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A skeleton-less sponge of Caribbean mangroves: invasive or undescribed?

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Abstract. Recent surveys of sponges occurring on Caribbean mangrove roots demonstrated the presence of a skeleton-less sponge of the genus *Halisarca,* very similar in its morphology to the temperate *H. dujardinii*. This study evaluated the possibility that the mangrove sponge was actually *H. dujardinii* that had been introduced into the Caribbean mangroves. Detailed histology revealed differences between the mangrove sponge and *H. dujardinii* in cuticle thickness, and in characteristics of the choanocytes, spherulous, and granular cells. Also, phylogenetic reconstruction and genetic distance estimates based on cytochrome oxidase I gene sequences clearly differentiated the mangrove *Halisarca* sp. from *H. dujardinii*. Therefore, we rejected the hypothesis of the invasion of *H. dujardinii*, recognizing instead the mangrove *Halisarca* sp. as a new species and naming it *H. restingaensis* sp. nov. Estimated levels of genetic variation in the ribosomal internal transcribed spacers indicated that populations of *H. restingaensis* sp. nov. are highly differentiated between Venezuela and Panama (F_{st}=0.71). This level of population differentiation is consistent with the short larval competence period that is common in members of the genus *Halisarca*.

Additional key words: genetic variability, histology, *Halisarca*, Porifera, phylogeny

Sponge taxonomy relies primarily on morphological features such as overall body structure, shape, size, and color, and on skeletal traits such as the shapes and sizes of spicules or spongin fibers (Hooper et al. 2002). Therefore, species differentiation in Halisarcidae—whose members do not possess spicules—poses a major challenge. This family is represented by only one genus, *Halisarca*; the number of species within this genus is uncertain, with estimates ranging between 15 and 22, in part because of the lack of skeletal characters and because of limited interspecific differentiation in histological and cytological traits (Bergquist & Kelly 2004; Ereskovsky 2007). For instance, the species *Halisarca dujardinii* Johnston 1842 is considered cosmopolitan because of its apparent worldwide distribution in temperate zones (Bergquist 1996; Bergquist & Cook 2002; Bergquist & Kelly 2004). However, Ereskovsky et al. (2011) hypothesized that the "cosmopolitan" *H. dujardinii* actually represents a species complex, basing their argument on morphological differences in cells with inclusions (e.g., spherulous, granular, vacuolar, and microgranular cells) among samples from the White Sea, North Sea, Barents Sea, Bering Sea, and Japan, as well as on up to 0.5% pairwise sequence divergence in the mitochondrial cytochrome oxidase subunit I (COI) gene between White Sea and North Sea samples.

The difficulty in delineating species of skeleton-less sponges has implications extending beyond the realm of systematic inquiry, affecting ecological studies and management initiatives. In particular, reliable taxonomy is crucial when dealing with

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potentially invasive organisms. Biological invasions are on the rise, prompted by global environmental change and unnoticed transportation associated with increasing international trade and other human activities (Levine & d'Antonio 2003; Occhipinti-Ambrogi & Savini 2003). Invasive species tend to have wide, disjunct distributions and are often very abundant in their non-native habitats; as they are often ecological generalists, it is the chance of arriving at a particular locality rather than environmental conditions that limits their abundance and distribution (Lloret et al. 2004).

The only species recognized within the genus *Halisarca* in the Caribbean Sea (excluding the Gulf of Mexico) is the star encrusting sponge, *H. caerulea* Vacelet & Donadey 1989 (Collin et al. 2005). This sponge forms thin layers (~0.2 cm), covers areas of 2–500 cm^2, and is readily distinguishable due to its cobalt blue to violet coloration and a conspicuous star-shaped canal system that surrounds each osculum. *Halisarca caerulea* inhabits coral reefs, growing cryptically in crevices, overhangs, and coral undersides, mostly between 2 and 15 m deep, and is also found occasionally on mangrove roots (Díaz & Rützler 2009). Recent studies revealed the presence of a second Caribbean species of *Halisarca,* almost identical in external features (growth form, coloration, texture, oscula size, and arrangement) to those described for *H. dujardinii*, inhabiting mangrove ecosystems in the Caribbean (Rützler et al. 2000; Díaz 2005; Díaz & Rützler 2009). This mangrove *Halisarca* sp. is very abundant and conspicuous in the surveyed mangroves of Belize, Panama, and Venezuela. Thus, it is surprising that it has not been reported previously in other studies of Caribbean Porifera (Hechtel 1965; Wiedenmayer 1977; van Soest 1978; Alcolado 2002). One possibility could be that this mangrove *Halisarca* sp. is a member of *H. dujardinii* species complex that has invaded the Caribbean Sea only recently. Alternatively, it could be an undescribed species whose presence has remained unnoticed, due either to limited study of mangrove subtidal communities (Rützler et al. 2004) or to a sudden increase in its abundance, possibly in connection with environmental change.

Molecular markers (based on DNA sequences) are required to define species boundaries in sponges when morphological, histological, and biochemical characters do not provide sufficient discriminatory power, as in the genus *Halisarca* (Borchiellini et al. 2000; Boury-Esnault & Solé-Cava 2004; Heim et al. 2007). The genetic marker most used in sponge taxonomy and systematics is the mitochondrial gene COI, which, in most cases, shows levels of variation that can be correlated with species boundaries (e.g., Lopez et al. 2002; Wörheide et al. 2002b; Duran et al. 2004a; Álvarez et al. 2007; Redmond & McCormack 2009). The internal transcribed spacer (ITS) regions located between nuclear ribosomal genes also present levels of genetic variation that have allowed detection of species boundaries and patterns of population differentiation among geographic samples in sponges (e.g., Wörheide et al. 2002b).

The aim of this study was to establish whether the skeleton-less sponge of the Caribbean mangroves is a new species of the genus *Halisarca* or whether its presence in this ecosystem constitutes an invasion by *H. dujardinii*. For this purpose, samples of this sponge were collected from mangrove roots in Venezuela, Belize, and Panama and were used to conduct a comparative morphological analysis, including ultrastructural observations, a phylogenetic analysis using COI sequences, and an analysis of intraspecific genetic variation using ITS sequences.

Methods

Morphological analysis

Observations of living specimens (color, shape, size, surface features, oscules, and consistency) and underwater photographic records were used to describe the external morphology and habitat of the mangrove *Halisarca* sp. from Belize, Venezuela, and Panama (Fig. 1). Three individuals (1–3 cm^2) were collected at Laguna de La Restinga (10°59′N, 64°9′W), Margarita Island, Venezuela, and fixed immediately in Bouin's solution (picric acid 0.9%, formaldehyde 9%, and acetic acid 5%) for 4 h, then dehydrated in a series of ethanol washes (70%, 75%, 80%, 90%, 97%, and 100%) (Muricy & Pearse 2004). Final clearing was performed in three changes of xylene (100%), 1 min each. A Leica microtome was used to make sections (5–8 μm) after embedding in paraffin. The deparaffinized tissue was stained according to a hematoxylin-eosin protocol (Luna 1968).

Cytological data related to specimens from Twin Cays, Belize (16°50′N, 88°06.3′W), were obtained. Samples were fixed in cold (4°C) buffered glutaraldehyde (1.5% in 0.2 mol L^{-1} cacodylate with 0.1 mol L^{-1} sodium chloride and 0.4 mol L^{-1} sucrose, pH 7.2) and stored in the same solution for up to 1 week. Small subsamples were post-fixed in 2% osmium tetroxide in seawater, and then dehydrated. The embedding medium was Spurr low-viscosity epoxy resin (Polysciences, Inc.) mixed for "firm" consistency. Sections (1 μm) for reference light microscopy were stained in 1% aqueous toluidine

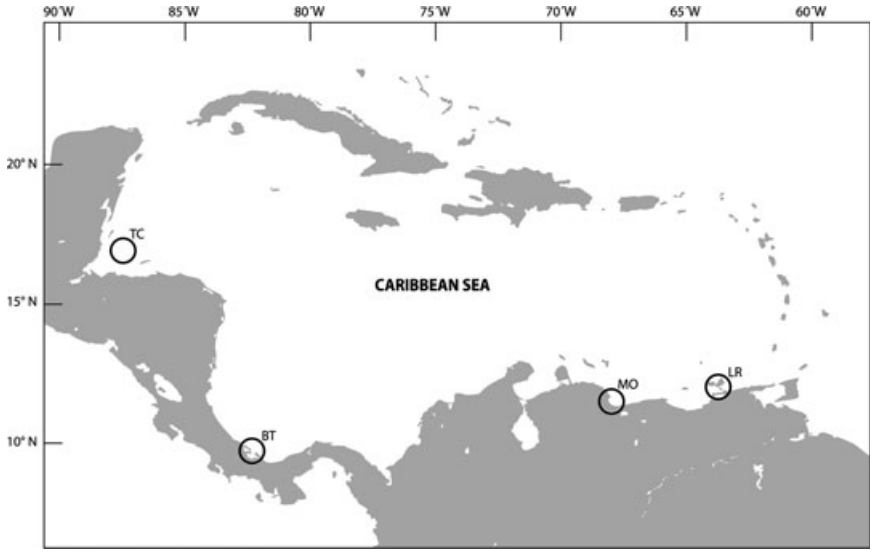

Fig. 1. Map showing the sampling localities where the mangrove *Halisarca* sp. (*H. restingaensis* sp. nov.) was collected. National Park Laguna de La Restinga (LR), National Park Morrocoy (MO), Venezuela; Bocas del Toro (BT), Panama; Twin Cays (TC), Belize.

blue. Thin sections were stained in uranyl acetate-lead citrate and photographed at 3000–30,000× primary magnification using a JEOL 1200 EX transmission electron microscope (TEM) at the National Museum of Natural History, Washington DC.

DNA extraction and amplification

For DNA extraction, 20 samples of the mangrove *Halisarca* sp. were collected haphazardly at two sites (10 samples from each site) in the Laguna de la Restinga (LR; 10°59′N, 64°9′W), and three samples were collected from the National Park Morrocoy (MO; 10°47′N, 68°19′W) on mainland Venezuela, 270 km east of La Restinga National Park (Fig. 1). Three samples were collected from mangroves in Solarte, Panama (BT; 09°17′N, 82°10′W), a site 16 km west of the Bocas del Toro field station of the Smithsonian Tropical Research Institute. Samples of *H. caerulea* were also collected at Bocas del Toro, Panama, from Adriana's Reef (9°14′N, 82°10′W), Hospital Point (9°20′N, 82°13′W), and Buoy 19 (9°18′N, 82°18′W) (Fig. 1). Samples were collected at least 5 m apart to minimize the collection of clonemates. Samples were immediately preserved in 100% ethanol and stored at −20°C within a few hours or days of collection.

DNA was extracted using DNeasy Tissue Kits (Qiagen) following the standard manufacturer directions. From each sample, a fragment of ~650 bp of COI was amplified by the polymerase chain reaction (PCR) with universal metazoan invertebrate primers (Folmer et al. 1994) LCO1490 (forward 5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (reverse 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′). Primers RA2 (forward 5′-GTCCCTGCCCTTTGTACACA-3′) and ITS2.2 (reverse 5′-CCTGGTTAGTTTCTTTTCCTCCGC-3′) were used for amplifying the nuclear ribosomal ITS1-5.8S-ITS2 region (~850 bp) (Wörheide 1998). The 25 μL PCR reactions contained 0.5 μmol L^{-1} of each primer (forward and reverse), 0.2 mmol L^{-1} of dNTPs, 1 U of GoTaq Flexi (Promega) DNA polymerase and buffer provided by the manufacturer, 0.04% bovine serum albumin, 1.5 mmol L^{-1} of $MgCl_2$, and 2–3 ng of template DNA. Thermal cycling conditions were similar to those in Duran et al. (2004a), and consisted of an initial denaturing step of 3 min at 94°C, 37 cycles of denaturing at 94°C for 50 s, annealing (45°C for COI and 54°C for ITS) for 55 s, and extension at 72°C for 1 min, followed by a final extension step of 72°C for 7 min. PCR products were purified by standard isopropanol/ethanol precipitation and directly sequenced in both directions using ABI BigDye technology at the Center for Sequencing and Analysis of Nucleic Acids at the Venezuelan Institute for Scientific Research.

Phylogenetic analysis

Sequencher 4.10.1 (Gene Codes Corporation) was used for the editing and assembly of forward and reverse sequences. Sequences were checked for possible contamination by comparing them against sequences in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence alignment was

performed using ClustalW in Mega 5.0 (Tamura et al. 2011). In addition to the sequences obtained in this study, the alignment included sequences of COI from *Halisarca* species available from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), including one of *H. harmelini* Ereskovsky et al. 2011 from the Mediterranean (NC014876), and two sequences of *H. dujardinii*, one from the White Sea (NC010212) and the other from the North Sea (HQ607143).

Nucleotide diversity, the average substitutions per site, and the number and relative frequency of haplotypes were calculated with DnaSP 5.10.01 (Librado & Rozas 2009). The amount of divergence among sequences was estimated with p distance (Nei & Kumar 2000), which is the proportion of different nucleotides between two sequences, using Mega 5.0 (Tamura et al. 2011). A maximum parsimony analysis, also implemented in Mega 5.0, was used to explore phylogenetic relationships among the species of *Halisarca* for which COI sequences were available. Statistical support for the nodes in the phylogenetic tree was evaluated with a bootstrap re-sampling method of 1000 iterations, using the max-min branch-and-bound method, considering all parsimoniously informative sites. A COI sequence of *Chondrilla nucula* Schmidt 1862 (DQ086813.1) was used as outgroup for rooting the phylogenetic tree; this is the most closely related Myxospongiae for which there is an available COI sequence (Borchiellini et al. 2004; Sperling et al. 2007). The consensus tree was obtained using a 70% cut-off for node support and the majority rule criterion (Nei & Kumar 2000). A saturation index was estimated using FalseTest in DAMBE (Xia & Xie 2001; Xia et al. 2003). To corroborate the results of the maximum parsimony analysis, maximum likelihood and neighbor-joining phylogenies were constructed in Mega 5.0 using the T92+G model of nucleotide substitutions, selected with the "Find Best DNA/Protein Models" algorithm, with default parameters and 1000 iterations of bootstrap re-sampling.

Intraspecific analysis of genetic variation

A maximum parsimony network was constructed using TCS 1.21 (Clement et al. 2000) to study the connections among ITS types of the mangrove *Halisarca* sp. This method is based on an algorithm that estimates the maximum number of nucleotide differences between haplotypes, which can be attributed to a series of simple mutations at each site. This number is referred to as the limit of parsimony (Clement et al. 2000). The haplotypes are connected, forming a network; the distance among them corresponds to the number of mutational steps required to evolve from one to another, estimated using coalescent theory (Freeland 2005). Haplotypes that differ by a number of nucleotides exceeding the limit of parsimony will not be connected to the haplotype network. In addition, an analysis of molecular variance (AMOVA) implemented in ARLEQUIN 3.5 (Excoffier & Lischer 2010) was used to determine whether the largest component of variance occurred between geographic localities or among individuals within each locality.

Results

Morphological comparison

Comparisons of the morphological features of the mangrove *Halisarca* sp. with other species in the genus (Table 1) showed that it is distinct from *H. dujardinii* in nucleus dimensions, cuticle thickness, and characteristics of the choanocytes, spherulous, and granular cells. Additional details on these morphological features are given below in the systematic account.

Phylogenetic relationships

Twenty-six sequences of COI were obtained from samples of the mangrove *Halisarca* sp. collected in Venezuela and Panama. These sequences consisted of only two haplotypes (haplotype diversity=0.166±0.098) that differed at two positions; both differences were C-T transitions that result in synonymous substitutions (Table S1). All samples from Venezuela and one individual from Panama contained Haplotype 1 (H1, GenBank accession number HQ602685.2), whereas Haplotype 2 (H2, JN971065.1) was present only in samples from Panama. The nucleotide diversity (π) was 0.00051 (±0.00033), and the genetic distance between haplotypes was 0.2%.

The six COI sequences of *H. caerulea* obtained *de novo* from Panama were identical. The alignment of the COI sequences of the mangrove *Halisarca* sp. with those of the other three *Halisarca* spp. with COI sequences available in GenBank was 596 bp long and contained 107 variable sites, 75 of which were parsimony informative (Table 2, Table S1).

The maximum parsimony phylogeny grouped the *Halisarca* spp. into two major clades (Fig. 2a). Clade I included the star encrusting sponge of Caribbean reefs, *H. caerulea*, with *H. harmelini* from the Mediterranean Sea. Clade II contained two subclades, IIA and IIB, which contained respectively

Table 1. Comparative summary of traits that distinguish the mangrove *Halisarca* sp. (=*H. restingaensis* sp. nov.) and some other species in the genus *Halisarca*. Important differences among species are highlighted in bold. NA, data not available.

Trait	Mangrove *Halisarca* sp. (= *H. restingaensis* sp. nov.)	*H. dujardinii*	*H. caerulea*	*H. purpura*
Distribution	Caribbean Sea	**NE Atlantic, SW Pacific**	Caribbean Sea	N. Gulf of Mexico, Caribbean Sea
Habitat	**Roots of *Rhizophora mangle*, mollusks**.	Hard substrate, algae, ascidians, mollusks	Dead parts of corals; cryptic	Turtlegrass *Thalassia testudinum*
Depth (m)	1–2	1–70	2–15	1
Color in life	Cream to pale yellow	Yellow-beige to pale brown	**Cobalt blue to violet**	**Purplish-red**
Consistency	Soft, delicate, slightly elastic	Soft, gel-like, slightly elastic	Soft, resilient, fleshy	Soft, almost colloidal
Surface texture	Smooth, slippery	Smooth, slippery	Smooth, somewhat slimy	**Smooth, lobate**
Overall thickness (mm)	1–2	1–5	1.2–1.5	**0.4–0.8**
Ectosome thickness (μm)	25–100	>24; three layered	25–80; reticulate with fibrous collagen	**600–700**
Cuticle thickness (μm)	**2–6**	**0.5–2**	NA	NA
Choanocyte chamber length×diameter (μm)	**40–170×18–40**	**250–600×24–100**	**Up to 200×25**	**18–80x12–30**
Choanocyte length×diameter (μm)	**Cylindrical, 4–7×2.5–3**	**Cylindrical or trapeziform, 6–9.5×3.5–5**	**Irregular shape, 4–5×2.5–3**	NA
Number of microvilli	33–40	38–40	NA	NA
Nucleus dimensions (μm)	**Spherical, 1–1.8 diameter**	**Pyriform, 2.5×4.8**	**2 diameter**	NA
Granular cell diameter (μm)	**Irregular shape, 4.5**	**Up to 8**	**Rare, 6–8**	NA
Rhabdiferous cell diameter (μm)	Not present	Not present	**Rare, round, 6–7; inclusions 0.8–1.6×0.3–0.6 μm**	NA
Vacuolar cell dimensions (μm)	NA	6.9×10.6, vacuoles 1.7×2.9 μm	Not present	NA
Spherulous cell diameter (μm)	**Abundant in mesohyl, 6–8; spherules 2–5.5**	**Abundant, 8–12; spherules 2–8**	**12; spherules 3–4**	NA
Symbiotic bacteria size (μm)	Curved spiral form, 0.25–0.5	Curved spiral form, 0.45	**Small rods, 0.25**	NA
Bacteriocyte diameter (μm)	**Spherulous cells with oval bacteria, 1.3–5**	Not present	Not present	NA
References	This paper	Ereskovsky (2007), Gonobobleva & Maldonado (2009), Ereskovsky et al. (2011)	Vacelet & Donadey (1987)	Little (1963), van Soest (2012)

Table 2. Number of polymorphic sites (PS), number of conserved sites (CS), and genetic p distance (GD%) observed in mitochondrial cytochrome oxidase subunit I (COI) gene sequences among four species of *Halisarca*.

Species	(PS; CS; GD%) Mangrove *Halisarca* sp. (= *H. restingaensis* sp. nov.)	*H. caerulea*	*H. harmelini*
H. dujardinii	45; 610; 6.5	81; 574; 12.1	67; 517; 11.5
Mangrove *Halisarca* sp. (= *H. restingaensis* sp. nov.)		74; 581; 11.1	74; 581; 11.1
H. caerulea			35; 620; 5.5

the two haplotypes of mangrove *Halisarca* sp., and the two haplotypes of *H. dujardinii* (from the White and North Seas). This tree topology was well-supported, as indicated both by high bootstrap values for the nodes and by a high consistency index (0.95), indicating that most parsimony informative sites were congruent. The level of homoplasy was low, as indicated by a relatively high retention index of 0.8, along with an adequate saturation index (Iss=0.58<Issc=0.77; p<0.05).

The genetic distance between *H. caerulea* and *H. harmelini* was 5.5%. The distance between the haplotypes of mangrove *Halisarca* sp. (in clade IIA) was 0.2%, while the distance between the haplotypes of *H. dujardinii* (in clade IIB) was 0.5%. The distance between subclades IIA and IIB was 6.5%. Estimates of genetic differentiation between *H. dujardinii* and mangrove *Halisarca* sp. were $N_{st} = 0.95$ and $F_{st} = 0.94$.

Both the maximum likelihood and neighbor-joining reconstructions of the phylogeny showed similar topologies to the one just described (Fig. 2b). However, these analyses did not group *H. caerulea* with *H. harmelini*.

Intraspecific genetic variation

Within the mangrove *Halisarca* sp., the alignment of 26 sequences of ITS rDNA consisted of 815 bp, containing 36 variable sites, 13 of which were parsimony informative (π=0.00594±0.00211) (Table 3, Table S2). These sequences were grouped in 13 ribotypes (diversity=0.872±0.052) (GenBank accession numbers JX262407–JX262419). The genetic distance was smaller within Venezuela (Table 3), even though the sample size was much larger than in Panama (*N*=23 vs. *N*=3, respectively).

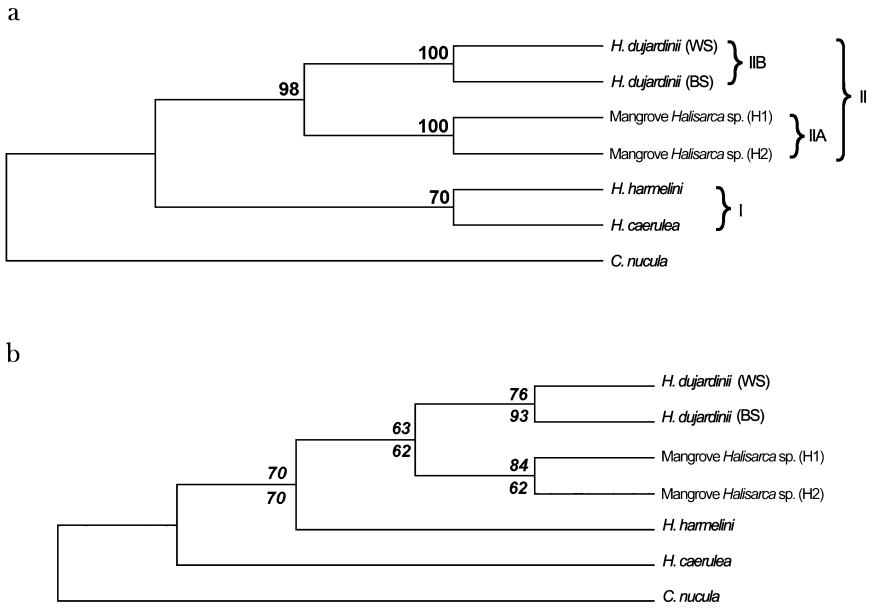

Fig. 2. Phylogenetic reconstruction of *Halisarca* species using the mitochondrial gene cytochrome oxidase I, constructed with T92+G model. **a**. Maximum parsimony tree. Bootstrap values are shown above each node. IC=0.95; IR=0.8; tree size=429. **b**. Maximum likelihood and neighbor-joining tree (the two methods yielded identical topologies). Maximum likelihood bootstrap values are shown above each node, and neighbor-joining bootstrap values are shown below each node.

Table 3. Number of individuals (*N*), number of ribotypes (*n*), ribotype (*r*) and nucleotide (π) diversity estimates, and genetic p distances (GD%) observed in the internal transcribed spacer rDNA region of the mangrove *Halisarca* sp. (=*H. restingaensis* sp. nov.) from three localities (LR=National Park Laguna de La Restinga, MO=National Park Morrocoy, BT=Bocas del Toro). Estimates of variance are standard deviations.

Locality	*N*	*n*	*r*	π	GD%
LR	20	9	0.789±0.086	0.0021±0.0004	0.2±0.001
MO	3	2	0.067±0.314	0.0008±0.0003	0.1±0.001
BT	3	3	1.0	0.023±0.007	2.4±0.004

The parsimony ribotype network indicated that the most ancestral ribotype in the sample was R4, from a sample collected in La Restinga, Venezuela, and that it was connected directly to other four ribotypes (Fig. 3). Ribotypes 11 and 12 were found in samples from Panama and were most distantly related to R4; R12 was connected to R3 by three mutational steps, and R11 was connected to R12 by 13 mutational steps. Ribotype 13 corresponded with the third sample from Panama and was so divergent that it could not be connected to the network (i.e., the number of nucleotide differences attributable to single step mutations in relation to the other ribotypes was above the parsimony limit).

The AMOVA indicated that the largest component of variation, 65.27%, was due to differences between the localities (Venezuela and Panama), while 28.14% of the variation was attributed to differences among individuals within the localities; the remaining 6.59% was due to differences between sampling points within localities (La Restinga vs. Morrocoy) (Table 4). The observed genetic differentiation between localities was high ($F_{st} = 0.71$) and statistically significant ($p<0.001$).

Systematics

Overall, morphology, phylogenetic analyses, and the genetic distances among species and clades reject the hypothesis that the mangrove *Halisarca* sp. is in fact a population of *H. dujardinii* that has invaded the Caribbean mangroves. These results support the alternative hypothesis that the mangrove *Halisarca* sp. constitutes a distinct, new species in the genus,

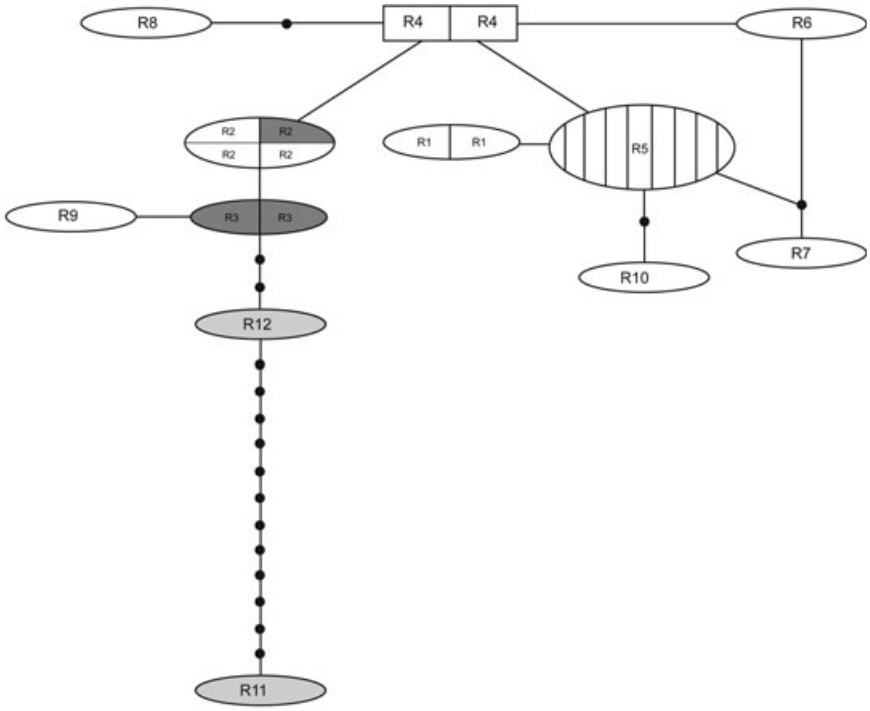

Fig. 3. Parsimony ribotype network using the internal transcribed spacer region of *Halisarca restingaensis* sp. nov. The area of the ellipses is proportional to the frequency of the ribotypes, and the number of internal subdivisions indicate the exact number of sequences from individual samples that shared that ribotype; the square denotes the inferred ancestral ribotype. Small dots along lines indicate unsampled ribotypes. Shading denotes the locality at which samples were collected: white corresponds to La Restinga, Venezuela; light gray to Bocas del Toro, Panama; and dark gray to Morrocoy, Venezuela.

Table 4. Results of analysis of molecular variance (AMOVA) among and within localities where the mangrove *Halisarca* sp. (=*H. restingaensis* sp. nov.) was sampled. Df=degrees of freedom, Π_{CT}=variance among groups, Π_{SC}=variance among population within groups, Π_{ST}=variance within populations.

Source of variance	Df	Covariance	% Total	Fixation indices	p value
Among localities	1	3.58	65.27	Π_{CT}=0.65	<0.001
Among sampling points within localities	1	0.36	6.59	Π_{SC}=0.19	<0.01
Among individuals	24	1.55	28.14	Π_{ST}=0.72	<0.001
Total	26	5.49			

more closely related to *H. dujardinii* than to the other Caribbean species, the star encrusting sponge *H. caerulea*. Given that we first distinguished mangrove *Halisarca* sp. from *H. dujardinii* with individuals from "Laguna La Restinga," Venezuela, we here name it *Halisarca restingaensis*.

Phylum Porifera Grant 1836
Class Demospongiae Sollas 1885
Subclass Verongimorpha Erpenbeck et al. 2012
Order Chondrosida Boury-Esnault & Lopès 1985

Diagnosis. Encrusting to massive, with a marked ectosome or cortex enriched by a highly organized fibrillar collagen. The skeleton is composed of nodular spongin fibers or aster microscleres, or is absent. Collagen is always very abundant (Cárdenas et al. 2012).

Family Halisarcidae Schmidt 1892
Genus *Halisarca* Johnston 1842

Diagnosis. "Halisarcidae with choanocyte chambers are tubular, branched, and wide mouthed. Larvae are incubated dispherulae (a larval form found only in *Halisarca*) with simple undifferentiated histology and cilia of uniform length. Skeleton is composed of fibrillar collagen only, there are no fibrous or mineral elements present; ectosomal and subectosomal collagen is highly organized and structurally diversified" (Ereskovsky et al. 2011).

Type species. *Halisarca dujardinii* Johnston 1842

Halisarca restingaensis sp. nov.

Material examined. Holotype: MMM 145 (Museo Marino de Margarita), Laguna de La Restinga, Margarita Island, Venezuela (10°59′N, 64°9′W), 1 m depth, attached to roots of *Rhizophora mangle* L., April 2010, collector A. Alvizu. Paratypes: MMM 146-147, Laguna de La Restinga, Venezuela (10°59′ N, 64°9′W), 1.5 m depth, attached to roots of *R. mangle*, April 2010, collector A. Alvizu. MMM 148-149, Solarte Island, Bocas del Toro, Panama (9º21′ N, 82°15′W), attached to roots of *R. mangle*, July 2010, collector M.C. Díaz. USNM (Smithsonian National Museum of Natural History) 42954 [KR86-40], Grouper Gardens, Twin Cays, Belize (16°49′48″N, 88°06″11″W), 0.5 m depth, on mangrove prop root, April 1986, collectors K. Rützler and K.P. Smith. KR00-25, Hidden Creek, Twin Cays, Belize (16°49′48″N, 88°06″11″W), 1 m depth, on mangrove prop root, November 2000, collector K. Rützler. USNM KR97.08-10, Hidden Creek, Twin Cays, Belize (16°49′48″N, 88°06″11″W), 1 m depth, on mangrove prop root, August 1997, collector K. Rützler.

Synonymy. *Halisarca* sp.: Díaz et al. 2004: 5; Rützler et al. 2004: 27; Collin et al. 2005: 643; Díaz 2005: 468; Díaz & Rützler 2009: 162.

Etymology. This species is named after the coastal lagoon of La Restinga (Margarita Island, Venezuela), due to its abundance among the epibionts of *R. mangle* there, and given that we discovered its distinctness using material collected there.

Description. The sponges examined form extensive sheets (>10 cm^2 in area coverage per specimen) over the submerged stilt roots of the red mangrove, *R. mangle*. The tissue body is 1–2 mm thick and very delicate; its surface is smooth and slippery. Oscula, when visible, are rounded, small (1–3 mm in diameter), and slightly elevated (Fig. 4). Radiating canals may be

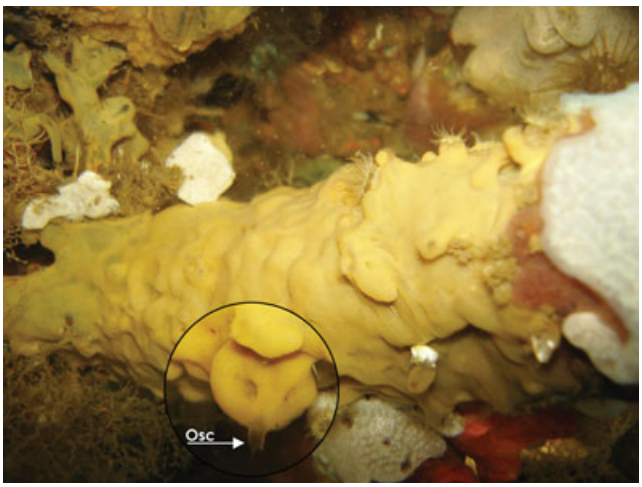

Fig. 4. *Halisarca restingaensis* sp. nov. *in situ* growing over a root of *Rhizophora mangle*. An osculum is visible in the enlarged inset (Osc). The image is of a sponge in the National Park Laguna de La Restinga, Venezuela.

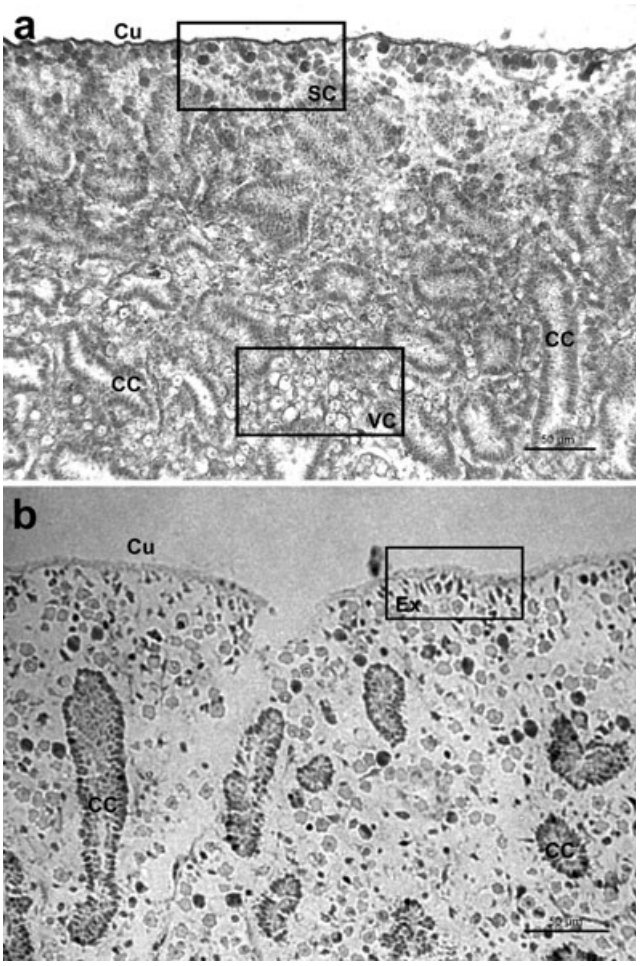

Fig. 5. Histological sections of the ectosome and choanosome of *Halisarca restingaensis* sp. nov. stained with (**a**) hematoxylin-eosin and (**b**) toluidine blue. CC, choanocyte chambers; Cu, cuticle; Ex, exopinacocytes; SC, spherulous cells; VC, vacuolar cells.

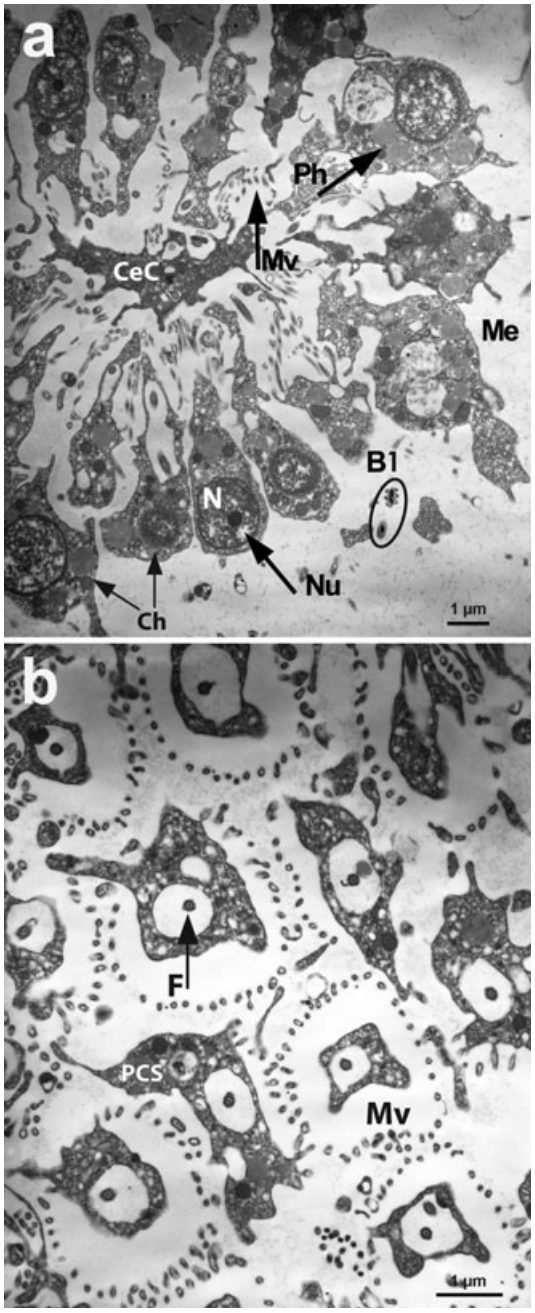

Fig. 6. Transmission electron micrographs of *Halisarca restingaensis* sp. nov. **a**. Transverse section of a choanocyte chamber. **b**. Cross-section of choanocytes. B, bacteria; CeC, central cell; Ch, choanocyte; F, flagellum; Me, mesohyl; Mv, microvilli; N, nucleus; Nu, nucleolus; PCS, periflagellar cytoplasmic sleeve; Ph, phagosomes.

distinguished *in situ* and are very thin and shallow (<1 mm wide). Color in live specimens ranged from cream to pale yellow, both internally and externally.

Histological observations. A heavily stained collagenous layer 2–6 µm in thickness covers the sponge surface (Fig. 5a). In material stained with toluidine blue, this cortical layer appears to be made up of fibrillous collagen, with a low cell density (Fig. 5b). In this section and underneath the cortical layer, the elongated cell bodies of the exopinacocytes (0.5–3 µm in diameter) are observed (Fig. 5b). Underneath and between the exopinacocytes, cells with dark or transparent inclusions (spherulous and vacuolar cells, respectively) are abundant (Fig. 5a,b). In the choanosome, tubular elongated choanocyte chambers and the mesohyl with various mesohylar cells can be distinguished. The mesohyl is predominantly occupied by cells with dark inclusions (spherulous cells), as well as other cells with clear inclusions that could be vacuolar cells. Some of these cells are described more fully in the cytology section below. The choanocyte chambers are abundant and tubular, 40–170 µm in length and 18–40 µm in width, and branch rarely (Fig. 5a,b).

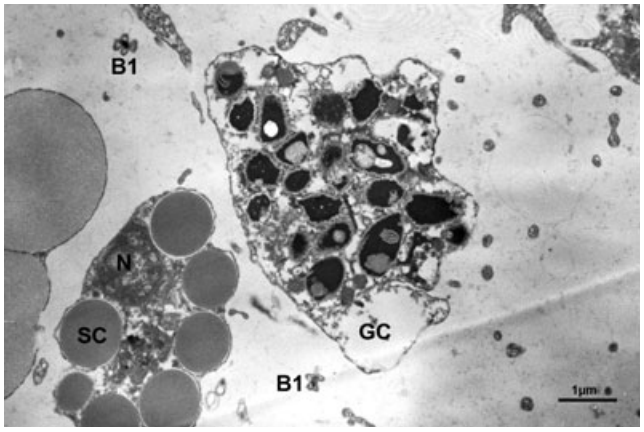

Fig. 7. Transmission electron micrograph of an inclusion-containing cell of *Halisarca restingaensis* sp. nov. B, bacteria; GC, granular cell; SC, spherulous cell; N, nucleus.

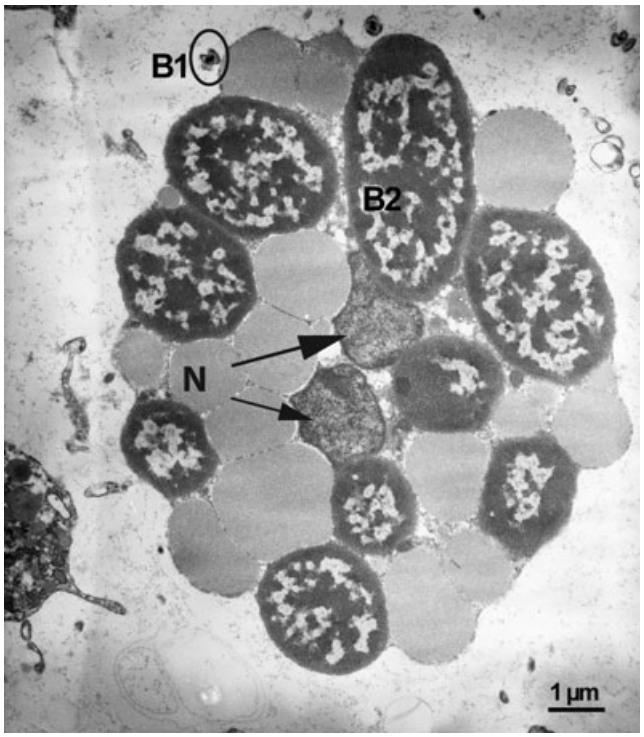

Fig. 8. Transmission electron micrograph of a spherulous cell of *Halisarca restingaensis* sp. nov. with bacteria-like inclusions (a bacteriocyte). B1, symbiotic extracellular bacteria; B2, intracellular bacteria; N, nucleus.

Cytological observations. Choanocytes are cylindrical, measuring 4–7 µm in length and 2.5–3 µm in width. Their collars consist of 33–40 microvilli surrounding the apical flagellum (Fig. 6a). Most choanocytes have a periflagellar cytoplasmic sleeve (Fig. 6b). The choanocyte cytoplasm contains abundant phagosomes, and the cell nucleus is spherical (1–1.8 µm in diameter), and occupies a basal position. In some instances, the nucleolus could also be seen (Fig. 6a).

The exopinacocytes lying underneath the collagenous cortical layer (Fig. 5b) are T-shaped in cross-section, and measure 7.9–10 µm in length and 0.5–3.8 µm in diameter (Fig. S1).

Spherulous cells are abundant in the ectosomal and mesohyl regions; they are irregular in shape (6–8 µm in diameter) and are filled with electron-dense inclusions (2–5.5 µm in diameter). The nuclei and nucleoli of the spherulous cells are sometimes visible (Fig. 7). Some spherulous cells also contain intracellular oval bacteria (1.3–5 µm in diameter) (Fig. 8). These cells are identified here as bacteriocytes, a term that refers to cells containing spherule-like inclusions and oval bacteria. These bacteriocytes have not previously been reported in any of the three *Halisarca* spp. on which ultrastructural studies have been carried out. Another type of bacteria found is a symbiotic spiral-curved bacterium (0.25–0.5 µm in diameter; Figs. 6a, 7, and 8), which seems to be exclusively extracellular.

Granular cells are less abundant, but also present in the mesohyl. Their shape is irregular, with a mean diameter ~4.5 µm, and dark, oval or irregular electron-dense homogeneous granules 0.5–3.5 µm in diameter fill their cytoplasm (Fig. 7).

Distribution. *Halisarca restingaensis* sp. nov. has been reported associated with the roots of the red mangrove, *R. mangle*, in Venezuela, Panama, and Belize (Rützler et al. 2004; Collin et al. 2005; Díaz & Rützler 2009). *Halisarca restingaensis* sp. nov. is one of the most abundant species in the Caribbean mangroves, and its abundance does not fluctuate greatly through time like that of other dominant species (Díaz & Rützler 2009).

Discussion

The results of the analysis of morphology confirm that *H. restingaensis* sp. nov. is similar to *H. dujardinii*, and very different from other species from the Caribbean Sea (Table 1). However, *H. restingaensis* sp. nov. differs in ecological characters (e.g., habitat and geographical distribution) from all species of *Halisarca* listed by Ereskovsky et al. (2011) in their comparative table. Histological observations also indicate important differences between *H. restingaensis* sp. nov. and other known species in the genus. The histological traits unique to *H. restingaensis* sp. nov. are a thin ectosome, a thick cuticle, the absence of a cortex, and smaller choanocyte

chambers (Table 1). Members of *H. restingaensis* sp. nov. also have some unique cytological characteristics (Table 1), including smaller choanocytes and their nuclei, smaller granular and spherulous cells, and the presence of intracellular bacteriocytes. There are some similarities between *H. restingaensis* sp. nov. and other members of the genus, of course, including the presence of a periflagellar cytoplasmic sleeve in *H. restingaensis* sp. nov., *H. dujardinii* and *H. caerulea* (Vacelet & Donadey 1987; Ereskovsky et al. 2011), and the presence of the symbiotic bacteria like those described by Ereskovsky et al. (2011) in both *H. restingaensis* sp. nov. and *H. dujardinii*.

The phylogenetic analysis grouped *H. restingaensis* sp. nov. with *H. dujardinii*, and separated these two species from the star encrusting sponge *H. caerulea* from the Caribbean coral reefs, which in turn was grouped together with *H. harmelini* from the Mediterranean. This phylogeny was congruent with that proposed by Ereskovsky et al. (2011), which also separated *H. dujardinii* and *H. harmelini* in different clades. This topology alone could have been interpreted as confirmation of the idea that *H. restingaensis* sp. nov. was indeed *H. dujardini*. However, the genetic distance observed here between *H. restingaensis* sp. nov. and *H. dujardinii* was 6.5%. This level of genetic difference is 13 times that estimated between the two haplotypes of *H. dujardinii* (0.5%) currently deposited in GenBank, which Ereskovsky et al. (2011) argued should be considered different species. Moreover, the genetic distance between *H. restingaensis* sp. nov. and *H. dujardinii* was larger than the distance between the star encrusting sponge *H. caerulea* from the Caribbean and *H. harmelini* from the Mediterranean Sea (5.5%), which undoubtedly constitute separate species. In terms of number of base pairs, the difference between *H. restingaensis* sp. nov. and *H. dujardinii* was 45 bp, while the difference between *H. caerulea* and *H. harmelini* was 35 bp. These differences between *Halisarca* spp. are much larger than those among the species in the *Cliona celata* complex (0.1–0.5%, Xavier et al. 2010), between the two species of Caribbean fire sponges (*Tedania ignis* Duchassaing & Michelotti 1864 and *T. klausi* Wulff 2006: 0.9%) and between species in the sponge genus *Ircinia* (2.7%, Pöppe et al. 2010). However, the distance between species in the sponge genus *Scopalina* (13–22%, Blanquer & Uriz 2007) is larger than the genetic difference between *H. restingaensis* sp. nov. and *H. dujardinii*, although it has to be taken into account that the genus *Scopalina* is a paradigmatic example of highly structured populations and restricted dispersal (Uriz et al. 1998). The level of genetic differentiation (using COI) between *H. restingaensis* sp. nov. and *H. dujardinii* estimated by N_{st} (0.95) and F_{st} (0.94) also indicates that there is no gene flow among these biological entities.

In some instances, the current geographic distribution of sponge species is consistent with the phylogeny of a particular group, as in the genera *Scopalina* and *Aplysina*, where the Caribbean species form a separate clade from the species inhabiting the Mediterranean (Blanquer & Uriz 2007; Heim et al. 2007). The phylogeny of the genus *Halisarca* clearly does not follow this pattern, as the Caribbean species were grouped separately with species from the Mediterranean and other temperate regions. Unfortunately, the slow rate of change of most mitochondrial genes in Porifera, relative to other metazoans, has made it impossible to obtain a reliable molecular clock for this phylum (Huang et al. 2008). Thus, speciation in the genus *Halisarca* cannot be timed precisely, and it is currently impossible to associate it with geological events as in other animals.

The low level of COI variability within *H. restingaensis* sp. nov. (π=0.00051) is similar to that observed in other viviparous sponges, such as *Crambe crambe* Schmidt 1862 (π=0.00058; Duran et al. 2004a) and *Astrosclera willeyana* Lister 1900 (π=0.00049; Wörheide 2006). This is in contrast with other oviparous sponge species such as *Callyspongia vaginalis* Lamarck 1814 (π=0.0132; DeBiasse et al. 2010), *Cliona celata* Grant 1826 (π=0.057; Xavier et al. 2010), and *Xestospongia muta* Schmidt 1870 (π=0.00386; Lopéz-Legentil & Pawlik 2009), which display levels of variation in COI that are one to two orders of magnitude greater than those in *H. restingaensis* sp. nov.

On the other hand, the level of intraspecific genetic variation within *H. restingaensis* sp. nov. estimated with the ITS rDNA region (2%) fell within the range reported for other sponge species, e.g., *Leucetta changonesis* Dendy 1913 (2.16%; Wörheide et al. 2008), and *Haliclona cinerea* Grant 1826 and *H. oculata* Pallas 1766 (1.91% and 1.75%; Redmond & McCormack 2009). The level of variation observed in the genus *Halisarca* was higher than in *Axinella corrugata* George & Wilson 1919 (<1%; Lopez et al. 2002), *C. crambe* (1.2%; Duran et al. 2004a), and *A. willeyana sensu stricto* (0.6%; Wörheide et al. 2002a). However, this level of variability was ten times lower than the greatest intraspecific variation estimated for this marker in sponges, 29% in *Acanthella cavernosa* Dendy 1922 (Álvarez et al. 2007).

All of the genetic analyses presented here, including the parsimony ribotype network, the AMOVA,

and the estimate of genetic differentiation (F_{st}), indicate that the samples of *H. restingaensis* sp. nov. in Venezuela and Panama correspond with different populations that are genetically isolated from one another. High levels of genetic isolation are common among sponge populations and have been observed both in Mediterranean (*C. crambe* and *Scopalina lophyropoda*) Schmidt 1862 (Duran et al. 2004b; Blanquer & Uriz 2010) and Caribbean species (*Callyspongia vaginalis* and *Xestospongia muta*) (Lopéz-Legentil & Pawlik 2009). This phenomenon has been attributed to the short larval dispersal distances and short competency periods reported in most species of sponges (Ilan & Loya 1990; Ereskovsky & Gonobobleva 2000; Mariani et al. 2000; Whalan et al. 2005; Maldonado 2006; Richards et al. 2007). The competency period for the larvae of *H. dujardinii* is <24 h (Mukhina et al. 2007). Although similar studies are still needed, it is likely that *H. restingaensis* sp. nov. has a similarly short competency period.

Conclusions

The results presented here indicate that the skeleton-less sponge of the Caribbean mangroves is not an invasive population of the widely distributed and morphologically similar *H. dujardinii*, but is in fact a new species described and named here as *H. restingaensis* sp. nov. Nevertheless, this study is limited, as it only compared *H. restingaensis* sp. nov. with the Mediterranean-Eastern Atlantic species and with co-occurring species in the Caribbean. Genetic information is lacking for most species in the genus. We expect that future studies will provide more detailed information on the cytology, biology, and ecology of *H. restingaensis* sp. nov., and will determine its affinities with the other ~15 species in the genus (Ereskovsky et al. 2011).

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Supporting information

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

Table S1. Variable sites in the cytochrome oxidase I gene in species of the spicule-less sponge genus *Halisarca* and in *Chondrilla nucula*.

Table S2. Variable sites of the ribosomal DNA types (ribotypes) of *Halisarca restingaensis*.

Fig. S1. Cross-section of exopinacocytes with transversal polygonal shape. Exopinacocyte (Ex), cuticle (Cu), choanocyte chambers (CC).