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MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF Isostichopus sp. IN THE COLOMBIAN CARIBBEAN SEA

CARACTERIZACIÓN MORFOLÓGICA Y MOLECULAR DE Isostichopus sp. EN EL MAR CARIBE COLOMBIANO

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ABSTRACT

Isostichopus sp. (Aspidochirotida: Stichopodidae) are sea cucumbers widely distributed in the Caribbean Sea. Amongst them, *Isostichopus badionotus* is one of the most harvested species. It shows a wide range of morphotypes widespread in the Caribbean region including different habitats (muddy, sandy and rocky bottoms, and sea grass beds). In Colombia, three morphotypes can be distinguished; two of them live in sea grass beds while the third one is found on rocky substrates. The present study describes the morphological characteristics of these morphotypes and analyzes their genetic structures through 16S rDNA and COI data. Our phylogenetic analyses show that the morphotype living on rocky substrates is morphologically and genetically distinct from the two other morphotypes and might not correspond to *I. badionotus*, the only species of the *Isostichopus* genus previously reported for this region.

Key words: cucumbers, COI, 16S rDNA, Isostichopus badionotus

RESUMEN

Isostichopus sp. (Aspidochirotida:Stichopodidae) son pepinos de mar ampliamente distribuidos en el mar Caribe. Entre ellos, *Isostichopus badionotus* es una de las especies más cosechadas. Muestra un amplio rango de morfotipos extendidos en la región caribeña, incluyendo diferentes hábitats (fondos barrosos, arenosos y rocosos, y bancos de pastos marinos). En Colombia, se pueden distinguir tres morfotipos; dos de ellos viven en los bancos de pastos marinos mientras que el tercero se halla sobre sustratos rocosos. En el presente estudio se describen las características morfológicas de esos morfotipos y se analizan sus estructuras genéticas mediante datos de 16S rDNA y COI. Nuestros análisis filogenéticos muestran que el morfotipo que vive sobre sustratos rocosos es morfológica y genéticamente distinto de los otros dos morfotipos y puede no corresponder a *I. badionotus*, la única especie del género *Isostichopus* previamente reportada para esta región.

Palabras clave: pepinos, COI, 16S rDNA, Isostichopus badionotus

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INTRODUCTION

Sea cucumbers are over-exploited in the three main oceans, and started to be recently targeted in the Caribean sea (Purcell *et al.*, 2012). *Isostichopus badionotus* is one of the sea cucumber species widespread in the Caribbean region (Guzman *et al.*, 2003), ranging from North Carolina to northern Brazil. In addition, this species has been previously reported in the east to the middle-Atlantic and in the Gulf of Guinea (western Africa) (Hendler *et al.*, 1995). It is found at depths from 0-55m (Miller and Pawson, 1990). *I. badionotus* is a common shallow water species that inhabits rocky bottoms, as well as sea grass beds (Hendler *et al.*, 1995). In Colombia, there is an illegal, unregulated and unquantified fishery (Toral-Granda, 2008), and it is of potential commercial interest in Florida, Puerto Rico and the U.S.Virgin Islands (Bruckner, 2006).

At present, Colombian Caribbean Sea cucumbers have not been well studied (Rodríguez *et al.*, 2013; Agudelo and Rodríguez, 2015) and there are many gaps in their knowledge especially related to their taxonomic characterization (Honey-Escandon *et al.*, 2012; Smirnov, 2012). The advances in this regard allowed the reporting of around 44 species, most of which have been captured by exploratory fishing, and identified mainly as deep-sea species (Caycedo, 1978; Borrero *et al.*, 2003; Borrero-Pérez *et al.*, 2012; Toral-Granda, 2008; Toral-Granda *et al.*, 2008).

Since sea cucumbers are an important source of animal proteins, studies in Colombia are beginning to develop the conditions for their production under controlled environments (Rodríguez *et al.*, 2013; Agudelo and Rodríguez, 2015; Vergara and Rodríguez, 2015; 2016). In this way, part of the present team studied the gonad morphology and the larval development of a species, *Isostichopus* sp. aff *badionotus*, including its spawning period (Agudelo-Martínez and Rodríguez-Forero, 2017).

According to Guzmán and Guevara (2002) and Toral-Granda (2008), there are three species of *Isostichopus* including *I. badionotus*, *I. fuscus*, and *I. macroparentheses* distributed in Central and South America. However, the observation of various morphotypes inside the species groups suggests the occurrence of more than three species for this region. In Colombia, three morphotypes can be distinguished in *I. badionotus*. The morphotypes studied are similar because they all have a robust body, a mouth located ventrally, twenty pelleted tentacles, anus in terminal position, gonads in the form of tufts, podias or ambulatory feet distributed in three rows, one or two poly vesicles, all these, distinctive characteristics of the Stichopodidae family. Preliminary phylogenetic analyses showed two morphotypes that cluster together and one that is well separated from the others. Two of them live in sea grass beds while the third is found on rocky substrates. Also, histological studies revealed a different structure of the body wall. The present study describes the morphological characteristics of these morphotypes and analyzes their genetic structures through *16S* rDNA and *COI* data.

This research can be very useful to increase the knowledge about the taxonomy status of some Colombian sea cucumbers and to find out the morpho-anatomical and genetic differences between species of genus *Isostichopus*, from the native populations of the Santa Marta, Caribbean Sea. This information will also be useful for conservation and management purposes.

MATERIAL AND METHODS

Collection of animals

During one year, two hundred sea cucumbers were purchased from local artisanal fishermen in Rodadero Bay (11° 13' 22.73" N - 74° 13' 32.59" W), Airport Bay (11° 07' 10" N - 74° 13' 50" W), and Taganga Bay (11° 16' 03.4" N - 74° 11' 32.3" W), located in Santa Marta, Magdalena, Colombia (Figure 1) and transferred to the Aquaculture Laboratory (Universidad del Magdalena, Colombia). Most of the material was collected in shallow waters (1 to 20 m depth). One hundred and thirty sea cucumbers were collected between Rodadero and Airport bays while seventy were obtained from the bay of Taganga. Individuals from the three morphotypes were weighed with an analytical scale Ohaus (0.001 g), and their length was measured with a standard measuring board (mm). Photographs of the dorsal and ventral sides of the body were taken by using digital camera (Cannon EOS, Rebel XTi). Besides the external and internal anatomical observations, skin samples, tentacles and podia were taken for reviewing ossicles. Samples were dissolved in 10% sodium hypochlorite for 10 min and rinsed with water as described by Lambert (1985), and examined microscopically through slide preparations. For this, the animals were anesthetized by immersion in sea water with ice and then sacrificed. The characterization was made by comparison with Tommasi (1969) and Purcell et al.,



Figure 1. Marine and coastal areas of Colombia Caribbean Sea (modified by José Viillacob). Blue points in the studied areas.

(2012). Our results showed that the specimens of the three morphotypes came from two lineages of *Isostichopus* species (*Isostichopus badionotus* and *Isostichopus* sp., characterized in this report) that were recorded in the Center of Biological Collections of Universidad de Magdalena (Santa Marta, Magdalena, Colombia) (CBUMAG:ECH:00001, CBUMAG:ECH:00002, CBUMAG:ECH:00003).

DNA extraction, amplification and sequencing

Twenty-seven individuals were dissected and preserved in absolute ethanol (99.5%). DNA extraction was performed from up to 15 mg of muscle tissue using the DNeasy Blood & Tissue Kit (QIAGEN). The quality of the DNA was verified on a 1% agarose gel stained with GelRed (Biotium). PCR amplification of the *COI* gene was performed using the primers co1eF (5'-ATAATGATAGGAGGRTTTGG-3') and co1eR (5'-GCTCGTGTRTCTACRTCCAT-3') (Arndt *et al.*, 1996), and of the *16S* gene with the primers 16Sas (5'-CGCCTGTTTATCAAAAACAT-3') and 16Sbr (5'-CTCCGGTTTGAACTCAGATCA-3'). PCR reactions were performed with 2 μ L template in a 25 μ L volume with final concentrations of 2 mM MgCl2, 5X PCR buffer (no MgCl2 BIOLINE®), 0.4 μ M of each primer, 0.4 μ M of each dNTP, and 2 units Taq (BIOLASETM, BIOLINE®). PCR amplifications were performed under the following conditions: 1 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, 1 min at 40 °C, 1.5 min at 72 °C, and there was a final extension period of 5 min at 72 °C. The PCR products were verified on a 2% agarose gel stained with GelRed (Biotium). Following amplification, PCR products were purified using the MACHEREY-NAGEL kit (NucleoSpin® Extract II), and both strands were sequenced in both directions.

Sequence analyses and molecular phylogenetic analyses

Primer sequences were removed from the start and the end of the obtained sequence and sequence ambiguities were resolved by comparing the electropherograms using the program BioEdit v. 7.0.5.3 (Hall, 1999). After trimming, forward and reverse sequences for each specimen were assembled. Each assembled sequence was examined and edited by hand, and each sequence was checked for stop codons (in the case of the COI data file). Finally the consensus sequence from each contig was verified using the Blast tool on NCBI (www.ncbi.nlm.nih.gov). The obtained sequences and downloaded sequences from GenBank were aligned using the ClustalW algorithm (Thompson et al., 1997) in MEGA 6 (Tamura et al., 2013). The downloaded sequences were, for the COI data set, one sequence of I. badionotus (EU848276.1), one COI sequence of Isostichopus sp. (FJ971400.1), five sequences of I. fuscus (AF486424.1/28.1), three sequences of S. herrmanni, and one sequence of Holothuria leucospilota (KC405565.1); for the 16S data set, one sequence of I. macroparentesis (AY338415.1), two sequences of I. badionotus (JN207495.1, EU822435.1), six sequences of I. fuscus (AY153494.1, AY153499.1), three sequences of Stichopus herrmanni (EU822451.1, EU856636.1, FJ223863.1), and one sequence of Holothuria leucospilota (JQ657266.1). The COI final alignment was of 665 bp, and the 16S final alignment was of 508 bp. Sequences were submitted to GenBank under accession numbers KX383967-KX384018.

Sequence divergences were calculated using the K2P distance model (Kimura, 1980) following the barcoding approach suggested by Hebert *et al.* (2003; 2004). Phylogenetic analyses were made performing a Neighbour-Joining (NJ) tree with a distance matrix generated by using MEGA 6 (Tamura *et al.*, 2013). Bootstrap values were obtained by making 1,000 replicates. Bayesian analyses were also performed using MrBayes (Ronquist and Huelsenbeck, 2003), by considering the best substitution models for each region using MrModelTest 3.7 (Nylander, 2004). Two independent runs of 2,000,000 generations, 25% of the trees were discarded as burning. Convergence was validated by the standard deviation of split frequencies (<0.01), by plotting the likelihood values over time, and

by using the sump command in MrBayes. The percentage of trees recovering a particular clade was used as a measure of that clade's posterior probability (Huelsenbeck and Ronquist, 2001).

RESULTS

Morphological analyses

Morphotype I

Description

Seventy adults with average weight 232.4 ± 44.7 g, one specimen of 1 kg. Body is elongated, cylindrical, completed in rounded ends. The species presents varieties in body colorations (brown, reddish, yellow), size and in the ending of the body edges (Figure 2).

Specimens have brown coloration and numerous conical warts colouring brown. The dorsal side of the body presents a variable number of rectangular shape protuberances caused by depressions at the corners. This morphotype has an organization similar to four rows of low projections with small central spots that give it an appearance of breasts. In the two dorso-lateral margins there is only one row of warts and two more in the ventrolateral body. The ambulacral feet are of brown color with three lines of longitudinal podiums.

Calcareous ring composed of projections in the form of pyramid and tower by way of a wheel, with a marked invagination at the base, with two small mountains or bumps on its outer edge. A respiratory tree and one or two poly vesicles are present. No presence of tubules of Cuvier. Gonads are placed in clusters, coloring whitecreamy, depending on the maturity gonad stage. Mature from July to November. Digestive tract is two times longer than body length, cylindrical beige, which occupies three times the length of their body.

Ossicles: Towers, canes, in the shape of s and c, in badges perforated (Figure 3).

Habitat (or ecology)

These specimens have been found in Colombia Caribbean Sea in Rodadero beach (11° 12′ 25.5″ N – 74° 13′ 54.8″ W), Airport beach (11° 09′ 20.2″ N – 74° 13′ 56.6″ W), and mainly in Taganga bay (11° 15′ 53.0″ N – 74° 11′ 32.0″ W). They inhabit sandy bottoms and have cryptic behavior. They feed on particulate material that includes algae, bacteria and marine sediment. Mature from July to November.





Figure 2. Isostichopus badionotus. a) Morphotype I. Typical morphotype, solid beige in the dorsal side, which is covered with dark chocolate round warts. b) Morphotype II. 1) Orange sea cucumber. The dorsal side is covered by orange warts that end in a dark color. 2) Dark sea cucumber surrounded by yellow round warts. 3) Ambulacral feet organized in three rows with podias that end in a dark color.



Figure 3. Isostichopus badionotus ossicles. a) Perforated tables, b) larger plates, c), d) and e) C-shaped rods, f) S-shaped rods.

Morphotype II

Description

Adults with average weight 470.4 ± 162.8 g, one specimen of 1 kg. Body is elongated, cylindrical, completed in rounded ends. These specimens present varieties in colorations (brown, reddish, yellow), size and in the ending of the edges (Figure 2).

The body is light brown and on its surface has tiny pale spots with small, almost imperceptible dark papillae. They showed elongated body, cylindrical or fusiform, robust, with blunt edges. The ventral region is flat and the dorsal region is concave. The ventro-lateral margin is devoid of fleshy projections. The podiums are dark coffee arranged in three longitudinal rows, the middle one being thicker than those of the lateral ones. They have dark brown coloration with tiny conical pinnules, which colouring reddish-brown, whose endings are yellow in colour (Table 1). The mouth position is ventral and the anus, terminal. Eighteen to twenty peltates tentacles and anal opening without tooths. Presence of tube feet arranged in three rows, which become six rows when observed in the water. Their terminal colour is brown (Figure 2).

Table 1. Comparative characters among sea cucumber species

Species	New proposed species (MIII)	Isostichopus badionotus MI	Isostichopus badionotus MII
Total length (mm)	20	≥25	≥30
Average weight (g)	192.9 ± 61.6	243.3 ± 70	470.4 ± 162.8
Body shape	Thin and rigid	Robust and flexible	Robust
Body color	Brown with coffee, orange or reddish dermal spots	From yellow to diverse brown coloration	Most of them have brown coloration
Spots	Irregular	Rounded	Rounded
Dermal papillae shape	Sharp-pointed	Rounded with of chocolate chip terminations	Very small rounded endings with yellow terminations
Podia	3 beige rows	3 beige rows	3 rows, with brown terminal color
Mouth	Ventral	Ventral	Ventral
Anus	Terminal	Terminal	Terminal
Respiratory tree	Presence	Presence	Presence
Gonads	Arranged in clusters, beige, green and yellow-greenish coloration	Arranged in clusters, white- creamy coloration	Arranged in clusters, white- creamy coloration
Mature broodstock	July to November	July to November	July to November
Digestive tract	Long, cylindrical digestive tube,	Long, cylindrical digestive tract,	Long, cylindrical digestive tract,
	beige, with reddish pharynx.	beige. Occupies three times the	beige. Occupies three times the
	Twice the length of its body	length of its body	length of its body
Digestive tract weight (g)	7.9 ± 3.0	8.2 ± 4.0	12.1 ± 5.0
Habitat	Sandy bottoms	Rocky bottoms	Rocky bottoms

Calcareous ring composed of projections in the form of pyramid and tower by way of a wheel, with a marked invagination at the base, with two small mountains or bumps on its outer edge. A respiratory tree and one or two poly vesicles without tubules of Cuvier are present. Gonads are placed in clusters, coloring white-creamy, depending on the maturity gonad stage. Mature from July to November. Digestive tract long, three times longer than body length cylindrical beige, which occupies three times the length of their body.

Ossicles:Towers, canes, in the shape of s and c, in badges perforated (Figure 3).

Habitat (or ecology)

These specimens have been found in the Colombia Caribbean Sea, mainly in Taganga bay (11° 15' 53.0" N - 74° 11' 32.0" W), inhabiting sandy bottoms. They feed on particulate material that includes algae, bacteria and marine sediment. Mature from July to November.

Morphotype III

Description

Adults with average weight around 192.9 ± 61.6 g. Body is elongated, cylindrical, completed in blunt ends. The species presents varieties in colorations in the dorsal



Figure 4. New proposed species. a) Variations in irregular spots. Trapezoidal-shaped body. 1cm bar scale. b). Variations in body color. c) Close view of the peltates tentacles of *Isostichopus isabellae n* sp. d) Peltates tentacles contracted of the new proposed species. Lateral view. e) Ambulacral feet organized in three rows (arrows) with most of the podia ending in a cream color rather than in black (white line). Mouth (+), pinules (*).

region, which are cream-yellow with irregular spots: coffee, orange or reddish. Its ventral region and the tube feet are beige (Figure 4) (Table 1).

Mouth is ventral with 16-20, peltate tentacles, anus is terminal without teeth (Figure 4). Presence of ambulacral feet arranged in three rows. The dorsal region was slightly curved while the ventro-lateral margin of the wall was thick and provided with extensions called papillae.

The calcareous ring has a pyramid form and tower with two small bumps on its top edge. Presence of respiratory tree. Presence of poly (1-2) vesicles without tubules of Cuvier. Gonads arranged in clusters, beige, green and yellow-greenish coloration, depending on the maturity gonad stage. Female gonads tubules reaching a diameter of 405.07 ± 248.19 µm. Male gonads with thinner tubules which diameter was around of 272.18 ± 56.37 µm. Mature oocyte diameter: 125.24 ± 13.11 µm. Cuvierian organ absent. Long, cylindrical digestive tube of beige colour, with reddish pharynx, twice the length of its body (Figure 5).

Ossicles: tables terminated in a crown, perforated by four central holes, c and s shaped rods, rounded and larger plates whit several holes (Figure 6).

Habitat (or ecology)

It inhabits rocky and sandy and feeds on particulate material that includes algae, bacteria and marine sediment. The species have been found in Rodadero beach, Airport beach, Taganga bay. Gonochoric species with bifurcated gonad attached to dorsal mesentery. Mature from July to November.

Remarks

Most of them are smaller in size than Morphotype I and II. They have a trapezoidal-shaped body, which is thin and rigid. They show dorsal irregular spots and different colours that varies from coffee, to red, orange and beige, depending on the individual. Conical pinnules. Ossicles are similar to that other *Isostichopus*. Seasonal spawning from July to November.

Sequence and phylogenetic analyses

Tables 2 and 3 show the divergent nucleotide sequences of the *COI* and *16S* nucleotide alignments. For the *COI* gene three haplotypes corresponding to the three morphotypes where found (Table 2). The 16S gene is more variable, as expected, and we found more than one haplotype for morphotype III (Table 3). The results of our phylogenetic analyses are presented in Figure 7 and Figure 8. Both the NJ and the Bayesian analyses, for both genes (*COI* and *16S*), recovered the same tree topology.

The phylogram obtained by Neighbor-joining and Bayesian analysis derived from *COI* sequences (Figure 7) showed that the three morphotypes of the so-called *Isostichopus badionotus* species form two well separated clades: one with all morphotype III (M3) individuals and the other including all morphotypes I (MI) and II (MII). The "morphotype III" clade also includes a sequence retrieved from GenBank identified as *Isostichopus* sp., the sister group of the "morphotype III" clade *I. fuscus*. The "morphotype I+II" clade also includes a sequence retrieved from GenBank identified as *Isostichopus badionotus*. This clade forms the sister group of the clade formed by morphotypes III and *I. fuscus*.

The *COI* distance matrix indicated that the average distance percentage for *Isostichopus* was 0.20%, while the average between species distance value was 7.80%. Comparisons with *S. herrmanni* sequences indicated that the between genera distance average value was 16% (Table 2).

For the 16S distance matrix we obtained similar results, however, the sequence of *I. macroparentheses* available in Genbank is genetically very different from other *Isostichopus* sequences. In this case the average within species distance percentage for *Isostichopus* was of 0.5%, while the average between species distance value was of 13.78%. The distance values between *I. macroparentheses* and the other *Isostichopus* species were higher than the distance values obtained between the other *Isostichopus* species and *Stichopus* herrmanni (Table 3). One 16S sequence of *I. badionotus* (JN207495.1) clusters within the "Morphotype III" clade.



Figure 5. Internal morphology of the new specie proposed. 1) Pigmented polian vesicle; 2) Respiratory tree; 3) Gonads; 4) Intestine; 5) Internal muscle; 6) Skin.



Figure 6. Ossicles of the dorsal body wall of the new specie proposed. a) Perforated tables with a single central hole, four smaller peripheral holes that end in crown as towers b) Rod with small holes, c) Rod of tentacles d) C-shaped rod, e) Perforate round plates of the dorsal body wall, f) Panoramic view of ossicles samples: very small tables, larger plates, rods some with holes.

Table 2. Comparison of Santa Marta Isostichopus COI sequences from the three found morphotypes and other sequencesavailable in Genbank. Numbers indicate divergent nucleotide positions along the alignment. Dots correspond to
conserve nucleotides. Only sequences representative of each different haplotype are shown.

Organism	Nu	cleot	ide p	ositi	on																							
	9	12	36	42	48	64	69	75	84	96	114	120	138	141	144	153	159	160	162	165	177	195	199	205	208	214	229	253
FJ971400.1 <i>Isostichopus</i> sp.	С	С	А	С	С	Т	Т	Т	G	А	С	Т	Т	Т	G	Т	С	Т	Α	С	С	С	Α	Т	С	С	Т	Т
A5 co1 (Morpho 3- Isostichopus sp.)																												
EU848276.1 I. badionotus	Т	Α	G	Α	Т	С	С	С	А			Α	С	С	Α	С	Т	С	Т	Т	Т		G	С	Т	Т	С	
Br1 co1 (Morpho 1-1. badionotus)	Т	Α	G	Α	Т	С	С	С	Α			Α	С	С	Α	С	Т	С	Т	Т	Т		G	С	Т	Т	С	
H2 co1 (Morpho 2-1. badionotus)	Т	А	G	Α	Т	С	С	С	А	G		Α	С	С	А	С	Т	С	Т	Т	Т		G	С	Т	Т	С	
AF486428.1 <i>I. fuscus</i>			G	Α	Т	С	С		Α		Т	Α		С	Α		Т	С	G		Т	Т			Т			Α
AF486425.1 <i>I. fuscus</i>			G	Α	Т	С	С		Α			Α		С	Α		Т	С	G		Т	Т			Т			Α



Table 3. Comparison of Santa Marta Isostichopus 16S sequences from the three found morphotypes and other sequences available in Genbank. Numbers indicate divergent nucleotide positions along the alignment. Dots correspond to conserve nucleotides. Only sequences representative of each different haplotype are shown.

Organism	Nucle	eotide p	osition	1																				
	1	2	7	49	89	91	93	95	110	111	114	115	116	117	128	129	144	145	146	147	148	149	150	151
JN207495.1 Isostichopus sp.	Т	Т	С	С	Т	Т	Т	G	Т	Т	С	Т	Т	Т	Т	Т	Α	Α	Т	Т	Т	С	Α	С
SPA5 16S (Morpho 3 - Isostichopus sp.)																								
SPA4 16S (Morpho 3 - Isostichopus sp.)																								
SPA2 16S (Morpho 3 - Isostichopus sp.)		С																						
SPA1 16S (Morpho 3 - Isostichopus sp.)																								
A4 16S (Morpho 3 - Isastichapus sp.)																								
R2 16S (Morpho 3 - Isostichopus sp.)																								
2 16S (Morpho 3 - Isastichapus sp.)																								
A1 16S (Morpho 3 - Isostichopus sp.)																								
EU822435.1 I. badionotus				Т					С	С												Т	G	Α
H1 16S (Morpho 2 - I. badionotus)				Т					С	С												Т	G	G
Br2 16S (Morpho 1 - I. badionotus)				Т					С	С												Т	G	Α
AY338415.1 I. macroparentesis	Α	С	Α	Т	С	С	Α	Α	Α	Α	G	С	Α	G	Α	G	С	Т	Α	Α	Α	Α		Α
AY153499.1 I. fuscus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
AY153498. 1 l. fuscus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
	152	181	183	184	185	193	194	195	196	197	198	200	201	203	204	205	206	207	208	211	213	214	215	218
JN207495.1 <i>Isostichopus</i> sp.	Α	С	G	С	Т	G	G	-	-	Т	Т	С	-	Т	Α	С	Т	Т	Т	Т	Т	Т	Α	Α
SPA5 16S (Morpho 3 - Isastichopus sp.)								-	-				-											
SPA4 16S (Morpho 3 - Isastichopus sp.)								-	-				-											
SPA2 16S (Morpho 3 - Isostichopus sp.)								-	-				-											
SPA1 16S (Morpho 3 - Isostichopus sp.)								-	-				-											
A4 16S (Morpho 3 - Isostichopus sp.)								-	-				-											
R2 16S (Morpho 3 - Isastichopus sp.)								-	-				-											
2 16S (Morpho 3 - Isastichopus sp.)								-	-				-								С			
A1 16S (Morpho 3 - Isastichapus sp.)								-	-				-											
EU822435.1 I. badionotus	С					Α		Т	С	С	С		Т	С		Т			С	С	С	Α	G	
H1 16S (Morpho 2 - I. badionotus)	С					Α		Т	С	С	С		Т	С		Т			С	С	С	Α	G	
Br2 16S (Morpho 1 - I. badionotus)	С					Α		Т	С	С	С		Т	С		Т			С	С	С	Α	G	
AY338415.1 I. macroparentesis	G	Т	Α	Т	С		Α	-	-	Α	Α	Α	С	Α	Т		Α	Α	Α	С	С	С	G	Т
AY153499.1 <i>I. fuscus</i>	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
AY153498. 1 l. fuscus	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?

- c	0		n			t			i	i		1	n		ι	1		e				s		-
Organism	Nuc	leotide	positio	n																				
	222	223	224	225	227	228	229	231	232	237	238	240	241	242	265	267	270	271	273	281	284	286	289	290
JN207495.1 Isostichopus sp.	Α	Т	Α	Α	Т	Т	С	Т	С	С	Т	Α	G	С	С	Α	G	С	G	Α	Т	С	G	Т
SPA5 16S (Morpho 3 - Isastichopus sp.).																							
SPA4 16S (Morpho 3 - Isostichopus sp).																							
SPA2 16S (Morpho 3 - Isostichopus sp.).																							
SPA1 16S (Morpho 3 - Isostichopus sp.).																							
A4 16S (Morpho 3 - Isostichopus sp.)																								
R2 16S (Morpho 3 - Isostichopus sp.)																								
2 16S (Morpho 3 - Isostichopus sp.)																								
A1 16S (Morpho 3 - Isostichopus sp.)																								
EU822435.1 I. badionotus		G	Т		Α	G	Т	С	Т	Α	С				Т		Α			Т				
H1 16S (Morpho 2 - I. badionotus)		G	Т		Α	G	Т	С	Т	Α	С				Т		Α			Т				
Br2 16S (Morpho 1 - I. badionotus)		G	Т		Α	G	Т	С	Т	Α	С				Т		Α			Т				
AY338415.1 I. macroparentesis	-	-	-	G	G	Α	Т					G	Α	Т	Α	С		Α	С	Т	Α	Т	Α	Α
AY153499.1 I. fuscus	?	?	?	?	?	?	?	?	?	?	?	?	?		Т					Т				
AY153498. 1 l. fuscus	?	?	?	?	?	?	?	?	?	?	?	?	?		Т					Т				
	291	294	295	296	298	300	303	304	309	310	311	312	314	316	318	319	320	321	329	330	331	335	378	379
JN207495.1 Isostichopus sp.	Α	Α	Α	Α	С	Т	Т	С	G	-	Α	Т	С	-	С	Т	Т	Α	Α	Α	Α	Т	Т	Т
SPA5 16S (Morpho 3 - Isastichopus sp.).									-				-										
SPA4 16S (Morpho 3 - Isastichopus sp).									-		С		-										
SPA2 16S (Morpho 3 - Isastichapus sp).									-				-										
SPA1 16S (Morpho 3 - Isostichopus sp).									-				-										
A4 16S (Morpho 3 - Isostichopus sp.)										-				-										
R2 16S (Morpho 3 - Isostichopus sp.)										-				-										
2 16S (Morpho 3 - Isostichopus sp.)										-				-										
A1 16S (Morpho 3 - Isostichopus sp.)										-				-										
EU822435.1 I. badionotus					Т				Α	Т				-	Т			Т						
H1 16S (Morpho 2 - I. badionotus)					Т				Α	Т				-	Т			Т						
Br2 16S (Morpho 1 - I. badionotus)					Т				Α	Т				-	Т			Т						
AY338415.1 I. macroparentesis	Т	G	Т	С	Т	С	Α	Α	-	-	-	-	Α	С	Α	С	Α	G	G	Т	Т	С	С	С
AY153499.1 <i>I. fuscus</i>									Α	Т		С		Α			С							
AY153498. 1 I. fuscus									Α	Т		С		Α			С							

Table 3. (continues)

Organism	Nucleotide position													
	383	389	391	401	402	407	437	438						
JN207495.1 Isostichopus sp.	Α	Т	Т	Α	Α	Т	С	G						
SPA5 16S (Morpho 3 - Isostichopus sp.)														
SPA4 16S (Morpho 3 - Isostichopus sp.)														
SPA2 16S (Morpho 3 - Isostichopus sp.)														
SPA1 16S (Morpho 3 - Isostichopus sp.)		С					?	?						
A4 16S (Morpho 3 - Isostichopus sp.)						С								
R2 16S (Morpho 3 - Isostichopus sp.)						С								
2 16S (Morpho 3 - Isostichopus sp.)														
A1 16S (Morpho 3 - Isostichopus sp.)						С								
EU822435.1 I. badionotus	G	С					Α							
H1 16S (Morpho 2 - I. badionotus)	G	С					А							
Br2 16S (Morpho 1 - I. badionotus)	G	С					А							
AY338415.1 <i>I. macroparentesis</i>	G		А	G	G		Т	А						
AY153499.1 I. fuscus	G	С					А							
AY153498. 1 I. fuscus	G	С					А							



0.08

Figure 7. Tree topology obtained by Neighbor-joining and Bayesian analyses derived from COI (cytochrome c oxidase I) sequences showing two genetically distinct lineages of *Isostichopus* in Colombia. Sequences downloaded from GenBank are coded with each accession number. Asterisks represent posterior probabilities/booststraps that were > 0.90/90%, diamonds represent posterior probabilities/booststraps that were > 0.80/80%.



Figure 8. Tree topology obtained by Neighbor-joining and Bayesian analyses derived from *16S rRNA* sequences showing two genetically distinct lineages of *Isostichopus* in Colombia. Sequences downloaded from GenBank are coded with each accession number. Asterisks represent posterior probabilities/booststraps that were > 0.90/90%, diamonds represent posterior probabilities/booststraps that were > 0.80/80%.

DISCUSSION

For years, there have been great controversy around the categorization of species worldwide and Colombian sea cucumber is no exception. Genetic studies are definitive for the understanding of the taxonomic identification of sea cucumbers. A species under a certain area can be easily confused or mistakenly classified if only its macroscopic characters are observed. There are pronounced differences between the two lineages studied in this work. One of the main features is that *I. badionotus* has strong chocolate spots with well pronounced edge, while *Isostichopus* sp. (morphotype III) presents different brownish spots distributed on the dorsal surface of the body. This morphotype was characterized by having a trapezoidal-shaped body, which is in contrast to *I. badionotus* who

has the ventral region flat and the dorsal region concave. *Isostichopus* sp. (morphotype III) was found always in rocky bottoms while *I. badionotus* was found in sandy bottoms as was state by Purcell *et al.*, (2012). Additionally, the morphotype III digestive tract length, which occupies twice the length of its body in contrast to three times the length for *I. badionotus*. Our findings suggest morphotype III, could be a different species of *Isostichopus*.

Genetic evidence for taxonomic divergence

Since its appearance the DNA barcoding approach has been proposed as a strategy that could help on species characterization and discovery by allowing taxonomists to rapidly sort specimens and by highlighting divergent taxa that may represent new species (Hebert and Gregory, 2005). The increase in DNA-barcoding initiatives has led to the realization that, for a significant proportion of species, there is an actual correlation between genetic divergence and taxonomic status (Hebert et al., 2004). For the application of genetic barcodes we need to analyze the genetic divergence between and within species (interand intra-genetic distances), so that, for a given genetic marker (typically the COI gene), the lowest interspecific value does not overlap with the highest intraspecific value, maintaining a gap also called the "barcoding-gap" (Hebert et al., 2003). This threshold, depending on the organisms and the study, seems to lie around 2-4% (Hebert et al., 2003; Meyer and Paulay, 2005). Although the COI has proven to be a useful tool for the characterization of species in the Holothuroidea (Uthicke et al., 2010), the 16S rRNA gene has also been successfully tested for this purpose (Kamarudin, 2015; Wen and Zeng, 2014). In this study we evaluated the potential use of the COI and 16S rRNA genes for the characterization of Isostichopus species in Colombia.

In Uthicke's et al. (2010) barcoding study with COI, conspecific sequence variation averages of 1.3% (numbers between 2.0 and 4.5%) and a congeneric average value of 16.9% were obtained, showing a clear "barcoding gap". In our study, the average COI within-species distance value was of 0.20%, while the average between-species distance value was of 7.80%. We also obtained a clear "barcoding gap". Comparisons with S. herrmanni sequences indicated that the average between-genera distance value was of 16%. Both for the COI and the 16S genes we found larger genetic distances values between Isostichopus sp. morphotype III and I. badionotus (morphotypes I and II) than between either of those with I. fuscus. Unfortunalty there is very little genetic information published in Genbank for the group. Although we tried to include the only 16S sequence available for I. macroparentheses, this sequence clearly does not belong to this species. When the sequence is compared to others in the dataset using the Blast tool, it matches another Istichopodid, Astichopus multifidus. Our tree reconstructions (both genes) showed the presence of two Isostichopus well differentiated groups. These barcoding results, corroborated by the morphological analyses, suggest that there might be two Isostichopus lineages or species in our Colombian samples.

Furthermore, although we initially thought that the morphotype II could also be different to *I. badionotus* due to its external morphology, as proposed in Vergara and Rodríguez (2015), the genetic results revealed that it is part of the variety of morphotypes of the *I. badionotus* species. Nevertheless, its external characteristics, as well as the ossicles conformation directed the studies to analysis of genetic sequences that allowed us to confirm the presence of two lineages of *Isostichopus* in the Colombian Caribbean.

Isostichopus includes ten nominal Atlantic species: I. assimilis, I. maculatus, I. acanthomela, I. badionotus, I. diaboli, I. errans, I. haytiensis, I. macroparentheses, I. moebii and I. xanthomela (Heilprin, 1888). While in the present literature only two Atlantic species are recognized, it is quite possible that the morphotype III encountered here represents a described species currently relegated into the synonymy of I. badionotus. In order to confirm the taxonomical status of the Colombian Isostichopus, all these nominal species need to be evaluated on the basis of their descriptions and types.

It is well known that studies that confirm the taxonomic classification of the species are an essential tool for the implementation of management plans and conservation programs for sea cucumbers. This study can be a tool to establish the plans that currently do not exist in the country.

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