Zoological Journal of the Linnean Society, 2009, **157**, 51–69. With 7 figures

The taxonomic status of some Atlanto-Mediterranean species in the subgenus *Holothuria* (Echinodermata: Holothuroidea: Holothuriidae) based on molecular evidence

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Received 4 June 2008; accepted for publication 24 September 2008

Molecular and morphological data were used to evaluate the taxonomic status of the species *Holothuria tubulosa* Gmelin, 1790, *Holothuria stellati* Delle Chiaje, 1823, *Holothuria mammata* Grube, 1840, and *Holothuria dakarensis* Panning, 1939, belonging to the nominate subgenus *Holothuria (Holothuria)* (family Holothuriidae) from the Mediterranean Sea and Atlantic Ocean. A 16S rRNA marker distinguished three well-supported clades with clear genetic differentiation amongst them. The morphometric characters, although they reflected the clades, showed great variability, and some specimens from different clades overlapped. The morphological data and the literature suggest that the clades correspond to *H. dakarensis* (from Cape Verde Islands), *H. mammata* (from the Atlanto-Mediterranean area) and *H. tubulosa* (from the Mediterranean Sea). *Holothuria stellati* is considered here to be a junior subjective synonym of *H. tubulosa*. Great morphological intraspecific variation within *H. tubulosa* and *H. mammata* explains the confusion in the literature. *Holothuria tubulosa* includes specimens with distinctive ossicles, but others are similar to *H. mammata*. In these cases, the presence or absence of Cuvierian tubules proved a reliable indicator to the identity of these species; unfortunately this character is difficult to assess in preserved material. According to the results of discriminant analysis we propose a set of ossicle morphometric variables that permit the optimum assignation of individuals to the clades. Our results present a new perspective on the taxonomic status of species in *Holothuria (Holothuria)*, and show how a molecular approach, combined with a morphological approach, can solve taxonomic problems.

doi: 10.1111/j.1096-3642.2009.00529.x

ADDITIONAL KEYWORDS: 16S rRNA gene – Macaronesia – Mediterranean Sea – ossicle morphometry.

INTRODUCTION

The family Holothuriidae represents 11% of the total diversity of the class Holothuroidea, and includes about 185 species. The species are classified into five genera: *Actinopyga* Bronn, 1860, *Bohadshia* Jaeger, 1833, *Holothuria* Linnaeus, 1767, *Labidodemas* Selenka, 1867, and *Pearsonothuria* Levin, Kalinen & Stonik, 1984. *Holothuria* comprises about 148 species, which are classified into 18 subgenera (Samyn, Appeltans & Kerr, 2005). Eleven subgenera, amongst them the subgenus *Holothuria*, are distributed throughout the Atlantic Ocean. Eight species are recognized in this subgenus: *Holothuria (Holothuria) fungosa* Helfer, 1912 and *Holothuria (Holothuria) massaspicula* Cherbonnier, 1954 from the Red Sea (Samyn, 2003); *Holothuria (Holothuria) caparti* Cherbonnier, 1964 from West Africa; *Holothuria (Holothuria) stellati* Delle Chiaje, 1823 from the Mediterranean Sea; *Holothuria (Holothuria) helleri* von Marenzeller, 1877, *Holothuria (Holothuria) tubulosa* Gmelin, 1790 and *Holothuria (Holothuria) mammata* Grube, 1840 from the Mediterranean Sea and the eastern Atlantic; and *Holothuria (Holothuria) dakarensis* Panning,

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1939 from the eastern and western Atlantic – Gulf of Mexico (Pawson & Shirley, 1977).

The status of *H. dakarensis*, *H. tubulosa*, *H. mammata*, and *H. stellati* has been the subject of much discussion and many changes. According to Koehler (1921, 1927) and earlier authors (Lampert, 1885; Perrier, 1902), *H. tubulosa*, *H. mammata*, and *H. stellati* are distinct species. Panning (1934) found similarities amongst these species, maintained the specific rank of *H. mammata*, but considered *H. stellati* to be a variety of *H. tubulosa*. In 1939, Panning considered *H. stellati* to be a superspecies made up of four species: *H. stellati stellati, H. stellati tubulosa, H. stellati mammata*, and *H. stellati dakarensis*. The reasoning behind this proposal was based on similarities in external morphology and the shape of the tables.

Cherbonnier (1950: 106) noted that it would have been preferable to use *H. tubulosa*, a name established in 1790 by Gmelin, rather than *H. stellati*, established in 1823 by Delle Chiaje, for the superspecies. Cherbonnier recognized *H. tubulosa*, *H. mammata*, and *H. dakarensis* as valid taxa that warrant specific status and pointed out the characteristics that differentiate them: presence of Cuvierian tubules, the size of the tables, and the size of the dorsal papillae. Cherbonnier unfortunately failed to incorporate *H. stellati* into his comparative study because he had no material available. Cherbonnier (1956) identified all the specimens collected as *H. tubulosa*, but described the great polymorphism of this species in terms of the ossicle shape, disposition and size of dorsal papillae, and disposition and number of the ventral podia. For that reason the specimens were distinguished from *H. mammata* only on the basis of body size, and the absence of Cuvierian tubules. For *H. stellati*, Cherbonnier mentioned the ossicle shape from aberrant samples that did not allow individuals to be identified as this species. Finally, Cherbonnier (1960) concluded that it is impossible to confuse juveniles of *H. mammata* with *H. tubulosa* and *H. stellati* because the last two species have a large number of ventral tube-feet, the ventral buttons are never almost solid as they are in *H. mammata*, and they do not have Cuvierian tubules.

Tortonese (1965) studied Italian material of this complex and identified *H. mammata* and *H. tubulosa*. He also discussed the taxonomic value of *H. stellati* and he concluded that it was difficult to establish the value of *H. stellati*, pointing to the need for further study in order to define the relationships with *H. tubulosa*. The differences cited by Tortonese are related to the tegument, length of papillae, and body wall coloration.

Rowe (1969) concluded that it is possible to distinguish the species by the size of the ossicles, *H. dakarensis* having the largest buttons and tables and *H. stellati* the smallest, whereas the ossicles of intermediate size occur in *H. mammata* and *H. tubulosa*. He proposed as a specific character the large mammillate dorsal papillae in *H. mammata* and many more elongate, almost solid, buttons (up to about 250 μm long) in the walls of the ventral podia in *H. tubulosa*. He considered that the presence or absence of Cuvierian organs is difficult to assess because the specimens usually eviscerate before reaching the laboratory.

Gustato & Villari (1977) studied the systematics and frequency of occurrence of the species of *Holothuria* in the Gulf of Naples, and attention was focused on defining the taxonomic status of *H. stellati*. They recognized *H. tubulosa, H. stellati*, and *H. mammata*, although they did not collect specimens of the last species. These same authors in 1980 presented a detailed comparison between *H. stellati* and *H. tubulosa*, finding important differences in the general body morphology and the morphology and percentage distribution of ossicles. *Holothuria stellati* was characterized by the high abundance of type B buttons that are 'virtually' absent in *H. tubulosa*. Buttons type A and C are present in both species.

Zavodnik (1999) mentioned that in spite of Gustato & Villari (1980) the systematic position of *H. stellati* was still dubious. The same author in 2003 recognized *H. stellati, H. tubulosa*, and with some doubt also *H. mammata*. The variability and the confusion of the three species were discussed and it was concluded that although most authors treat *H. stellati*, *H. tubulosa*, *H. mammata*, and *H. dakarensis* as valid species, doubts remain about their status as species.

The aim of the present work was to evaluate the taxonomic status of these species on the basis of molecular evidence and discuss the usefulness of the traditional taxonomic characters in light of the molecular evidence.

MATERIAL AND METHODS

Material studied

The specimens were collected from areas where the species have been recorded, or obtained from museum collections. A total of 49 individuals representing four species, *H. tubulosa, H. stellati, H. mammata*, and *H. dakarensis*, from eight geographical locations were studied: three localities in the Mediterranean Sea (Gulf of Tunis, Tunisia; Cabo de Palos, and Aguilas, Spain) and five in the Atlantic Ocean (Algarve and Azores, Portugal; Cape Verde Islands; Canary Islands, Spain and the Gulf of Mexico, Texas, USA) (Fig. 1, Table 1). Specimens from Tunisia and the Gulf of Mexico came from the National Museum of Natural History, Washington, D.C. USA. Others were collected by SCUBA at depths of 10–30 m. The specimens were transported on ice to the laboratory, relaxed by

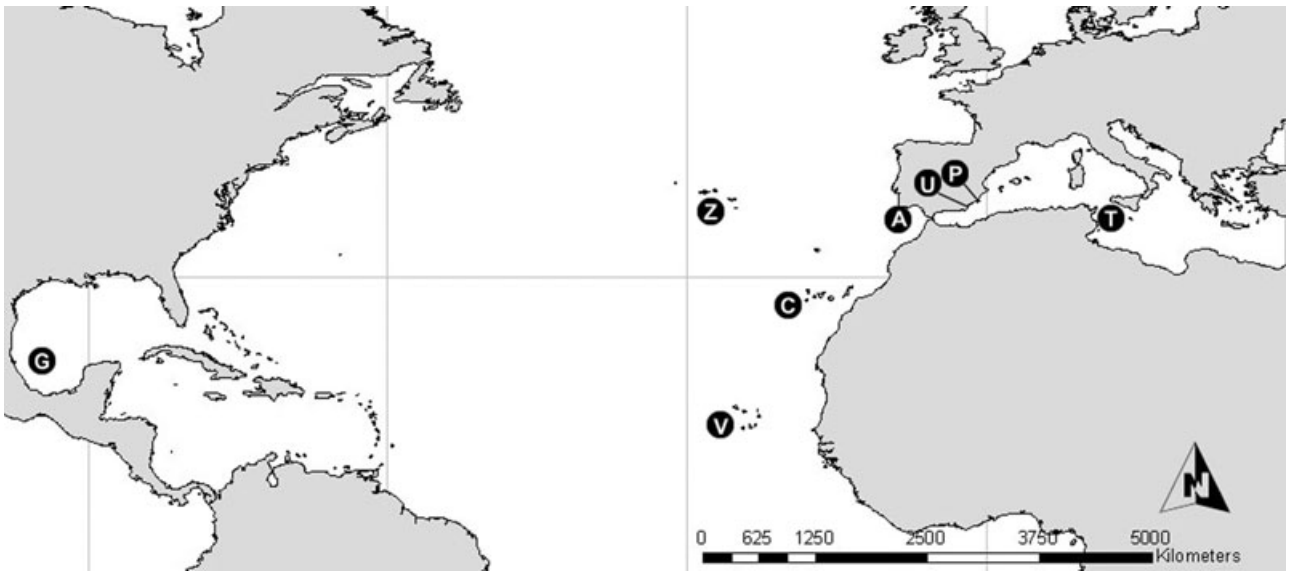

Figure 1. Collection localities: Mediterranean Sea: T, Tunisia; P, Cabo de Palos; U, Aguilas. Atlantic Ocean: C, Canary Islands; A, Algarve; Z, Azores Islands; V, Cape Verde Islands, G, Gulf of Mexico.

cooling them to temperatures close to freezing and dissected. Tissue samples of muscle and body wall were removed from each specimen and preserved in 100% ethanol. Individuals were preserved in 70% ethanol for morphological and ossicle examination. Preliminary identification of specimens was carried out on the basis of external and internal morphology and ossicles, following criteria in the literature (Lampert, 1885; Perrier, 1902; Koehler, 1921, 1927; Panning, 1934, 1939; Cherbonnier, 1950, 1960; Tortonese, 1965; Rowe, 1969; Gustato & Villari, 1977). The coloration and the number of stone canals were recorded; the presence or absence of Cuvierian tubules was determined using scanning electron microscopy and ossicles from the tentacles were qualitatively reviewed in some specimens in order to determine presence or absence of large thick rods with side complex ramifications, described by Koehler (1921) as a diagnostic character of *H. stellati* (Table 1).

Molecular analysis

Thirty-six specimens were genetically analysed, extracting the DNA from muscle tissue using the Sambrook, Fritsch & Maniatis (1989) protocol and DNAzol reagent (GIBCO BRL, Carlsbad, CA).

DNA preparation

1 : 10 to 1 : 500 dilutions were used to amplify the DNA, according to the extraction method used and the quality of the tissue. The primers used for amplification of the 16S gene were 16SAR 5′-CGCCTGT TTATCAAAAACAT-3′ and 16SBR 5′-CTCCGGTTTG AACTCAGATCA-3′ (Palumbi, 1996). The complete PCR cycle was: 94 °C for 5 min, then 40 cycles of denaturation at 94 °C for 20 s, annealing at 46 °C for 20 s, and extension at 72 °C for 20 s followed by a 2 min final extension time at 72 °C (Clouse, Janies & Kerr, 2005), in a PTC 100 Thermal Cycler (MJ Research). PCR products were purified using Exo/SAP-IT (USB Co., Cleveland, OH). Purified DNA was sequenced with an ABI sequencing kit (Big Dye Terminator Cycle Sequencing v. 2.0-ABI PRISM, Applied Biosystems) and then analysed with an ABI 3700 automated sequencer.

mtDNA data analysis

Phylogeny was estimated with three methods: maximum likelihood (ML), maximum parsimony (MP), and a distance-based method. The program PhyML (Guindon & Gascuel, 2003; Guindon *et al.*, 2005) was used for ML, which was carried out using the Hasegawa Kishino-Yano (HKY) evolutionary model and a transition/transversion (ti/tv) ratio of 2 (500 bootstraps). PAUP* 4b10 (Swofford, 2002) was used for the MP and distance-based method. For the first analysis, an unweighted data set and a heuristic approach was used to search for the optimal tree (1000 bootstraps). The neighbor-joining (NJ) tree was inferred from F84 genetic distances (1000 bootstraps). Using the MEGA version 3.1 software (Kumar *et al.*, 2001), average Kimura two-parameter percentage distances between sequences were calculated. The outgroups were *Holothuria (Platyperona) sanctori* Delle Chiaje, 1823 and *Holothuria (Panningothuria) forskali* Delle Chiaje, 1823.

Table 1. Sampling sites and general information about each specimen

Nu. Ind.	Cat. number	Locality	Size (cm)	Preliminary identification	Colour pattern	Cuvierian tubules	Stone canal right/left	Tentacle rods	Molecular clade	Haplotype
1	Ho-1222	Canaries	15	*mammata*	1	p?	2/4	–	1	A
2	Ho-1293	Canaries	12.7	*mammata*	1	p?	1/0	–	1	B
3	Ho-1803	Azores	11	*mammata*	1	p?	2/2	×	1	C
4	Ho-1504	Canaries	14	*mammata-tubulosa*	1	d	2/1	–	1	C
5	Ho-1507	Canaries	7.5	*mammata*	1	p?	1/1	–	1	D
6	Ho-1698	Canaries	17	*mammata*	1	p	1/4	–	1	E
7	Ho-1764	Azores	16	*mammata*	1	p	1/2	–	1	F
8	Ho-1208	Canaries	15.5	*mammata*	1	p	3/1	–	1	F
9	Ho-1211	Canaries	16.5	*mammata-tubulosa*	1	d	2/2	–	1	G
10	Ho-1858	Algarve	19.5	*mammata*	1	p	1/0	–	1	G
11	Ho-1863	Aguilas	23	*mammata*	1	p	5/1	–	1	G
12	Ho-1864	Aguilas	18	*mammata*	1	p?	2/1	–	1	G
13	Ho-1855	Algarve	19	*mammata*	1	p	1/1	–	1	G
14	Ho-1204	Canaries	14.5	*mammata-tubulosa*	1	d	3/6	–	1	H
15	Ho-1361	Canaries	16	*mammata*	1	p?	1/2	–	1	H
16	Ho-1392	Canaries	9.5	*mammata-tubulosa*	1	d	1/1	–	1	I
17	Ho-1804	Azores	9	*mammata*	1	p?	2/0	–	1	J
18	Ho-1861	Algarve	9	*mammata*	1	p?	1/1	–	1	K
19	Ho-1477	Canaries	11.5	*mammata-tubulosa*	1	d	1/0	–	1	L
20	Ho-1831	Cabo de Palos	16	*mammata*	1	p?	2/0	+	1	M
21	Ho-1829	Cabo de Palos	11	*mammata*	1	p	1/0	–	1	N
22	Ho-1774	Azores	15	*mammata*	1	p?	1/1	–	1	O
23	Ho-1868	Aguilas	17	*mammata*	1	p?	2/4	–	1	P
24	Ho-1819	Cabo de Palos	16	*mammata-tubulosa*	1	d	3/1	–	1	Q
25	Ho-1769	Azores	19	*mammata*	1	p?	5/2	–	1	R
26	Ho-1830	Cabo de Palos	22	*stellati*	2	a	4/7	–	2	S
27	Ho-1865	Aguilas	26	*stellati*	2	a	7/9	+	2	S
28	Ho-1820	Cabo de Palos	18	*mammata-tubulosa*	1	d	2/0	+	2	S
29	Ho-1834	Cabo de Palos	26	*stellati*	2	a	19/12	–	2	T
30	Ho-1869	Aguilas	16	*mammata-tubulosa*	1	d	2/4	×	2	U

31	Ho-1827	Cabo de Palos	14	*stellati*	2	a	6/2	+	2	V
32	Ho-1866	Aguilas	25	*stellati*	2	d	16/9	–	2	V
33	Ho-1867	Aguilas	16	*stellati*	2	a	9/10	–	2	V
34	Ho-1828	Cabo de Palos	16	*stellati*	2	a	3/3	–	2	V
35	Ho-321	Cape Verde	7	*dakarensis*	3	d	1/0	–	3	X
36	Ho-312	Cape Verde	13.5	*dakarensis*	3	d	1/0	–	3	Y
37	Ho-1765	Azores	12.5	*mammata*	1	p?	2/1	–	–	
38	Ho-1782	Azores	16	*mammata*	1	p?	1/1	–	–	
39	Ho-1188	Canaries	13	*mammata-tubulosa*	1	d	1/1	–	–	
40	Ho-1197	Canaries	15.5	*mammata*	1	p?	3/2	–	–	
41	Ho-1202	Canaries	14	*mammata-tubulosa*	1	d	1/0	–	–	
42	Ho-1266	Canaries	16.5	*mammata-tubulosa*	1	d	2/0	–	–	
43	Ho-1818	Cabo de Palos	21	*mammata*	1	p	1/2	–	–	
44	Ho-1824	Cabo de Palos	16.5	*mammata*	1	p	1/0	–	–	
45	USNM E24215	Tunisia	9.2	*mammata*	1	nr	2/0	–	–	
46	USNM E24586	Tunisia	15.8	*mammata*	1	nr	8/6	–	–	
47	Ho-81	Cape Verde	8	*dakarensis*	3	d	1/0	–	–	
48	USNM E22870	Gulf of Mexico	9	*dakarensis*	3	nr	1/0	–	–	
49	USNM E29549	Gulf of Mexico	7.5	*dakarensis*	3	nr	1/0		–	
50	Ho-1194	Canaries	15	*sanctori*					–	
51	Ho-1224	Canaries	16	*sanctori*					–	
52	Ho-1256	Canaries	14.5	*sanctori*					–	
53	Ho-1310	Canaries	10	*sanctori*					Outgroup	

Preliminary identification was based on the columns indicated in grey. Colour pattern: 1: uniform coloration on the dorsal and ventral sides, with a slight difference between both sides, 2: sharp colour differentiation between the dorsal and ventral side, 3: dorsal speckled coloration. Cuvierian tubules: p, present; p?, present deteriorated; d, specimen deteriorated; a, absent; nr, not revised. Tentacle rods: +, complex tentacles rods present; ×, absent; –, not revised.

Ossicle morphometry

In order to test the traditional taxonomic characters and seek a detailed characterization of the ossicles in the four species, morphometric analyses were conducted on 49 specimens of *Holothuria (Holothuria)* and four specimens of *Holothuria (Platyperona) sanctori* as an external reference. Tissue samples were taken from the anterior dorsal and ventral body wall. Buttons and tables were morphometrically characterized by image analysis using MPI software (Consulting Image Digital, Barcelona, Spain). On the digitized images, the ossicles (buttons and tables) were selected and measured automatically; then, on the same image, the number of holes, their maximum and minimum diameters, and the diameter of the central orifice in the tables were recorded. In addition, the maximum diameters of the more elongate and almost solid buttons (up to about 250 μm long) from the walls of the ventral podia were measured in order to review this character described as diagnostic of *H. tubulosa* with respect to *H. mammata* by Rowe (1969). In addition, the frequency of appearance of this kind of buttons was registered.

Statistical analysis

The variables analysed were the mean, standard deviation, and maximum and minimum value of measurements from ten buttons and tables from the dorsal and ventral sides of each specimen. These measurements included size, number of holes in each ossicle, size of holes, and also some indices (indices of circular, elongate and wrinkled shape; and indices of slightness and slenderness) (Table 2). A total of 93 variables was obtained for the dorsal and ventral buttons and 124 for the dorsal and ventral tables (Supporting Information Appendix S1). The ossicles measured were randomly selected in the microscopic slide prepared for each specimen. Morphometric analyses were carried out for all the variables described, and also for the variables from tables or buttons separately. Morphometric data with a square root transformation were analysed by principal component analysis (PCA) using the CANOCO software package (Ter Braak & Smilauer, 1997). The original variables contributing most to the principal component scores were selected and Pearson correlations using Systat Software Inc. were calculated between them to select the main variables. A stepwise discriminant function analysis (Wilk's lambda method) using SPSS 15.0 software was performed on the same morphometric data used for the PCA to discriminate the clades defined in the *Holothuria* subgenus by the molecular analysis. Student's t-test was used to verify whether the frequency of the elongate, almost solid buttons (up to about 250 μm long) in the walls of the ventral podia differed significantly amongst the clades defined in the molecular analysis. Only specimens larger than 7 cm were considered for this analysis in order to avoid the effect of body size on ossicle morphology in our analysis.

RESULTS

Molecular analysis

In the analysed alignment of the 16S gene sequences from 38 specimens, a total of 468 sites was compared (GenBank accession numbers EU191948–EU191980, EU191982–EU191983, FJ231190–FJ231192). One hundred and sixteen sites were variable across all sequences and 65 of them were parsimony-informative. Most of the *Holothuria* subgenus sequences were unique and only six haplotypes, amongst a total of 24, were present in more than one specimen. All the analyses based on mtDNA data placed the sequences of *Holothuria* subgenus in three separate clades, supported by high bootstrap values (Fig. 2A, B). The first one includes specimens previously identified on the basis of morphological characteristics, such as *H. mammata* and *H. mammata-tubulosa* from all localities; the second includes specimens identified as *H. stellati* and *H. mammata-tubulosa* from Mediterranean localities, and the third clade is composed of *H. dakarensis* from Cape Verde (Fig. 2A, B). There was no clear geographical pattern amongst specimens from the different localities in clade 1 as judged from the 16S mtDNA data. Some individuals within clade 2 appeared separated in the analyses but they were not supported by high bootstrap values. The average Kimura's two-parameter (K2P; Kimura, 1980) genetic distances within each clade were less than 0.70% (Table 3). Pairwise distances between clades ranged from a minimum of 4.31% between clades 2 and 3 and a maximum of 5.34% between clades 1 and 2. Between the *Holothuria* subgenus clades and the species from other subgenera (outgroups) the distances ranged from 20.04% to 23.76% (Table 3).

Ossicle morphometry

Figure 3 shows the distribution of individuals (Fig. 3A) and the main variables that explain this distribution (Fig. 3B) in the space defined by the first two axes of the PCA performed on the morphometric matrix. The first and second principal component (PC1 and 2) axes jointly explained 79.2% of the total variance. The distribution in Figure 3A agrees basically with the results of the molecular analysis except for two individuals of clade 2 that are morphologically undifferentiated from the individuals of clade 1 (Fig. 3A). The specimens previously identified as *H. dakarensis*, including those of clade 3, are

Table 2. Morphometric variables and index considered for buttons and tables on the dorsal and ventral side

Variables of ossicle buttons or tables

Abbreviation	Variable description
A	Area of the button or the disc of the table.
P	Perimeter of the button or the disc of the table.
CP	Convex perimeter of the button or the disc of the table.
XD	Maximum diameter of the button or the disc of the table.
ND	Minimum diameter of the button or the disc of the table.
NH	Number of holes of the button or the disc of the table.
XDP*	Maximum diameter of the buttons from ventral podia
ArC**	Area of the central orifice in each table, calculated using the formula $\Pi*r^2$.
HTL**	Height of the table (lateral view).
WDL**	Width of the disc (lateral view).
WSL**	Width of the spire (lateral view).

Variables of holes

Abbreviation	Variable description
XDH	Mean maximum diameter of the holes in each button or table.
NDH	Mean minimum diameter of the holes in each button or table.
ArH	Mean area of the holes in each button or table. The area of each hole was calculated using the area of an ellipse (Π*a*b); 'a' is the minimum diameter and 'b' is the maximum diameter of the hole.
TArH	Total area of the holes in each button or table, calculated by adding the area of each hole in the button or in the disc of the table.

Indices

Abbreviation	Index	Formula	Description
CSI	Circular shape	$4\Pi A/P^2$	This is the ratio between the area of the button or the disc and the area of a circle with the same perimeter. Values ranged from 0 to 1. CSI = 1 indicates a circular button or tables with circular discs.
ESI	Elongation shape	ND/XD	This is the ratio between the minimum diameter and maximum diameter of the button or the disc. Values ranged from 0 to 1. Smaller values of ESI indicate more elongated buttons or tables with more elongated discs.
WSI	Wrinkled shape	CP/P	This is the ratio between the convex perimeter and the perimeter. Values ranged from 0 to 1. Smaller values of WSI indicate more wrinkled buttons or tables with more wrinkled discs.
SHI	Slightness H	TArH/A	This is the ratio between the total area of holes and the button or disc area. Values ranged from 0 to 1. Greater values of SHI indicate slighter buttons or tables with slighter discs, with respect to their holes.
SCI**	Slightness C	ArC/A	This is the ratio between the area of the central orifice in each table and the disc area. Values ranged from 0 to 1. Greater values of SCI indicate tables with slighter discs, with respect to the central orifice.
SLI**	Slenderness	(WDL/WSL) HTL	This is the ratio between the width of disc and the width of spire, multiplied by the height of the table. Greater values of SLI indicate slenderer tables.

In the analysis the mean, standard deviation, and the maximum and minimum values of each were used. A single asterisk (*) indicates the variables that were only measured in the buttons from ventral podia; two asterisks (**) indicate the variables that were only measured in the table ossicles (full details about variables in Appendix S1).

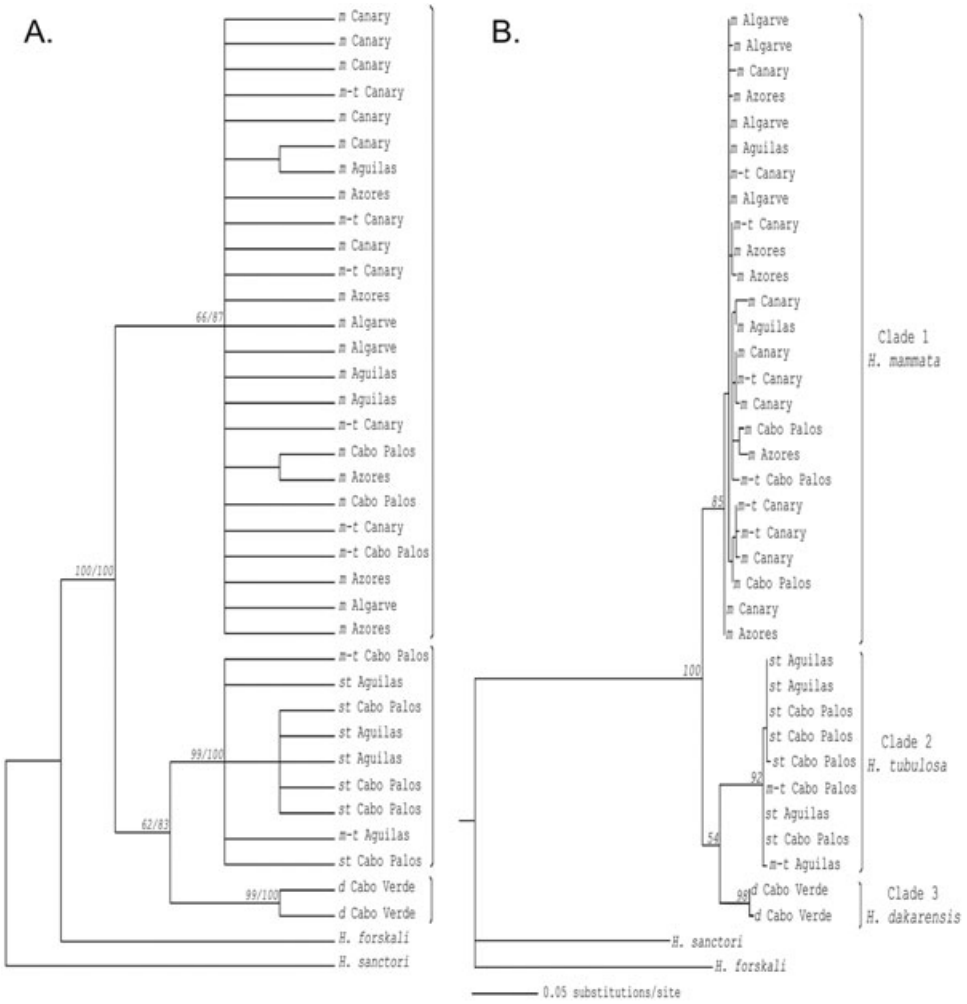

Figure 2. A, maximum parsimony (MP) tree obtained from 16S mtDNA sequences (215 steps; consistency index = 0.856, retention index = 0.896, homoplasy index = 0.144), numbers on branches represent per cent bootstrap support values of 1000 replicates from MP/neighbour-joining analyses. B, optimal maximum likelihood tree obtained from 16S mtDNA sequences (–ln likelihood = 1493.80208), numbers on branches represent per cent bootstrap support values of 500 replicates. In both figure parts, labels of operational taxonomic units indicate the preliminary species identified (m, *Holothuria mammata*; t, *Holothuria tubulosa*; st, *Holothuria stellati*; d, *Holothuria dakarensis*) and the locality. After these, the clade (1–3) and the name of the species are provided.

differentiated in the first quadrant. The individuals corresponding to *Holothuria sanctori* are separated in the positive part of axis 1.

Six main variables determined the separation observed in the positive part of the PC1 (Fig. 3B), whose contribution to the principal component score was > 0.85. The variables were the mean total area of the holes in the dorsal and ventral tables (mTArHdt, mTArHvt), which are represented in Figure 4A and B; the mean area of the disc in the dorsal and ventral tables (mAdt, mAvt) (Fig. 4A, B); the mean total area of the holes in the ventral buttons (mTArHvb), and the mean area of the ventral buttons (mAvb) (Fig. 4C). Three variables determined the groups in the negative part of the PC1 (Fig. 3B), whose contribution was > 0.50. These were the standard deviation of the maximum and minimum diameter of the holes in the tables (sdXDHdt, sdNDHdt) (Fig. 4A) and the mean elongation shape index in the ventral buttons (mESIvb) (Fig. 4D). In the PC2 the separation was determined mainly by three variables in the positive part and four in the negative part (Fig. 3B), whose contribution to the principal component score was > 0.52. The variables for the positive part were the mean and minimum elongation shape index of the dorsal buttons (mESIdb, nESIdb) (Fig. 4E) and the mean area of the central orifice in the ventral tables (mArCvt) (Fig. 4B). For the negative part, the

Table 3. Kimura two-parameter (K2P) mean percentage distances within each clade and between clades

Clades	Mean %	*N*
K2P mean percentage distances within clades		
Clade 1	0.70	25
Clade 2	0.23	9
Clade 3	0.22	2
Holothuria sanctori	–	1
Holothuria forskali	–	1
K2P mean percentage distances between clades		
Clade 1 – Clade 2	5.34	
Clade 1 – Clade 3	4.69	
Clade 1 – *H. sanctori*	20.80	
Clade 1 – *H. forskali*	21.58	
Clade 2 – Clade 3	4.31	
Clade 2 – *H. sanctori*	23.76	
Clade 2 – *H. forskali*	20.56	
Clade 3 – *H. sanctori*	22.19	
Clade 3 – *H. forskali*	22.73	
H. sanctori – H. forskali	20.04	

variables were the mean number of holes in the dorsal buttons (mNHdb) (Fig. 4E), the standard deviation of the perimeter of the ventral buttons (sdPvb), which is highly correlated with the standard deviation of the maximum diameter of the ventral buttons (sdXDvb) (Fig. 4F), and the mean perimeter of the dorsal buttons (mPdb) (Fig. 4E), which is also highly correlated with other size variables, such as the maximum diameter and convex perimeter of the dorsal buttons (not shown).

According to these results, *H. sanctori* has the largest ventral and dorsal tables, ventral and dorsal buttons, and the largest holes in each type of ossicle. Within the *Holothuria* subgenus, individuals from clade 3 and the other specimens from Cape Verde and the Gulf of Mexico were characterized by having the largest dorsal and also ventral tables with large holes (mAdt, mAvt, mTArHdt, mTArHvt) (Fig. 4A, B) and a large central orifice in the ventral tables (mArCvt) (Fig. 4B). Individuals from clade 1 and clade 2 possessed smaller tables and holes than those of clade 3 (Fig. 4A, B), and the size of the holes in the dorsal tables varied according to the standard deviation of the mean maximum and minimum diameter of table holes (sdXDHdt, sdNDHdt) (Fig. 4A). Clade 3 and clade 1 presented the largest ventral buttons (mAvb) (Fig. 4C). As regards the size of the holes in ventral buttons (mTArHvb), the largest holes are in clade 3 (Fig. 4C). In clade 1 a gradient that finished in specimens showing large buttons without holes was observed (Fig. 4C). The smallest ventral buttons (mAvb) and dorsal buttons (mPdb) belonged to the specimens of clade 2 clearly separated in the upper left-hand side (Fig. 4C, E), which is characterized by the shortest dorsal and ventral buttons (mESIvb, nESIdb) (Fig. 4D, E). Individuals from clade 1 are distinguished in having the largest dorsal buttons (mPdb) (Fig. 4E), the greatest number of holes in the dorsal buttons (mNHdb) (Fig. 4E), and the greatest variability in ventral button size, as seen from the variable standard deviation of the perimeter (sdPvb) and standard deviation of the maximum diameter (sdXDvb) (Fig. 4F).

A similar distribution of individuals was obtained when only the button data were used (data not shown). However, a separate analysis of dorsal and ventral tables did not discriminate between individuals from clade 1 and clade 2. The Student's t-test performed between the molecular clades 1 and 2 showed no significant differences for the frequency of the elongate, almost solid buttons (up to about 250 μm long) in the walls of the ventral podia.

The stepwise discriminant function analysis selected 12 variables as the most important in the discrimination of the individuals from the three clades (Table 4). 77.6% of the variance is accounted for by function 1 and 22.4% by function 2. Wilk's lambda showed each function is significant ($P = 0.000$). The interpretation of group separation, based on the structure coefficients (Table 4), indicates that characters of height and size of tables were the key variables characterizing function 1, which separate clearly clade 3 from clades 2 and 1 (Table 5, Fig. 5A–F). The key variables in function 2 that separate clades 2 and 1 are related to size and elongation shape of buttons (Table 5, Fig. 5G–L). The classification statistics for each clade showed that 100% of originally grouped specimens were correctly classified.

DISCUSSION

The *Holothuria* subgenus is a group of sea cucumbers with few taxonomically informative morphological characteristics and with a high degree of intraspecific phenotypic diversity, as in other holothurians (Clouse *et al.*, 2005). In the literature, *H. tubulosa*, *H. stellati*, *H. mammata*, and *H. dakarensis* have been considered as varieties, subspecies, or species, and although some authors have proposed solutions to this taxonomic problem, the reality is that confusion has remained. Because morphologically defined taxa are still the basis for most ecological, physiological, and anatomical research (Flowers & Foltz, 2001), genetic analysis has been used in many marine invertebrates in order to help remove doubts concerning the status of species based on morphological characters alone

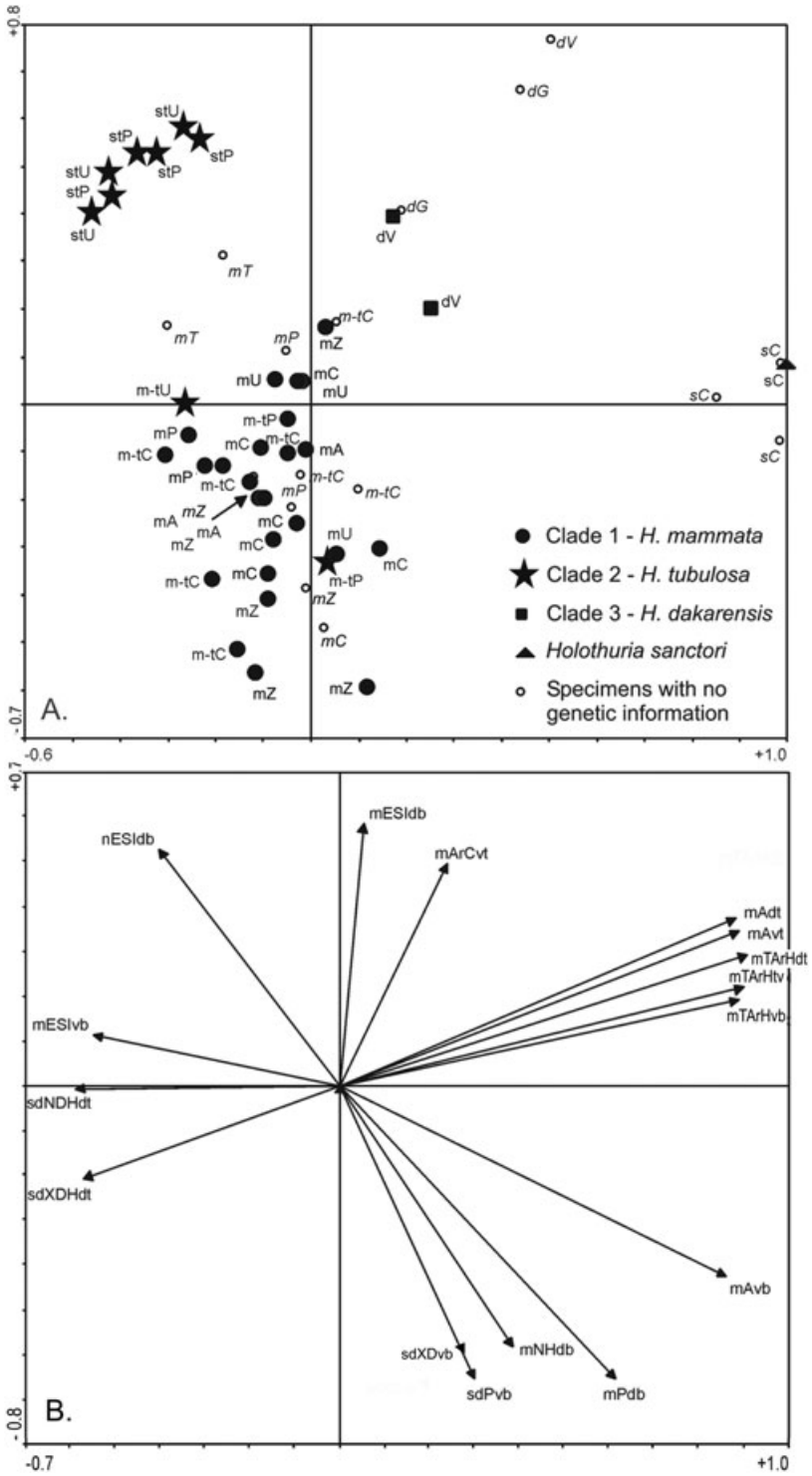

Figure 3. A, distribution of 49 individuals of the *Holothuria* subgenus and four of *Holothuria sanctori* in the space defined by the first two axes of the principal components (PC) analysis on the basis of morphometric variables of the ossicles. The symbols indicate assignment to the three identified clades; labels of the individuals indicate the preliminary species identified (st, *Holothuria stellati*; m, *Holothuria mammata*; t, *Holothuria tubulosa*; d, *Holothuria dakarensis*; and s, *Holothuria sanctori*) and the locality according to Figure 1. B, the most important variables are presented. PC1 (+): mTArHdt, mTArHvt, mAdt, mAvt, mTArHvb. PC1 (–): sdXDHdt, sdNDHdt, mESIvb. PC2 (+): mESIdb, nESIdb, mArCvt. PC2 (–): mNHdb, sdPvb, sdXDvb, mPdb (see Table 2 and Appendix S1 for the meaning of the abbreviations of the variables).

Table 4. Structure and standardized discriminant function coefficients for the 12 morphometric variables selected by the stepwise discriminant function analysis

	Structure coefficients		Standardized coefficients	
	Discriminant function		Discriminant function	
Variable	1	2	1	2
Mean of the slenderness index of dorsal tables (mSLIdt)	0.269*	–0.052	3.982	–1.216
Minimum value of the height of ventral tables in lateral view (nHTLvt)	0.172*	0.039	1.262	0.851
Mean width of dorsal table discs in lateral view (mWDLdt)	0.160*	–0.048	–4.033	1.094
Mean perimeter of dorsal table discs (mPdt)	0.152*	–0.008	1.292	0.211
Maximum value of the elongation shape index of dorsal table discs (xESIdt)	–0.101*	–0.043	–0.758	–0.442
Minimum value of the width of ventral table discs in lateral view (nWDLvt)	0.094*	–0.066	0.575	–0.854
Mean of the elongation shape index of dorsal buttons (mESIdb)	–0.051	0.357*	2.348	1.861
Minimum value of the minimum diameter of ventral buttons (nNDvb)	0.027	–0.332*	–1.880	–1.122
Mean minimum diameter of ventral buttons (mNDvb)	0.016	–0.298*	2.462	–1.059
Standard deviation of the elongation shape index of dorsal buttons (sdESIdb)	–0.050	0.271*	–2.650	0.024
Minimum value of the area of dorsal buttons (nAdb)	0.070	–0.207*	0.319	2.201
Standard deviation of the slightness H index[1] of dorsal table discs (sdSHIdt)	–0.004	–0.054*	–0.695	0.383

The asterisk (*) indicate the largest absolute correlation between each variable and any discriminant function. [1]The meaning of slightness H index appear in the Table 2.

(Knowlton, 2000; Williams, 2000; Flowers & Foltz, 2001; Clouse *et al.*, 2005; Uthicke, Purcell & Blockmans, 2005).

In our results, the molecular information pointed to three divergent clades supported by high bootstrap values, corresponding to different species according to the mean K2P percentage distances. The differences amongst the three clades are greater than those registered by Kerr *et al.* (2005) between *Bohadshia argus* (Semper, 1868) and *Bohadshia marmorata* (Jaeger, 1833) (4.35%) (Table 3). The distances between the outgroups *H. sanctori* and *H. forskali* and the *Holothuria* subgenus clades are up to one order of magnitude higher than those betwen *Holothuria* subgenus clades. These results are similar to those of Uthicke *et al.* (2005) and Kerr *et al.* (2005), who reported distances between subgenera of 16 and 20.5%, respectively, and also supported the need to make a revision of the entire genus *Holothuria*.

Species and pattern of morphological variation

Clade 3, together with specimens from Cape Verde and the Gulf of Mexico, is a group well supported by button and table morphometry (Figs 3A, 4A–B, 5A–F), and morphological characters, such as colour pattern (pattern 3, Fig. 7F) and the number of stone canals (1/0, Table 1), closely coincide with the description of *H. dakarensis* (Panning, 1939; Cherbonnier, 1950; Rowe, 1969). It is necessary to obtain sufficient fresh material from the Gulf of Mexico and other localities in the east Atlantic such as West Africa, including Dakar (its type locality; Panning, 1939) and Angola (Cherbonnier, 1965), in order to evaluate the status of distant populations.

All specimens from clade 1 and 2 belong to *H. mammata*, *H. stellati*, or *H. tubulosa*, according to the preliminary identification. Nevertheless, the phylogenetic results suggest that there are only two species, each of which shows great morphological variability. These two species would be *H. tubulosa* and *H. mammata* for the following reasons.

The morphological variability in the specimens from clade 2 covers the range of diagnostic characteristics of *H. stellati* and *H. tubulosa* as provided in the literature. According to our molecular results, the variability in these characters should be considered as intraspecific variation, so the name *H. tubulosa* would prevail and *H. stellati* would be a junior subjective synonym of *H. tubulosa*. Clade 2 includes some specimens with small and rounded buttons and others with larger and more elongated buttons (Fig. 3A); some individuals have large complex rods in the tentacles and others do not (Fig. 6C–F); the ventral side of some specimens is lighter in colour than the dorsal side (pattern 2, Fig. 7E), whereas others show similar coloration on both surfaces (pattern 1, Fig. 7D) and

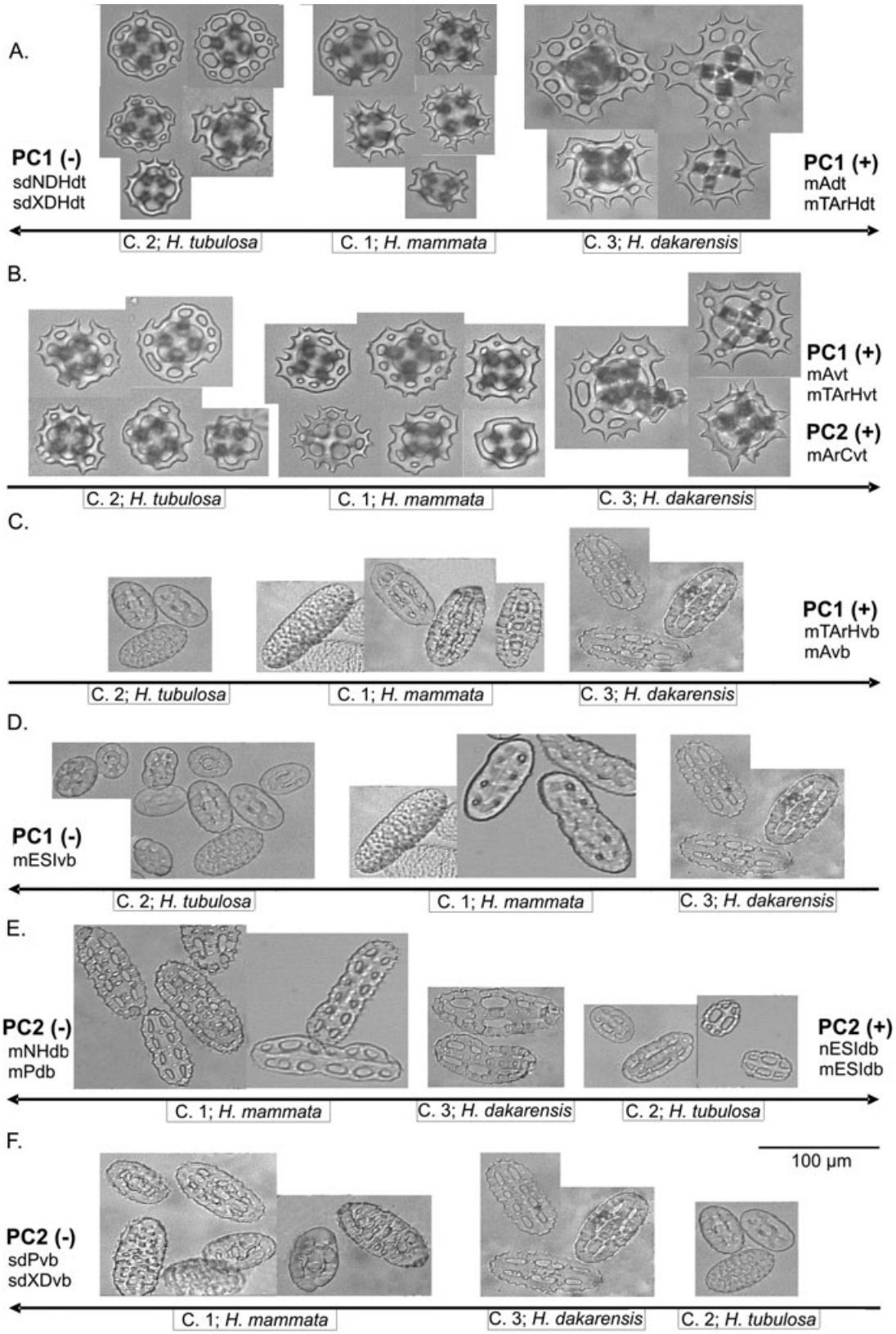
A.
PC1 (-)
sdNDHdt
sdXDHdt
PC1 (+)
mAdt
mTArHdt
C. 2; H. tubulosa
C. 1; H. mammata
C. 3; H. dakarensis
B.
PC1 (+)
mAvt
mTArHvt
PC2 (+)
mArCvt
C. 2; H. tubulosa
C. 1; H. mammata
C. 3; H. dakarensis
C.
PC1 (+)
mTArHvb
mAvb
C. 2; H. tubulosa
C. 1; H. mammata
C. 3; H. dakarensis
D.
PC1 (-)
mESlvb
C. 2; H. tubulosa
C. 1; H. mammata
C. 3; H. dakarensis
E.
PC2 (-)
mNHdb
mPdb
PC2 (+)
nESldb
mESldb
C. 1; H. mammata
C. 3; H. dakarensis
C. 2; H. tubulosa
F.
100 µm
PC2 (-)
sdPvb
sdXDvb
C. 1; H. mammata
C. 3; H. dakarensis
C. 2; H. tubulosa

Figure 4. Representation of the main variables used to separate the groups, following Figure 3. In each part the molecular clade (C) is represented and the species name. The images representing clade 2 – *Holothuria tubulosa* describe the specimens clearly separated. A, mAdt, mTArHdt, sdXDHdt, sdNDHdt. B, mAvt, mTArHvt, mArCvt. C, mTArHvb, mAvb. D, mESIvb. E, mESIdb, nESIdb, mNHdb, mPdb. F, sdPvb, sdXDvb. Scale bar = 100 μm (A–F) (see Table 2 and Appendix S1 for the meaning of the abbreviations of the variables).

Table 5. Functions at groups (clades) centroids

	Discriminant function	
Clades	1	2
1	–1.091	–2.527
2	–3.568	6.496
3	29.697	2.352

the number of stone canals varies (1–19 + 0–12). The specimens with small and rounded buttons would be the typical individuals of *H. stellati* in accordance with Koehler (1921, fig. 131 p. 176) and Rowe (1969), who mention that this species possesses the smallest buttons of the three species, whereas *H. tubulosa* has larger buttons and more variability in their shape and size (Koehler, 1921, fig. 130, p. 175). According to Rowe (1969), the buttons of *H. tubulosa* are similar to *H. mammata*, being intermediate between *H. stellati* and *H. dakarensis*. Gustato & Villari (1980) attempted to clarify the systematic position of *H. stellati* and *H. tubulosa*, although in our opinion, their conclusions were incorrect. These authors considered that *H. stellati* has longer buttons (B-type buttons) than *H. tubulosa*, which is contrary to what the previous authors maintained. In addition, B-type buttons were not observed in the individuals examined in our study or in Zavodnik (2003), who considered that his specimens had a confusing mixture of ossicles characteristic of *H. mammata* and of *H. stellati*. The large complex rods in the tentacles, described by Koehler (1921) as a differentiating character of *H. stellati* with respect to *H. tubulosa*, appear in some specimens from clade 2, but others do not present this type of rod (Table 1, Fig. 6C–F), and also this type of button appears in some individuals from clade 1 (Fig. 6B). Both colorations observed in clade 2 have been used to distinguish these species. The ventral side being lighter than the dorsal side (pattern 2, Fig. 7E) was recorded by Koehler (1921) and Tortonese (1965) as being typical of *H. stellati*, although in the same work Tortonese cited the confusion resulting from the similar coloration of both surfaces (pattern 1, Fig. 7D). Gustato & Villari (1980) considered as diagnostic coloration the uniform colour in *H. stellati* and the ventral side being lighter than the dorsal side in *H. tubulosa*. Mezali (2002), who reported *H. stellati* and *H. tubulosa*, did not mention the coloration. The number of stone canals possessed by individuals in clade 2 includes the numbers described for *H. tubulosa* [three to five, but up to ten (Koehler, 1927)] and for *H. stellati* [two to three (Lampert, 1885)].

The specimens from clade 1 are the only ones to possess Cuvierian tubules, a diagnostic characteristic of *H. mammata* (Fig. 7A–C). In addition, they possess large unperforated buttons, mainly on the ventral side, described by Perrier (1902) and considered diagnostic by Cherbonnier (1960). However, the Cuvierian tubules in *H. mammata* are always short, sometimes present in low numbers (Fig. 7A–C), are never expelled through the anus, do not elongate, are weakly resistant, and do not stick when in contact with a solid surface (VandenSpiegel & Jangoux, 1988). These characteristics make it difficult to confirm their presence, especially if the specimens are collected a long time before being analysed, if they are not well preserved, or if they have eviscerated. However, if it is possible to verify the presence of the Cuvierian tubules, these are the clearest character to distinguish *H. mammata* from *H. tubulosa*. As regards the large ventral buttons without perforations, according to our results there is a clear gradient from individuals possessing mostly unperforated buttons to those mainly having buttons with holes (Fig. 4D). This variation makes it difficult to identify specimens based on this characteristic alone. In addition, the great variation in size (Fig. 3A, B) would explain the results of Rowe (1969), who did not describe a limited range in the size of buttons in *H. tubulosa* and *H. mammata*. To distinguish between these species Rowe proposed that '*H. tubulosa* has in general many more elongate, almost solid, buttons (up to about 250 μ long) in the walls of the ventral podia than *H. mammata*'. In our study this type of button was present in all the specimens reviewed and their maximum diameter was not an important variable for defining the groups; as regards the frequency of occurrence of these buttons, there was no significant difference between clades 1 and 2. Another character considered diagnostic in *H. mammata* are the large dorsal mammillate papillae (Rowe, 1969); our observations suggest that these papillae can change with the preservation process and they are a variable character (Fig. 7D) that may well be present in

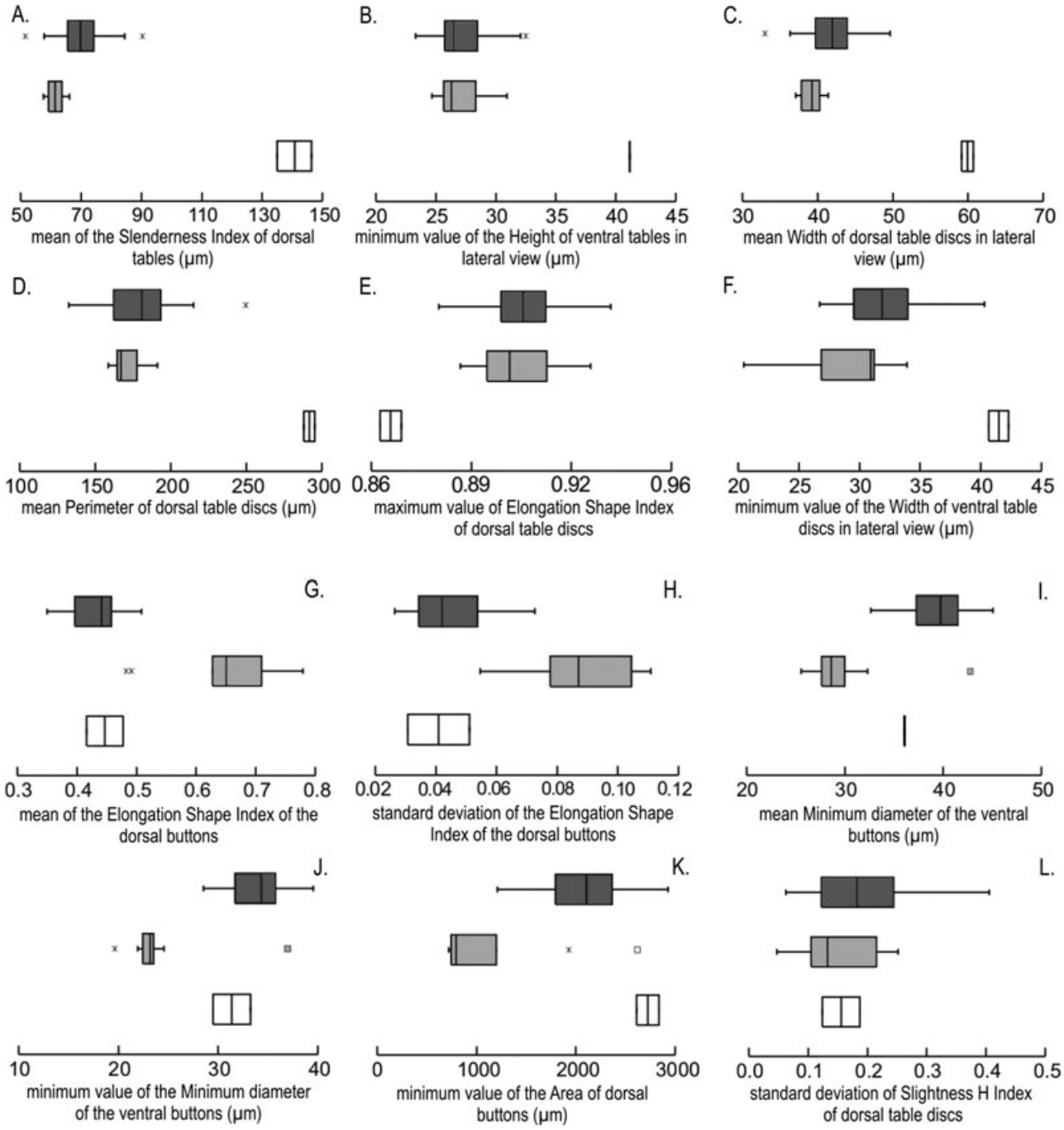

Figure 5. Main variables selected by the stepwise discriminant function analysis to distinguish clade 1 – *Holothuria mammata* (upper box, dark grey colour), clade 2 – *Holothuria tubulosa* (middle box, clear grey colour), and clade 3 – *Holothuria dakarensis* (bottom box, white colour). A–F, variables that characterize function 1. G–H, variables that characterize function 2. The centre lines of the boxes mark the median value, the hinges the lower and upper quartiles, respectively, and the whiskers the range of data between values smaller/greater than the lower/upper quartile minus/plus 1.5 times the interquartile range; asterisks represent outliers (data values outside of this range); and squares represent unusually small or large values outside of the outer fences.

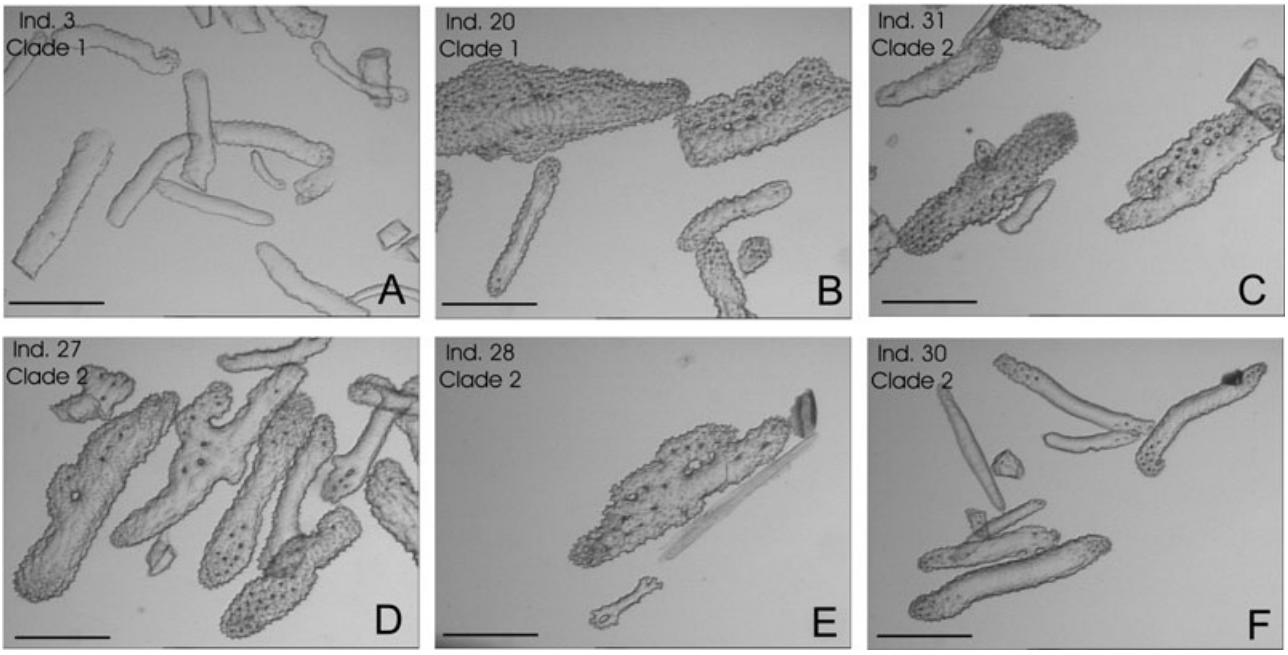

Figure 6. Tentacle rods of six specimens from *Holothuria mammata* – clade 1 (A, B) and *Holothuria tubulosa* – clade 2 (C, D, E, F). Scale bars = 100 μm (A–F). The number on the upper left-hand side of each image represents the individual number in accordance with Table 1.

specimens that are clearly not *H. mammata* (Fig. 7E). Cherbonnier (1956) who only identified *H. tubulosa* in his paper, also mentioned that some individuals, alive as well as in alcohol, presented the characteristic aspect of *H. mammata* illustrated by Koehler (1927). This may have been the case with the individuals described by Zavodnik (2003), who identified them as *Holothuria* sp. cf. *mammata* mostly on the basis of this character. However, we consider Zavodnik's specimens to be *H. tubulosa*, because the size of the most common buttons, their rounded shape, the coloration of both specimens, and the size and shape of dorsal papillae are similar to most of our specimens from clade 2 (Figs 4C–F, 7E). The measurements of the most common buttons reported by this author are within the range described for *H. stellati* (considered in this study *H. tubulosa*) by Rowe (1969). Although the number of stone canals has never been considered as a diagnostic character, we did observe some sort of pattern: few canals in *H. mammata* (1–5 + 0–6) and many in *H. tubulosa* (1–19 + 0–12) (Table 1). This pattern would also support the identification of Zavodnik's individuals as *H. tubulosa*, which show 8 + 10 (individual 1) and 12 + 8 (individual 2) stone canals.

As regards the tables, they are poorly described in the first taxonomic works on this subgenus. Panning (1939) described them in greater detail and proposed that the tables were one of the reasons for changing all of the studied species to subspecies of *H. stellati*, unlike Cherbonnier (1950), who considered them to be clearly different in shape and size (largest tables belonging to *H. dakarensis*, intermediate size to *H. mammata*, and the smallest to *H. tubulosa*), although this author did not include *H. stellati*. In our results the tables vary greatly in shape at the individual and species levels, and although they discriminate *H. dakarensis* individuals, they do not characterize *H. tubulosa* and *H. mammata* (Figs 4A, B, 5A–F, Table 4). The tables in these last species show discs perforated by four large central holes (rarely only three central holes) and four pillars forming the spire (rarely three pillars). The disc may have no external orifices or few holes or a complete ring of holes; it is also possible to find spinose or smooth discs (Fig. 4A, B). Indeed, all these variations may occur in the same individual. The size of the holes and the central orifice are variable and represent a randomly distributed characteristic. The spires usually have two cross beams ending in a crown of spines. However, the shape of this crown is variable, so that the Maltese-cross shape described by Gustato & Villari (1980) in order to distinguish *H. stellati* from *H. tubulosa* is not valid.

Characteristics of species

Based on the classification statistics, the discriminant function analysis classified correctly 100% of the

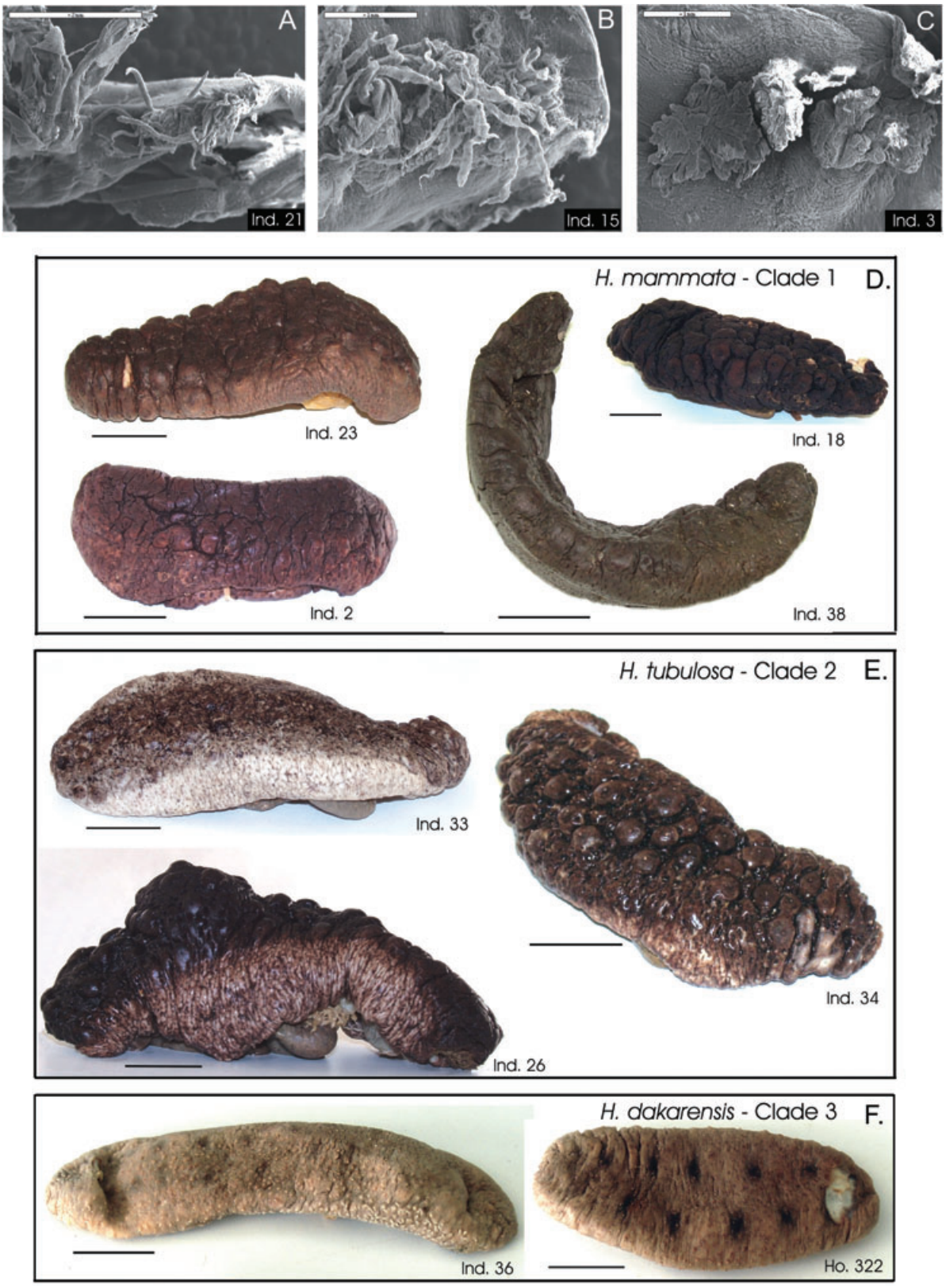
A
Ind. 21
B
Ind. 15
C
Ind. 3
H. mammata - Clade 1
D.
Ind. 23
Ind. 18
Ind. 2
Ind. 38
H. tubulosa - Clade 2
E.
Ind. 33
Ind. 34
Ind. 26
H. dakarensis - Clade 3
F.
Ind. 36
Ho. 322

Figure 7. Internal and external morphological characteristics. A, B, C, Cuvierian tubules from three specimens of clade 1 – *Holothuria mammata*. D, coloration pattern 1, clade 1 – *Holothuria mammata*. E, coloration pattern 2, clade 2 – *Holothuria tubulosa*. F, coloration pattern 3, clade 3 – *Holothuria dakarensis*. Scale bars = 2 mm (A); 1 mm (B, C); 2 cm (D, E, F). The number on the lower right-hand side of each image is the specimen number in accordance with Table 1, except that the second specimen in Figure 6F is identified by its catalogue number.

original grouped cases on the basis of the characteristics of Table 4. However, they must be observed in detail taking into account their variability (Fig. 5), because they all overlap, with the exception the characteristics from function 1 that discriminate *H. dakarensis*.

In the following account we present a summary of the characteristics that could help to distinguish the species on the basis of our results:

Holothuria dakarensis can be distinguished from *H. mammata* and *H. tubulosa* by its slenderest and highest dorsal and ventral tables (Fig. 5A, B) and its large dorsal and ventral table discs (Fig. 5C, D, F). This species possesses a dorsal speckled coloration (Fig. 7F) and one stone canal on the right side. There is no morphological difference between the specimens from Cape Verde and the Gulf of Mexico. According to our data, in the area studied, the species should be restricted to Cape Verde and would not be distributed in other Macaronesian archipelagos; this is in agreement with Pawson & Shirley (1977). In the east Atlantic, in accordance with the revised literature, the species should also be distributed in West Africa, including Dakar, which is its type locality, and Angola (Panning, 1939; Cherbonnier, 1965).

Holothuria tubulosa can be distinguished by its dorsal buttons in the body wall, which are smaller (Fig. 5K) and less elongated (Fig. 5G–H) than those in *H. mammata*; also its ventral buttons are smaller than those in this species (Fig. 5I–J). *Holothuria tubulosa* is also clearly distinguishable from *H. mammata* because of the sharp colour differentiation between the dorsal and ventral sides, the latter being lighter and sometimes with irregular light coloured spots (Fig. 7E). However, two specimens of *H. tubulosa* (Fig. 3A) not only overlapped in terms of size and shape of the buttons with *H. mammata* (Fig. 5G–L), but also showed the same coloration. The number of stone canals in *H. tubulosa* ranges from two to 19 and zero to 12 on the right and left sides, respectively. According to our data *H. tubulosa* occur only in the Mediterranean Sea.

Holothuria mammata has a clear diagnostic character: the presence of Cuvierian tubules, few in number, small, and never expelled. Nevertheless, if it is not possible to examine them, the following characteristics can be used: larger dorsal and ventral buttons (Fig. 5I–K) and more elongated shape of dorsal buttons (Fig. 5G–H) than those in *H. tubulosa*. It also has uniform coloration on the dorsal and ventral side, with only a slight difference between sides (Fig. 7D). The number of stone canals in *H. mammata* ranges from one to five and zero to six for the right and left sides, respectively. According to our data, *H. mammata* is from the east North Atlantic and the Mediterranean Sea.

CONCLUSION

Based on molecular evidence we have identified three species and, based on morphological data and the scientific literature, we refer to these species as *H. mammata*, *H. tubulosa*, and *H. dakarensis*. *Holothuria stellati* is considered a junior subjective synonym of *H. tubulosa* following the principle of priority defined by the International Commission on Zoological Nomenclature. Although the stepwise discriminant function analysis discriminated *H. dakarensis*, *H. tubulosa*, and *H. mammata*, the morphometric description of the ossicles by the PCA and other morphological characters permitted the differentiation of *H. dakarensis*, but not all the individuals of *H. tubulosa* and *H. mammata*. The large variability observed in these two species meant that some individuals overlapped and correct identification was hindered. Amongst the analysed characters, only the slenderness index of dorsal tables, the height of ventral tables, the size of dorsal and ventral tables, and the dorsal speckled coloration are diagnostic of *H. dakarensis*, and the presence of Cuvierian tubules of *H. mammata*. Nevertheless, this diagnostic character of *H. mammata* is sometimes difficult to assess. Our results show the importance of a molecular approach, compared with a morphological approach, in addressing this taxonomic problem because molecular evidence provides a reference to confirm the range of morphological variability in each species.

ACKNOWLEDGEMENTS

We thank Aaron Herrero Pérez and Jose Antonio García Charton for field assistance; David Pawson, Cynthia Ahearn, and John Ahearn for providing samples from the National Museum of Natural History, Smithsonian, Washington D.C. and facilities for their study; Carolina Espejo and Laura Entrambassaguas for initial ossicle preparation; Fernando Canovas for helping with the analysis; Gustav Paulay

and Didier VandenSpiegel (Aspidochirotid Working Group) for collaborating with methodological and bibliographical information; and in particular all partners of the research group at Murcia University for their many faceted collaboration. We also thank anonymous reviewers' comments on earlier version of this paper. Financial support was provided for the research group 'Ecología y Ordenación de Ecosistemas Marinos Costeros' (University of Murcia, Spain) and supported by the Programme AlBan, the European Union Programme of High Level Scholarships for Latin America, scholarship no. E04D037725CO.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Complete name of the morphometric variables measured for buttons and tables on the dorsal and ventral side. The first lower case letters used for the variables mean the following: m, mean; sd, standard deviation; x, maximum; and n, minimum. These are followed by the variable or index abbreviation (capital letters, Table 2). Finally, dorsal and ventral variables are distinguished by the letters 'd' and 'v', whereas buttons and tables are identified by the letters 'b' and 't'. Example: mAdt is the mean area of the dorsal table disc; mAvtd is the mean area of the ventral tables disc; mAdb is the mean area of the dorsal buttons; mAvb is the mean area of the ventral buttons.

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