703-646-7_6.

TAMURA K, STECHER G, PETERSON D, FILIPSKI A, NEI M KUMAR S. 2013. MEGA 6: Molecular Evolutionary Genetic Analyses version 6. Molecular Biology and Evolution. 30: 2725–2729.

UTHICKE S, BENZIE JAH. 2003. Gene flow and population history in high dispersal marine invertebrates: mitochondrial DNA analysis of *Holothuria nobilis* (Echinodermata: Holothuroidea) populations from Indo-Pacific. Molecular Ecology 12: 2635–2648.

UTHICKE S, PURCELL S. 2004. Preservation of genetic diversity in restocking of sea cucumber *Holothuria scabra* investigated by allozyme electrophoresis. Can J Fish Aquat Sci 61: 519–528.

UTHICKE S, PURCELL S, BLOCKMANS B. 2005. Natural hybridization does not dissolve species boundaries in commercially important sea cucumbers. Biological Journal of the Linnean Society 85: 261–270.

UTHICKE S, BYRNE M, CONAND C. 2010. Genetic barcoding of commercial Bêche-de-mer species (Echinodermata: Holothuroidea). Molecular Ecology Resources 10: 634–646.

WARD RD, HOLMES BH, O'HARA TD. 2008. DNA barcoding discriminates echinoderm species. Molecular Ecology Resources 8: 1202–1211.

YUE GH, ORBAN L. 2005. A simple and affordable method for high-throughput extraction from animal tissues for polymerase chain reaction. Electrophoresis 16: 3081–3083.

APPENDICES

Appendix Table I. List of economically important Philippine sea cucumbers collected and identified by collaborators and used in this study.

Species name	Province	Agency	Collector	GenBank accession no.	BOLD ID
Order: Dendrochirotida					
Family: Phylophoriidae					
1. *Neocucumis proteus* 1	Iloilo	NIPSC	RT	MH834563	BECHE049-20
2. *Neocucumis proteus* 2	Iloilo	NIPSC	RT	MH834566	BECHE050-20
3. *Neocucumis proteus* 3	Iloilo	NIPSC	RT	MH834564	BECHE051-20
4. *Neocucumis proteus* 4	Iloilo	NIPSC	RT	MH834565	BECHE052-20
5. *Neocucumis proteus* 49	Iloilo	UPV	FN	MH834567	BECHE053-20
6. *Neocucumis proteus* 56	Iloilo	UPV	FN	MH834568	BECHE054-20
7. *Neocucumis proteus* 57	Iloilo	UPV	FN	MH834569	BECHE055-20
8. *Neocucumis proteus* 59	Iloilo	UPV	FN	MH834570	BECHE056-20
9. *Neocucumis proteus* 64	Iloilo	UPV	FN	MH834571	BECHE057-20
10. *Neocucumis proteus* 30	Bohol	HNU	CB	MH834572	BECHE058-20
11. *Neocucumis proteus* 2	Cebu	CTU	ST	MH834573	BECHE059-20
12. *Neocucumis proteus* 3	Cebu	CTU	ST	MH834574	BECHE060-20
Order: Aspidochirotida					
Family: Stichopodidae					
1. *Stichopus horrens*	Ilocos Norte	MMSU	VG	MH834561	BECHE047-20
2. *Stichopus horrens* gray	Ilocos Sur	ISPSC	VS	MH834562	BECHE048-20
3. *Stichopus horrens*	Pangasinan	UPMSI	TE	MH834556	BECHE042-20
4. *Stichopus horrens* 1	Cagayan	UPMSI	FP	MH834558	BECHE044-20
5. *Stichopus horrens* 10	Cagayan	UPMSI	FP	MH834559	BECHE045-20
6. *Stichopus horrens* 3	Palawan	WPU	RB	MH834560	BECHE046-20
7. *Stichopus horrens*	Cagayan	CSU	HB	MH834557	BECHE043-20

8. *Stichopus hermanii*	Iloilo	NIPSC	RB	MH834551	BECHE037-20
9. *Stichopus chloronotus*	Ilocos Sur	ISPSC	VS	MH834550	BECHE036-20
10. *Stichopus vastus*	Palawan	WPU	RB	MH834552	BECHE038-20
11. *Stichopus monotuberculatus* 1	Ilocos Sur	ISPSC	VS	MH834553	BECHE039-20
12. *Stichopus monotuberculatus* 2	Ilocos Sur	ISPSC	VS	MH834554	BECHE040-20
13. *Stichopus monotuberculatus*	La Union	DMMMSU	VM	MH834555	BECHE041-20
14. *Thelenota ananas*	Palawan	WPU	RB	MH834593	BECHE035-20
Family: Holothuriidae					
1. *Bohadschia marmorata* 12	Palawan	WPU	JJ	MH834543	BECHE006-20
2. *Bohadschia marmorata* 15	Palawan	WPU	JJ	MH834542	BECHE005-20
3. *Bohadschia marmorata* 16	Palawan	WPU	JJ	MH834541	BECHE004-20
4. *Bohadschia marmorata*	Ilocos Sur	ISPSC	VS	MH834545	BECHE008-20
5. *Bohadschia marmorata*	Misamis Or.	MSU	MQ	MH834540	BECHE003-20
6. *Bohadschia marmorata*	Cagayan	CSU	HB	MH834544	BECHE007-20
7. *Bohadschia koellikeri*	Ilocos Sur	ISPSC	VS	MH834546	BECHE009-20
8. *Bohadschia vitiensis*	Ilocos Sur	ISPSC	VS	MH834547	BECHE010-20
9. *Bohadschia vitiensis*	Misamis Or.	MSU	MQ	MH834548	BECHE011-20
10. *Bohadschia sp*. 1	Pangasinan	UPMSI	CE	MH834549	BECHE012-20
11. *Bohadschia argus*	Ilocos Sur	ISPSC	VS	MH834538	BECHE001-20
12. *Bohadschia argus*	Misamis Or.	MSU	MQ	MH834539	BECHE002-20
13. *Actinopyga lecanora* 1	Ilocos Sur	ISPSC	VS	MH834590	BECHE013-20
14. *Actinopyga lecanora* 20	Ilocos Sur	ISPSC	VS	MH834591	BECHE014-20
15. *Actinopyga echinites* 19	Bohol	HNU	CB	MH834592	BECHE017-20
16.*Holothuria impatiens*	Eastern Samar	UPMSI	FP	MH834580	BECHE023-20
17. *Holothuria impatiens* 1	Pangasinan	UPMSI	CE	MH834577	BECHE020-20
18. *Holothuria impatiens* 2	Pangasinan	UPMSI	CE	MH834578	BECHE021-20
19. *Holothuria impatiens* 3	Pangasinan	UPMSI	CE	MH834579	BECHE022-20
20. *Holothuria impatiens*	Iloilo	NIPSC	RB	MH834576	BECHE019-20
21. *Holothuria impatiens* 1	Negros Oriental	NORSU	VB	MH834581	BECHE024-20
22. *Holothuria impatiens* 2	Negros Oriental	NORSU	VB	MH834575	BECHE025-20
23. *Holothuria albiventer*	Misamis Or.	MSU	MQ	MH834586	BECHE029-20
24. *Holothuria albiventer*	Cebu	CTU	ST	MH834587	BECHE030-20
25. *Holothuria fuscogilva*	Misamis Or.	MSU	MQ	MH834582	BECHE025-20
26. *Holothuria fuscogilva*	Bohol	HNU	CB	MH834583	BECHE026-20
27. *Holothuria fuscogilva*	Cebu	CTU	ST	MH834584	BECHE027-20
28. *Holothuria scabra*	Mindoro	CCG	MP	MH834588	BECHE029-20
29. *Holothuria scabra*	Pangasinan	UPMSI	FP	MH834589	BECHE030-20
30. *Holothuria atra*	Palawan	WPU	RB	MH834585	BECHE028-20

Collaborators: UPMSI – University of the Philippines Marine Science Institute, Bolinao, Pangasinan; **CSU** – Cagayan State University, Aparri, Cagayan; **ISPSC**- Ilocos Sur Polytechnic State College, Narvacan, Ilocos Sur, **MMSU** – Mariano Marcos State University, Currimao, Ilocos Norte; **DMMMSU** – Don Mariano M. Marcos State University, Sto. Tomas, La Union; **WPU** – Western Philippines University, Puerto Princessa City, Palawan; **CCG** – Calapan City Government, Calapan City, Mindoro Oriental; **HNU** – Holy Name University, Tagbilaran City, Bohol; **CTU** – Cebu Technological University, Camotes Island, Cebu; **UPV** – University of the Philippines Visayas, Miag-ao, Iloilo; **NIPSC** – Northern Iloilo Polytechnic State College, Concepcion, Iloilo; **NORSU** – Negros Oriental State University, Dumaguete City, Negros Oriental; **MSU** – Mindanao State University, Naawan, Misamis Oriental; and **MSU** – Mindanao State University, Bongao, Tawi-Tawi.

Collectors: CE – Christine Edullantes and **FP** – Faith Paran (UPMSI); **VS** – Victor Sanidad (ISPSC); **VM** – Vicky Malaya (DMMMSU); **VG** – Veronica Grande (MMMSU); **HB** – Helen Bangui (CSU); **RB** – Rudolph Balisco and **JJ** – Jeanbeth Jontilla (WPU); **FN** – Frances Nievales (UPV); **MP** – Marius Panahon (CCG); **CB** – Corazon Batoy (HNU); **RB** – Romy Billones and **RT** – Ruby Tizon (NIPSC); **ST** – Serapion Tanduyan (CTU); **VB** – Venus Bantoto (NORSU); **MQ** – Mariefe Quiñones (MSU)

Numbers in the species name refer to the reference individual species identified by the collectors.

Appendix Table II. Genetic variation of *COI* DNA sequences of economically important Philippine sea cucumbers compared per species based on K2P-distances.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1 *Neocucumis proteus*		.027	.031	.028	.028	.025	.023	.026	.024	.024	.023	.023	.023	.024	.025	.024	.023	.025	.027
2 *Stichopus horrens*	.238		.015	.009	.014	.021	.025	.027	.028	.026	.026	.027	.028	.026	.026	.025	.026	.026	.029
3 *Stichopus vastus*	.268	.087		015	.008	.023	.028	.028	.029	.028	.026	.028	.031	.026	.026	.026	.028	.027	.029
4 *Stichopus monotuberculatus*	.257	.032	.079		.013	.020	.025	.029	.028	.027	.027	.028	.029	.026	.027	.026	.025	.026	.029
5 *Stichopus hermanni*	.244	.079	.030	.066		.022	.027	.027	.027	.028	.026	.028	.031	.027	.026	.026	.027	.027	.027
6 *Stichopus. chloronotus*	.232	.145	.169	.140	.154		.025	.030	.031	.029	.028	.027	.028	.025	.027	.028	.026	.026	.025
7 *Thelonata ananas*	.187	.211	.236	.205	.223	.217		.026	.025	.024	.026	.023	.026	.023	.025	.024	.026	.026	.025
8 *Bohadschia marmorata*	.236	.229	.232	.244	.222	.269	.230		.021	.023	.020	.022	.027	.025	.029	.025	.024	.026	.027
9 *Bohadschia vitiensis*	.207	.239	.246	.242	.232	.283	.217	.149		.020	.017	.020	.026	.022	.029	.022	.024	.024	.027
10 *Bohadschia argus*	.219	.232	.242	.241	.238	.263	.207	.162	.131		.020	.015	.026	.024	.027	.024	.025	.024	.024
11 *Bohadschia koellikeri*	.195	.226	.226	.236	.220	.253	.229	.129	.108	.143		.020	.024	.022	.026	.022	.023	.023	.025
12 *Bohadschia sp.* 1	.207	.238	.242	.248	.238	.244	.202	.162	.127	.090	.133		.025	.024	.027	.025	.024	.024	.023
13*Holothuria fuscogilva*	.190	.249	.282	.259	.275	.262	.219	.238	.213	.242	.213	.223		.026	.025	.026	.025	.027	.028
14 *Holothuria atra*	.190	.233	.227	.230	.220	.244	.187	.211	.175	.196	.182	.203	.219		.024	.004	.027	.024	.024
15 *Holothuria. impatiens*	.220	.223	.222	.223	.217	.238	.210	.262	.261	.241	.238	.232	.219	.208		.025	.028	.027	.025
16 *Holothuria scabra*	.193	.236	.230	.233	.223	.248	.190	.208	.178	.193	.179	.206	.222	.007	.211		.027	.024	.024
17 *Holothuria albiventer*	.201	.217	.243	.226	.233	.241	.225	.200	.202	.214	.205	.205	.219	.233	.254	.236		.024	.025
18 *Actinopyga lecanora*	.231	.234	.244	.234	.241	.234	.233	.228	.211	.211	.193	.221	.239	.208	.255	.205	.206		.023
19 *Actinopyga echinites*	.238	.260	.254	.250	.241	.219	.225	.240	.245	.217	.223	.205	.256	.205	.214	.208	.226	.170	

Legend: lower diagonal – genetic variation
upper diagonal – standard error

Appendix Table III. Nucleotide base composition in 449 bp *COI* gene of economically important Philippine sea cucumbers.

Species name	Nucleotide frequencies (%)				
	T	C	A	G	GC
1. *Neocucumis proteus*	25.8	27.8	30.1	16.3	44.1
2. *Stichopus horrens*	31.2	24.3	25.2	19.4	43.7
3. *Stichopus vastus*	33.2	22.0	24.9	19.8	41.8
4. *Stichopus monotuberculatus*	30.7	24.7	25.6	18.9	44.5
5. *Stichopus hermanni*	32.5	23.2	25.6	18.7	41.9
6. *Stichopus chloronotus*	31.4	23.6	26.7	18.3	41.9
7. *Thelenota ananas*	24.9	27.8	29.2	18.0	45.8
8. *Bohadschia marmorata*	28.3	25.6	26.3	19.8	45.4
9. *Bohadschia vitiensis*	27.6	26.5	28.5	17.4	43.9
10. *Bohadschia argus*	27.6	26.1	27.6	18.7	44.8
11. *Bohadschia koellikeri*	28.5	25.4	28.7	17.4	42.8
12. *Bohadschia* sp. *1*	26.5	26.7	28.5	18.3	45.0
13. *Holothuria fuscogilva*	24.9	27.8	28.7	18.5	46.3
14. *Holothuria atra*	25.2	27.4	29.8	17.6	45.0
15. *Holothuria impatiens*	26.5	26.3	28.5	18.7	45.0
16. *Holothuria scabra*	25.2	26.9	30.1	17.8	44.7
17. *Holothuria albiventer*	29.2	23.6	29.4	17.8	41.9
18. *Actinopyga lecanora*	30.3	21.4	31.2	17.1	38.5
19. *Actinopyga echinites*	29.2	23.6	29.4	17.8	41.4
Average	**28.4**	**25.3**	**28.0**	**18.3**	**43.6**