

Phenotypic plasticity in the Caribbean sponge *Callyspongia vaginalis* (Porifera: Haplosclerida)

SUSANNA LÓPEZ-LEGENTIL^{1,2}, PATRICK M. ERWIN¹, TIMOTHY P. HENKEL¹,
TSE-LYNN LOH¹ and JOSEPH R. PAWLIK¹

¹Center for Marine Science, University of North Carolina Wilmington, 5600 Marvin K. Moss Lane, Wilmington NC 28409, USA. E-mail: susanna@univ-perp.fr

²Current Address: Department of Animal Biology (Invertebrates), Faculty of Biology, University of Barcelona, 645 Diagonal Avenue, 08028 Barcelona, Spain.

SUMMARY: Sponge morphological plasticity has been a long-standing source of taxonomic difficulty. In the Caribbean, several morphotypes of the sponge *Callyspongia vaginalis* have been observed. To determine the taxonomic status of three of these morphotypes and their relationship with the congeneric species *C. plicifera* and *C. fallax*, we compared the spicule composition, spongin fiber skeleton and sequenced fragments of the mitochondrial genes 16S and COI and nuclear genes 28S and 18S ribosomal RNA. Phylogenetic analyses with ribosomal markers 18S and 28S rRNA confirmed the position of our sequences within the Callyspongiidae. None of the genetic markers provided evidence for consistent differentiation among the three morphotypes of *C. vaginalis* and *C. fallax*, and only *C. plicifera* stood as a distinct species. The 16S mtDNA gene was the most variable molecular marker for this group, presenting a nucleotide variability ($\pi = 0.024$) higher than that reported for COI. Unlike recent studies for other sponge genera, our results indicate that species in the genus *Callyspongia* maintain a high degree of phenotypic plasticity, and that morphological characteristics may not reflect reproductive boundaries in *C. vaginalis*.

Keywords: sponge, spicule, COI mtDNA, 16S mtDNA, 18S rRNA, 28S rRNA, morphotypes, *Callyspongia*, phenotype.

RESUMEN: PLASTICIDAD FENOTÍPICA DE LA ESPONJA *CALLYSPONGIA VAGINALIS* (PORIFERA: HAPLOSCLERIDA). – La gran plasticidad morfológica de ciertas esponjas dificulta una correcta clasificación taxonómica. En el Caribe, se han observado varios morfotipos de la esponja *Callyspongia vaginalis* a nivel de colores y formas. Con el fin de determinar su clasificación taxonómica, se muestrearon y analizaron tres morfotipos de *C. vaginalis* y sus especies congénicas *C. plicifera* y *C. fallax*. Para cada muestra, se observó la composición espicular y del esqueleto dermal y se secuenciaron parte de los genes mitocondriales 16S y COI y parte de los genes ribosomales 28S y 18S. Los análisis filogenéticos con los genes ribosomales 18S y 28S confirmaron la posición taxonómica de las secuencias obtenidas. Ninguno de los marcadores genéticos utilizados reveló diferencias consistentes entre los tres morfotipos de *C. vaginalis* y *C. fallax*, y sólo *C. plicifera* apareció en los análisis como una especie distinta. El gen mitocondrial 16S fue el marcador molecular más variable para este grupo, presentando una variabilidad nucleotídica ($\pi = 0.024$) superior a la descrita para COI. Nuestros resultados indican que las especies del género *Callyspongia* presentan una gran plasticidad fenotípica y que estas diferencias morfológicas no suponen barreras reproductivas para *C. vaginalis*.

Palabras clave: esponja, espícula, 16S mtDNA, 18S rRNA, 28S rRNA, COI mtDNA, morfotipo, plasticidad fenotípica, *Callyspongia*.

INTRODUCTION

Many sessile benthic marine invertebrates exhibit variability in size, shape and color. This intra-specific variability has been a long-standing source of

taxonomic difficulty and has important implications in associated fields, including ecological research, biodiversity management and the identification of new pharmacologically active substances from invertebrate tissues (Holland, 2000; Miller *et al.*, 2001). Intra-

specific morphological diversity is often associated with differences in local environmental conditions or with genetic divergence. The advent of molecular techniques has provided an objective means of testing these two hypotheses (e.g. Klautau *et al.*, 1999; Miller *et al.*, 2001; López-Legentil and Turon, 2005; Blanquer and Uriz, 2007), often revealing a genetic basis for variable morphology. In fact, studies have uncovered the presence of sibling species in several groups of marine organisms (reviewed in Knowlton, 2000).

Sponges are a particular group in which morphological simplicity and phenotypic plasticity has led to difficulties in species identification (Knowlton, 2000). Taxonomic methods for identification are generally based on skeletal features (e.g. spicule morphology and fiber arrangements) and external morphology (e.g. color, texture, and growth form). These characteristics are often not diagnostic beyond the genus level and show high levels of intra-specific variability (e.g. Maldonado and Uriz, 1996; Erwin and Thacker, 2007a).

The genus *Callyspongia* (Demospongiae: Haplosclerida) includes species found in both the Indo-Pacific and the Caribbean (Wiedenmayer, 1977; Zea, 1987; Voogd, 2004). The high degree of variability in structural characteristics—including spicule composition and spongin fiber arrangements—within the genus has resulted in considerable taxonomic confusion (Wiedenmayer, 1977; Voogd, 2004). On many coral reefs in the Caribbean, *C. vaginalis* (Lamarck 1814) is among the most abundant sponge species (Pawlik *et al.*, 1995), and is typically encountered as one to several grey tubes with small conical projections that are often covered by the zoanthid *Parazoanthus* sp. (Zea, 1987). However, other morphologies have been observed, varying in both surface coloration and growth form (Zea, 1987). In a recent survey of the artificial reef shipwreck *USS Spiegel Grove* in Key Largo, Florida (Pawlik *et al.*, 2008), three morphotypes of *C. vaginalis* were especially abundant: a grey morph with a smooth surface and small conical projections, identical to the usual morph found on nearby reefs, a red morph with a surface with convoluted ridges (similar to that seen in *C. plicifera*) and thicker tube walls than the grey morph, and an orange morph with the same convoluted surface and shape as the red morph.

In this study, we analyzed the variation in a fragment of the mitochondrial genes COI and 16S and the nuclear genes 28S and 18S rRNA from sponge tissue samples to determine the taxonomic status of three morphotypes of *C. vaginalis*, hereafter referred to as grey, red, and orange morphs. We also sequenced samples from the congeneric species *C. plicifera* (Lamarck, 1814) and *C. fallax* Duchassaing and Michelotti, 1864, and retrieved haplosclerid sequences from GenBank to confirm the phylogenetic position of our sequences within the Callyspongiidae. Spicule dimensions and spongin fibers were observed and compared with previous descriptions of *Callyspongia* species in the Caribbean.

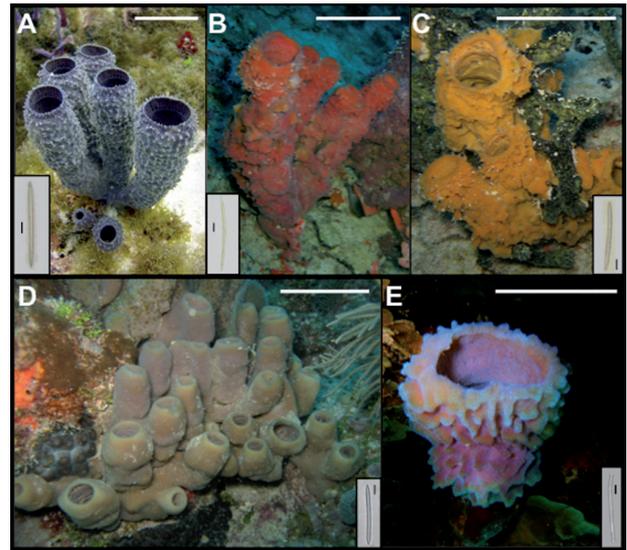


FIG. 1. – Specimens of *Callyspongia* analyzed from Key Largo, Florida and their spicule types: (A) grey *C. vaginalis*, oxeas; (B) red *C. vaginalis*, oxeas; (C) orange *C. vaginalis*, oxeas; (D) *C. fallax*, oxeas; and (E) *C. plicifera*, strongyles. Scale bar on sponge photos = 10 cm. Scale bar on spicule photos = 10 μ m.

MATERIALS AND METHODS

Samples

Three individuals from each morphotype of *C. vaginalis*, grey (common reef morph), red, and orange morphs (Fig. 1A-C respectively), and *C. fallax* (Fig. 1D) were collected from the shipwreck *USS Spiegel Grove* in Key Largo, Florida, at 30 m depth (N25°04'; W80°18.65). Additional samples of the grey and orange morphs, and their congeneric species *C. fallax* and *C. plicifera* (Fig. 1E) were collected from Conch Wall (N24°57.02'; W80°27.42') at 18 m depth (Table 1). Other samples of the red morph were collected from the Aquarius Habitat at 20 m depth (N24°57'; W80°27.22') in Key Largo, Florida (Table 1). Sampling was undertaken by SCUBA divers in November 2006 and May 2009. Species identifications were based on Wiedenmayer (1977) and Zea (1987).

Spicule morphology

Two subsamples of tissue, each including both the ectoderm and the endoderm, were analyzed from three individuals of each morphotype or species. Spicules were obtained by removing tissue with a 50% solution of chlorine bleach (2.5% sodium hypochlorite in water), which was subsequently rinsed in deionized water and stored in 100% ethanol. Lengths and widths of spicules were measured using light microscopy and the image analysis software ImageJ 1.41o. For each subsample, 5 to 28 photos were taken and 25 intact spicules were measured, yielding a total of 150 spicules measured per morphotype or species. Only

TABLE 1. – Number of individuals studied (N) and GenBank accession numbers (Acc. No.) for COI mtDNA, and 28S and 18S rRNA sequences from *Callyspongia vaginalis* (grey, orange and red morphs), *C. fallax* and *C. plicifera*.

	Species	<i>C. vaginalis</i> (Grey)	<i>C. vaginalis</i> (Orange)	<i>C. vaginalis</i> (Red)	<i>C. fallax</i>	<i>C. plicifera</i>
COI mtDNA	N	1	2	5	5	-
	Acc. No.	GQ415415	GQ415414	GQ415413	GQ415416	-
28S rRNA	N	4	5	7	6	4
	Acc. No.	EU863804	EU863805	EU863806	EU863802	EU863803
18S rRNA	N	1	4	5	3	3
	Acc. No.	EU863815	EU863813	EU863814	EU863812	GQ411355

TABLE 2. – 16S mtDNA haplotypes from three species and three morphotypes of *Callyspongia*, including collection location (Conch = Conch Wall, Habitat = Aquarius Habitat, Spiegel = Spiegel Grove Shipwreck), number of individuals sequenced (N), GenBank accession numbers (Acc. No.), haplotype name (Hapl. Name) and haplotype frequency encountered (Hapl. Frequency).

Sample	Location	N	Acc. No.	Hapl. Name	Hapl. Frequency
<i>Callyspongia fallax</i>	Spiegel, Conch	5			
<i>Callyspongia vaginalis</i> (Grey)	Spiegel	1	EU863810	H1	0.273
<i>Callyspongia plicifera</i>	Spiegel, Conch	4	EU863811	H2	0.182
<i>Callyspongia vaginalis</i> (Grey)	Spiegel	1			
<i>Callyspongia vaginalis</i> (Orange)	Spiegel	2	EU863809	H3	0.409
<i>Callyspongia vaginalis</i> (Red)	Spiegel, Habitat	6			
<i>Callyspongia vaginalis</i> (Grey)	Conch	1	GQ411356	H4	0.045
<i>Callyspongia vaginalis</i> (Grey)	Conch	1	GQ411357	H5	0.045
<i>Callyspongia vaginalis</i> (Grey)	Conch	1	GQ411358	H6	0.045

spicules present in all the subsamples were considered for statistical analyses. Nested analyses of variances (individuals nested within species or morphotypes) were conducted to compare spicule dimensions (length and width) among species and morphotypes. Pairwise Bonferroni post-hoc tests were run following significant ($P < 0.05$) ANOVA outcomes. Statistical analyses were performed using the software program Systat version 11.

Spongin fiber skeleton morphology

A 3 mm² tissue sample was obtained from both the ectoderm and the endoderm of three individuals from each sponge species or morphotype. The fragments were digested using 300 µl of cell lysis solution (Qiagen), 3 µl of Proteinase K (Sigma) and incubated at 55°C for 2 h. Samples were then washed three times in 95% ethanol and stored in 100% ethanol. Spongin skeletons were examined for branching patterns and fiber arrangement and width using light microscopy at x100 magnification.

DNA extraction and sequencing

Samples were kept at -20°C until processed. DNA was extracted using the Puregene kit (Gentra Systems). The primers 16SarL, 5'-CGCCTGTTTATCAAAA-CAT-3' and 16SbrH, 5'-CCGGTCTGAACTCA-GATCACGT-3' (Palumbi *et al.*, 1991), were used to amplify a fragment of the 16S mitochondrial gene. Nucleotide diversity was estimated with DnaSP v. 4 (Rozas *et al.*, 2003), and haplotype frequencies with

Arlequin v. 2000 (Schneider *et al.*, 2000). The universal primers LCO1490 and HCO2198, described in Folmer *et al.* (1994), were used to amplify a segment of the cytochrome *c* oxidase subunit I (COI) mitochondrial gene (5'End fragment). To amplify a fragment of the 18S rRNA gene, we used the forward primer 5'-CTGGTGCCAGCAGCCGCGG-3' and reverse primer 5'-TGTTGCCCTTCCGTC AATTCCT-3' modified from Kelly-Borges and Pomponi (1994) as described by Peterson and Addis (2000). Finally, the forward primer 28sCallyF 5'-TGCGACCCGAAA-GATGGTGA ACTA-3' and reverse primer 28sCallyR 5'-ACCAACACCTTTCCTGGTATCTGC-3' were designed based on 28s rRNA sequences of *C. plicifera* and *C. multiformis* available in Genbank (accession nos. AF441343 and AF441344 respectively). Amplification was performed in a 25 µL total-reaction volume with: 1.25 µL of each primer (10 µM), 0.5 µL dNTP's (10 mM), 2.5 µL 10x buffer, 2.5 µL MgCl₂, 0.5 µL Taq polymerase 5U, and 0.5 µL DNA. A single soak at 94°C for 5 min was followed by 40 amplification cycles (denaturation at 95°C for 30 sec; annealing at 45°C for COI, 50°C for 16S and 28S, and 60°C for 18S for 30 sec; and extension at 68°C for 2 min), and a final extension at 72°C for 5 min, in a Peltier PTC-200 gradient PCR.

PCR products were run in a 1% agarose gel and purified using the Wizard purification kit (Promega). Some PCR amplification products using 16S mtDNA and 18S rRNA primers were viewed as two distinct bands in the agarose gel. Both PCR fragments were gel cleaned using PerfectPrep Gel Cleanup (Eppendorf) and sequenced to confirm sequence identity. Sequenc-

ing reactions were carried out with the BigDye™ terminator v. 3.1 using the same primers as in the amplification step. Sequences were obtained on the ABI Prism 3100 automated sequencer. All sequences have been deposited in GenBank (accession nos. are listed in Tables 1 and 2).

Phylogenetic analysis

Consensus partial 18S and 28S rRNA gene sequences from *Callyspongia fallax*, *C. plicifera*, and the three morphotypes of *C. vaginalis* were compared with representative sequences from sponges in the order Haplosclerida to confirm the position of our sequences within this group (see Figs. 3 and 4 for GenBank accession numbers). Few 16S and COI (5' End fragment) mtDNA sequences of haplosclerid sponges were available in GenBank; therefore, these genetic markers were not used for phylogenetic analysis. Sequences were aligned using Clustal X (Thompson *et al.*, 1997) with default parameters. Neighbor-joining (NJ) and maximum parsimony analyses were conducted in MEGA 4 (Tamura *et al.*, 2007). For NJ analysis, the Kimura 2-Parameter model of nucleotide substitution was used and data were re-sampled using 10000 bootstrap replicates. For MP analysis, a heuristic search was performed with 10 random addition replicates. The Close-Neighbor-Interchange (CNI) branch swapping algorithm was implemented and data were re-sampled using 5000 bootstrap replicates (Felsenstein, 1985). MODELTEST 3.7 (Posada and Crandall, 1998) was used to select the best model of DNA substitution for the maximum likelihood (ML) analysis, which was the equal-frequency Tamura-Nei model (Tamura and Nei, 1993) with substitution rates varying among sites according to an invariant and gamma distribution (TrNef+I+G) for both 28S and 18S rRNA sequences. ML analysis was performed using GARLI v 0.951 (Zwickl, 2006) with the TrNef+I+G substitution model and default settings. Data were re-sampled using 100 bootstrap replicates. For Bayesian inference, MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) was used to calculate the posterior probabilities of branch nodes, implementing the TrNef+I+G likelihood model. The Monte Carlo Markov Chain length was set to 1 million generations with sampling every 100th generation and with a burn-in value of 2500. After 551000 (28S rRNA) and 510000 generations (18S rRNA), the average standard deviation of split frequencies between two independent chains reached less than 0.01.

RESULTS

Spicule morphology

Two types of spicules were present in oxidized tissue samples: strongyles in *Callyspongia plicifera* (Fig. 1E) and oxeas in all the other taxa: *C. fallax* (Fig. 1F), the common grey morph of *C. vaginalis* (Fig. 1A)

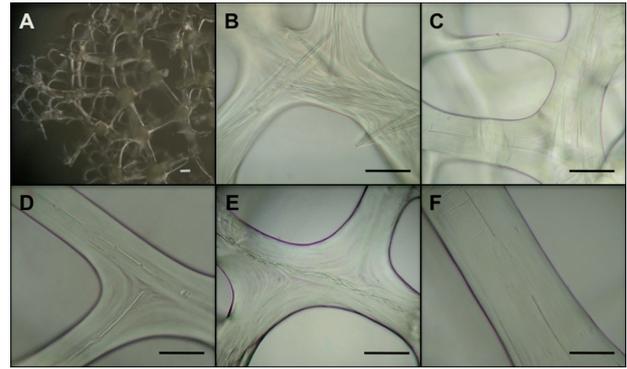


FIG. 2. – Fiber morphology and spicular tracts in *Callyspongia* spp. (A) Reticulated fiber network of *C. fallax* highlighting the dense mesh of the ectosomal skeleton (left) and more spaced mesh of the choanosomal skeleton (right). (B) Dense multispicular fiber tracts in primary and secondary fibers of *C. vaginalis* grey morph. (C) Pausispicular tracts in primary fibers and unispicular tracts in secondary fibers of *C. vaginalis* orange morph. (D) Unispicular tracts in *C. vaginalis* red morph. (E) Pausispicular tracts in primary fibers and unispicular tracts in secondary fibers of *C. fallax*. (F) Unispicular fiber tract in *C. plicifera* cored by strongyle spicules. Scale bars (A) = 100 μ m, and (B-F) 50 μ m.

and the red and orange morphs of *C. vaginalis* (Fig. 1B and 1C respectively). Strongyles from *C. plicifera* averaged 81.73 μ m in length (± 9.48 ; SD) and 1.19 μ m in width (± 0.33 ; SD). Oxeas from *C. fallax* averaged 77.95 μ m in length (± 7.67 ; SD) and 2.59 μ m in width (± 0.69 ; SD); the *C. vaginalis* grey morph 83.04 μ m (± 7.49 ; SD) and 2.76 μ m (± 0.73 ; SD); the orange morph 76.83 μ m (± 6.04 ; SD) and 2.05 μ m (± 0.63 ; SD); and the red morph 67.55 μ m (± 5.77 ; SD) and 1.85 μ m (± 0.59 ; SD). ANOVA analyses revealed significant differences among spicule lengths and widths among all species and morphotypes and among individuals within species and morphotypes ($P < 0.001$). For spicule length, all pairwise comparisons were significantly different ($P < 0.05$), except between *C. plicifera* and the grey morph of *C. vaginalis* ($P = 1.00$), and between the orange morph of *C. vaginalis* and *C. fallax* ($P = 1.00$). For spicule width, all pairwise comparisons were significantly different, except between the grey morph of *C. vaginalis* and *C. fallax* ($P = 0.16$).

Spongin fiber analysis

All species of *Callyspongia* displayed a reticulated network of fibers formed by a tangential intersection of primary and secondary fibers, with occasional tertiary fibers. Fiber networks formed rounded to triangular and rectangular meshes that became denser in the ectosomal region of the skeleton (Fig. 2A). All of the three morphotypes of *C. vaginalis* examined exhibited easily distinguishable primary and secondary fibers. The diameter of primary fibers averaged 62.68 μ m (± 12.93 ; SD) for the grey morph, 79.06 μ m (± 14.74 ; SD) for the orange morph, and 71.68 μ m (± 10.21 ; SD) for the red morph. Secondary fiber diameters averaged 30.86 μ m (± 9.84 ; SD) for

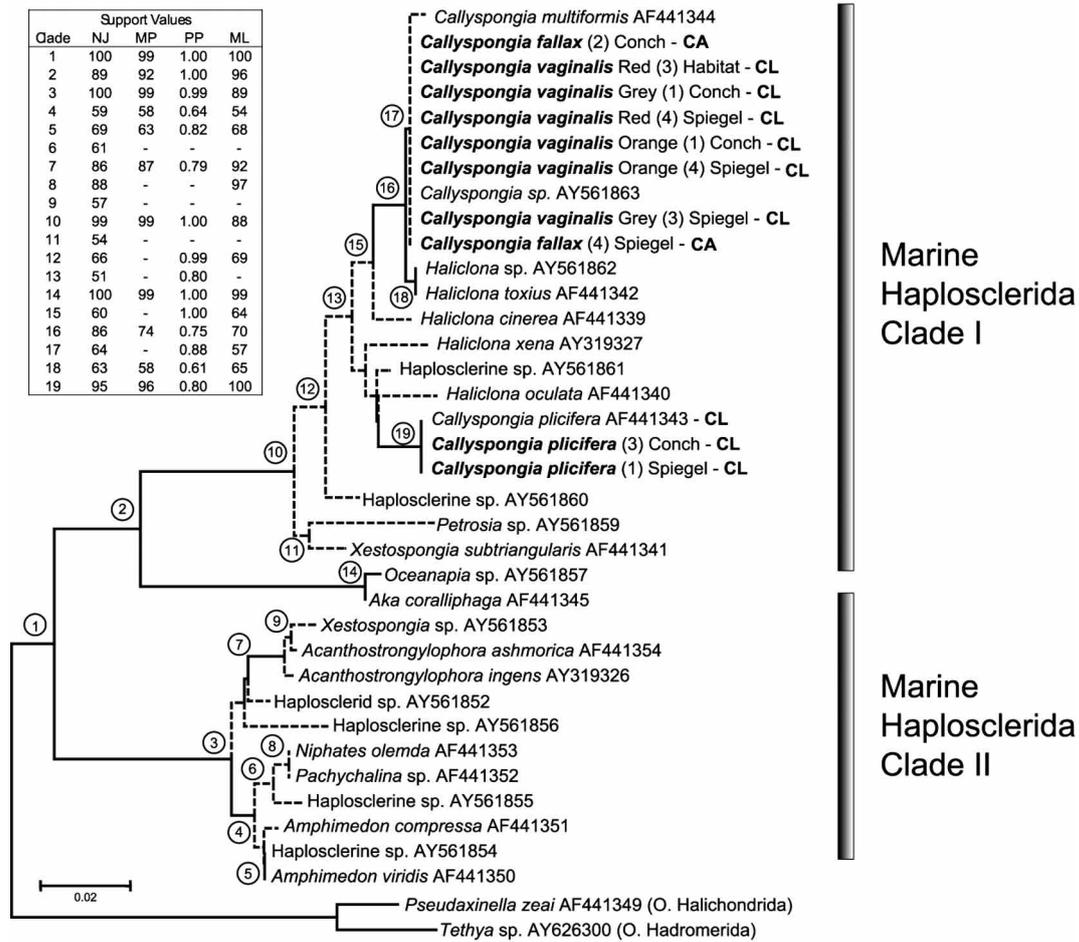


Fig. 3. – Phylogeny of partial 28S rRNA gene sequences from sponges in the order Haplosclerida highlighting the phylogenetic position of *Callyspongia* species and morphotypes from this study (bold lettering). Representatives of the orders Halichondrida (*Pseudaxinella* spp.) and Hadromerida (*Tethya* spp.) were used as outgroup taxa. Labels on terminal nodes of reference sequences indicate the sponge species and GenBank accession numbers. Labels on terminal nodes of sequences from this study also include morphotype (grey, red, and orange), number of sequences (in parenthesis) and collection location (Conch = Conch Wall, Habitat = Aquarius Habitat, Spiegel = Spiegel Grove Shipwreck). Subgenera of *Callyspongia* are shown in bold capital letters (CA = *Callyspongia*, CL = *Cladochalina*). Gradient bars and labels highlight the two major lineages of marine haplosclerid sponges. The tree topology was obtained from neighbor-joining (NJ) analysis. Individual bootstrap values from NJ, maximum parsimony (MP) and maximum likelihood (ML) analyses and posterior probabilities (PP) from Bayesian inference are located in the upper-left box and correspond to circled numbers on tree nodes. Solid lines indicate well-supported branches (support values greater than 75% for 3 of the 4 phylogenetic criteria or greater than 50% for all criteria) and dashed lines indicate weakly supported branches. Scale bar represents 0.02 substitutions per site.

the grey morph, 50.60 μm (± 13.16 ; SD) for the orange morph, and 56.16 μm (± 10.32 ; SD) for the red morph. The grey morph of *C. vaginalis* displayed a denser presence of spicules within the fiber network, with multispicular tracts (6-10 spicules) occurring in both primary and secondary fibers (Fig. 2B). The orange and red morphs exhibited mostly unispicular fiber cores, with occasional paucispicular tracts (2-4 spicules) in primary fibers only (Figs. 2C and 2D respectively). *Callyspongia fallax* displayed similar sized primary fibers (91.04 μm ± 16.96 SD) to *C. vaginalis*, although secondary fibers in this species (69.77 μm ± 14.22 SD) were difficult to distinguish from primary fibers based on width. Fibers of *C. fallax* were also cored by paucispicular tracts (5-6

spicules) in primary fibers and unispicular tracts in secondary fibers (Fig. 2E). *C. plicifera* exhibited much larger primary (169.82 μm ± 56.01 SD) and secondary fiber widths (96.79 μm ± 27.18 SD) than the other species examined. The fibers of *Callyspongia plicifera* were fasciculated and cored with sparse paucispicular tracts (2-3 spicules; Fig. 2F).

Genetic data and phylogenetic positioning

In the rare cases where two PCR products for 16S mtDNA were obtained from a single individual, sponge 16S mtDNA corresponded in all cases to a fragment of ~750 bp. Partial 16S mtDNA gene sequences (688 bp) revealed a total of six unique haplotypes (Table 2) and

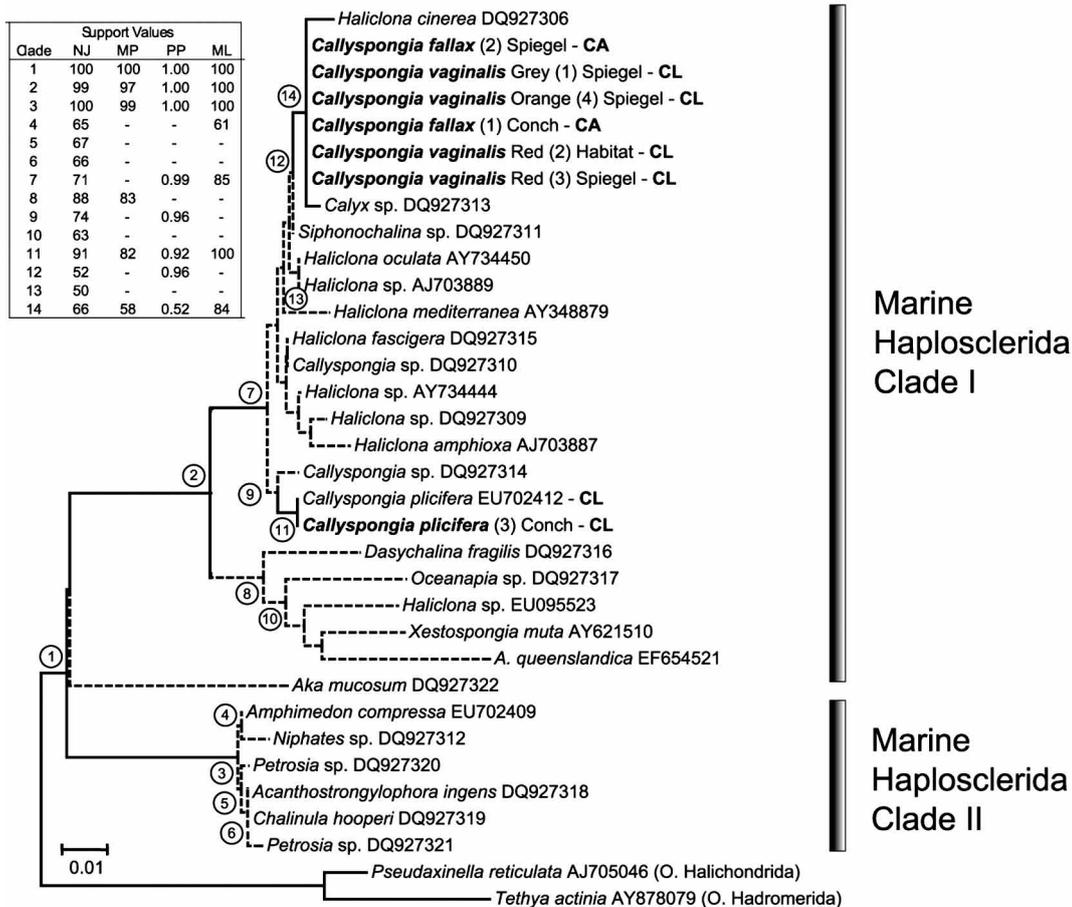


FIG. 4. – Phylogeny of partial 18S rRNA gene sequences from sponges in the order Haplosclerida highlighting the phylogenetic position of *Callyspongia* species and morphotypes from this study (bold lettering). Representatives of the orders Halichondrida (*Pseudaxinella* spp.) and Hadromerida (*Tethya* spp.) were used as outgroup taxa. Labels on terminal nodes of reference sequences indicate the sponge species and GenBank accession numbers. Labels on terminal nodes of sequences from this study also include morphotype (grey, red, and orange), number of sequences (in parenthesis) and collection location (Conch = Conch Wall, Habitat = Aquarium Habitat, Spiegel = Spiegel Grove Shipwreck). Subgenera of *Callyspongia* are shown in bold capital letters (CA = *Callyspongia*, CL = *Cladochalina*). Gradient bars and labels highlight the two major lineages of marine haplosclerid sponges. The tree topology was obtained from neighbor-joining (NJ) analysis. Individual bootstrap values from NJ, maximum parsimony (MP) and maximum likelihood (ML) analyses and posterior probabilities (PP) from Bayesian inference are located in the upper-left box and correspond to circled numbers on tree nodes. Solid lines indicate well-supported branches (support values greater than 75% for 3 of the 4 phylogenetic criteria or greater than 50% for all criteria) and dashed lines indicate weakly supported branches. Scale bar represents 0.01 substitutions per site.

an overall nucleotide diversity of 0.024 for this gene among the *Callyspongia* species and morphotypes analyzed. The most common haplotype (H3) was recovered from six individuals of the red morph, two of the orange morph, and one of the grey morph of *C. vaginalis* (Table 2). All specimens from *C. fallax* had the same haplotype (H1), also found in one individual of the grey morph of *C. vaginalis* (Table 2). Three unique haplotypes (H4, H5, H6) were also retrieved from samples of the grey morph of *C. vaginalis* at Conch Wall (Table 2). All samples of *C. plicifera* had haplotype H2, found exclusively in this species (Table 2). To confirm the nature of all the sequences we ran BLAST searches in GenBank. The best match for all our sequences was *C. plicifera* (95 to 99% identity depending on the blasted sequence; 100% coverage; GenBank accession no. EU237477).

Partial COI mtDNA gene sequences (550 bp) were identical for all three morphotypes of *C. vaginalis* and *C. fallax*. BLAST searches in GenBank showed that the best match for all the morphotypes of *C. vaginalis* and *C. fallax* was *Haliclona implexiformis* (93% identity, 100% coverage; GenBank accession no. EF519325). No COI mtDNA gene sequence could be obtained for *C. plicifera* using the universal primers of Folmer *et al.* (1994), which suggests that there was at least one mutation at the primer annealing sites. Our observation was further supported by analyzing the complete mitochondrion sequence for *C. plicifera* (GenBank accession no. EU237477), which revealed five mutations for LCO1490 annealing site and four for HCO2198.

Amplification using 18S rRNA primers resulted in two PCR products. The first product had less than 500bp while the second corresponded to a product of

~700bp. Identical 18S rRNA gene sequences (477 bp) were recovered from the longer PCR product of *C. fallax* and the three morphotypes of *C. vaginalis*, while *C. plicifera* (480 bp) exhibited 1.46% (5 bp) sequence divergence from *C. vaginalis* and *C. fallax*. Previously reported sequences from *Haliclona cinerea* (GenBank accession no. DQ927306) and an unidentified species of *Calyx* (GenBank accession no. DQ927313) were very similar to sequences from *C. vaginalis* and *C. fallax*, differing by 2 and 3 base pairs (0.29% and 0.59% divergence) respectively. The 18S rRNA sequence for *C. plicifera* obtained in this study was identical (0% divergence, 99% coverage) to the *C. plicifera* sequence available in GenBank (accession no. EU702412).

Partial 28S rRNA gene sequences (330 bp) recovered from *C. fallax* and the three morphotypes of *C. vaginalis* were also identical; however, *C. plicifera* (334 bp) exhibited 3.26% sequence divergence from *C. vaginalis* and *C. fallax*. Previously reported sequences from *C. multiformis* (GenBank accession no. AF441344) and an unidentified species of *Callyspongia* (GenBank accession no. AY561863) were very similar to sequences from *C. fallax* and *C. vaginalis*, differing by a single base pair (0.27% divergence). The 28S rRNA sequence for *C. plicifera* obtained in this study was identical (0% divergence, 93% coverage) to the *C. plicifera* sequence available in Genbank (accession no. AF441343). Phylogenetic analysis using 18S and 28S rRNA positioned all *Callyspongia* sequences in this study within the marine Haplosclerida Clade I, forming a monophyletic group with species from the genera *Haliclona* (n = 5), *Petrosia* (n = 1) and *Xestospongia* (n = 1) in the 28S phylogeny (Fig. 3) and the genera *Haliclona* (n=8), *Calyx* (n=1) and *Siphonochalina* (n=1) in the 18S phylogeny (Fig. 4). The genus *Callyspongia* was not upheld as monophyletic in either analysis, with *C. plicifera* consistently grouping separately from other species of *Callyspongia*. In the 28S phylogeny, *C. fallax*, *C. vaginalis*, and two additional species of *Callyspongia* formed a monophyletic clade closely related to *Haliclona toxius* and *Haliclona* sp. (Fig. 3). In the 18S phylogeny, *C. fallax* and *C. vaginalis* formed a monophyletic clade with *Haliclona cinerea* and *Calyx* sp., and *C. plicifera* grouped more closely with an unidentified Pacific species of *Callyspongia* (GenBank accession no. DQ927314) than with its Caribbean congeners (Fig. 4). The two subgenera of *Callyspongia* represented in this study were not monophyletic, as species from separate subgenera were more closely related to each other than to species from the same subgenus (Figs. 3 and 4).

DISCUSSION

In this study, we report a lack of genetic differentiation among three morphotypes of the sponge *Callyspongia vaginalis* that vary in color and shape, and their congeneric species *C. fallax*. Analyses of COI mtDNA and the nuclear genes 28S and 18S rRNA

resulted in identical partial sequences for all three *C. vaginalis* morphotypes and *C. fallax*. Different 16S mtDNA haplotypes were obtained for the grey morph of *C. vaginalis*, which had an overall nucleotide variability ($\pi = 0.024$) much higher than previously reported for COI in sponges (ranging from $\pi = 0.00049$ Wörheide, 2006; to $\pi = 0.0039$ López-Legentil and Pawlik, 2009). However, the grey morph shared one haplotype with the orange and red morph (H3) and also shared another haplotype with *C. fallax* (H1), suggesting that hybridization occurs among these forms. All genetic markers distinguished *C. plicifera* from *C. vaginalis* and *C. fallax*, which supports the species status of the first.

In a recent study, Blanquer and Uriz (2007) tested the resolution of the nuclear markers 18S and 28S rRNA and the mitochondrial COI in the sponge genus *Scopalina*. Sequence variation values were higher for the mitochondrial COI than for the nuclear ribosomal genes, and higher for 28S rRNA than for 18S rRNA (Blanquer and Uriz, 2007). Unlike the 28S rRNA, 18S rRNA, and mitochondrial COI, which have all been widely used as molecular markers for sponge taxonomy, mitochondrial 16S has primarily been sequenced in sponges to address issues of higher-level metazoan taxonomy (Wang and Lavrov, 2007). In the only previous work using mitochondrial 16S for phylogenetic analyses of sponges, Heim *et al.* (2007) reported that this gene was highly conserved and was not useful for species differentiation of the genus *Aplysina*. Yet, in the present study, only the mitochondrial 16S gene presented some degree of intra-species variability. Our results suggest that for at least some sponge genera, the 16S mtDNA gene has higher nucleotide diversity than traditionally used markers and may resolve some existing taxonomic conflicts at the species level and even prove to be a valid marker for population genetic studies.

Micro-morphological analyses revealed that *Callyspongia plicifera* was the only species with strongyle spicules, while *C. fallax* and the three morphotypes of *C. vaginalis* contained oxeas, and had spongin fibers much larger in diameter. These results were largely consistent with genetic data, as *C. plicifera* was genetically differentiated from *C. fallax* and *C. vaginalis* not only on the basis of 16S mtDNA sequences, but also based on 18S and 28S rRNA sequences. Spicule dimensions and spongin fiber data also revealed differences among the morphotypes of *C. vaginalis*, with the red and orange morphs of *C. vaginalis* containing significantly shorter and thinner spicules that were less dense in fiber cores than the common grey morph. However, these micro-morphological differences were not reflected in the molecular dataset. Spicule and fiber morphology measurements reported in this study are within the range described by Zea (1987), who also reported that oxeas from *C. vaginalis* varied in length, diameter and shape between and within locations and that this variation was unrelated to gross sponge morphology. Thus, although we found significant differ-

ences between the spicule lengths and widths of *C. fallax* and the grey morph of *C. vaginalis* compared with the orange and red morphs, additional sampling from other locations may still yield different results. *Callyspongia vaginalis* and *C. fallax* are widely distributed in the Caribbean and sampling of a single geographic region (i.e. Florida) may mask broader intra-specific variability in spicule morphology. As pointed out by Desqueyroux-Faúndez (1999), due to the high degree of variability within the Callyspongiidae, structural characteristics such as spicules may have limited utility for the identification of species within this group. The value of spicules as a diagnostic character has also been discussed for other demosponge taxa (e.g. *Chondrilla nucula*, Klautau *et al.*, 1999; *Latrunculia*, Miller *et al.*, 2001), suggesting that spicule shape and size may not be valid taxonomic characters to differentiate species within the same genera.

Phylogenetic analyses performed with 18S and 28S rRNA sequences confirmed that all our *Callyspongia* sequences were positioned within the marine Haplosclerida Clade I, but that they did not form a monophyletic grouping at the genus or subgenus level. *Callyspongia plicifera* consistently grouped separately from other Caribbean species of *Callyspongia*, and was more closely related to *Haliclona* spp. (28S rRNA data) and a Pacific species of *Callyspongia* (18S rRNA data). These results are contrary to the subgenus assignments of Wiedenmayer (1977), Van Soest (1980), and Zea (1987), who classified *C. vaginalis* and *C. plicifera* in the subgenus *Cladochalina* Schmidt 1870 (including *Spinosella* Vosmaer 1887) and *C. fallax* in the subgenus *Callyspongia* Duchassaing and Michelotti 1864 according to their spongin fiber skeleton (Desqueyroux-Faúndez and Valentine, 2002). As indicated by Desqueyroux-Faúndez and Valentine (2002), after observation of the high diversity of species and the subtle characters differentiating the different subgenera, our results also suggest that a thorough revision of the genus is necessary.

Much of the morphological variation in sponges formerly attributed to intra-specific adaptation to local environmental conditions has subsequently been found to reflect genetically distinct sibling species (e.g. Klautau *et al.*, 1999; Miller *et al.*, 2001; Duran and Rützler, 2006). Moreover, most studies focusing on taxonomically problematic groups have revealed genetic variation associated with subtle morphological differences that were generally not recognized as diagnostic in traditional sponge taxonomy (e.g. color; Boury-Esnault *et al.*, 1992; Klautau *et al.*, 1999; Miller *et al.*, 2001; Knowlton, 2000). In fact, few studies have reported a lack of genetic variability in sympatric species that differed in color and shape (e.g. Solé-Cava and Thorpe, 1986; Boury-Esnault *et al.*, 1992). In the present study, there was no genetic evidence of consistent differentiation among the three morphotypes of *C. vaginalis* and *C. fallax* for the molecular markers used. Further, cyanobacterial symbionts, a common source of

intra-specific color variation in sponges, have not been reported for this species (Erwin and Thacker, 2007b), suggesting that morphological plasticity is responsible for these differences.

No comprehensive data on the ecological distribution of the three morphs of *C. vaginalis* and *C. fallax* are available, but the grey morph is abundant and *C. fallax* uncommon on Caribbean coral reefs (Pawlik *et al.*, 1995), and the red and orange morphs are more abundant and co-exist with the grey morph and *C. fallax* on the shipwreck *USS Spiegel Grove* (Pawlik *et al.*, 2008). The young sponge community found on the decks of the *USS Spiegel Grove* clearly differed from that of the nearest reef (located 800 m NW) in terms of species present, abundance, and palatability to predators (Pawlik *et al.*, 2008). Intra-specific variability in secondary chemistry has often been related to color differences in marine invertebrates (e.g. Rogers and Paul, 1991; López-Legentil *et al.*, 2005). The orange and red morphs of *C. vaginalis* may be preferred by sponge-eating fishes (primarily angelfish and parrotfish) over the commonly occurring grey morph. If the sponge community changes over time to match that found on adjacent reefs (Pawlik *et al.*, 2008), the orange and red morphs may be preferentially grazed and their abundance consequently reduced. This may indicate some degree of ecological differentiation, with the red and orange morphs more opportunistically recruiting to uncolonized habitats. Clearly more studies of the distribution of morphs are necessary to determine whether they segregate spatially or temporally. Alternatively, the three morphotypes and the specimens identified as *C. fallax* may persist because they do not interbreed due to differentiation at loci other than those studied. If this is true, divergence of the three morphs of *C. vaginalis* and *C. fallax* must have been sufficiently recent that no genetic signature is apparent in the genes analyzed herein.

In summary, our study revealed a lack of genetic differentiation between three morphotypes of *C. vaginalis* that varied in color and shape, and *C. fallax*. Although the information retrieved from the four genetic markers used in this study yielded consistent results, some caution is necessary as further studies using other morphotypes of *C. vaginalis* or genetic markers (especially ribosomal internal transcribed spacer regions) may yield different results. Contrary to what has been found for other sponge genera (e.g. *Aplysina*), the 16S mtDNA was the most variable marker for this group and should be further investigated for studies of sponge population genetics. Phenotypic plasticity due to some degree of ecological differentiation is the most parsimonious explanation for the observed morphological variability in *C. vaginalis*, and the lack of genetic differentiation between these morphotypes and *C. fallax*.

ACKNOWLEDGEMENTS

Dr. Sven Zea kindly confirmed the identification of our specimens and provided helpful insights into

the taxonomy of the species reported here. Dr. Xavier Turon made useful comments on a draft of the ms. Steve McMurray and Dr. Sven Rohde helped with the sampling. This study was financially supported by grants from NOAA's Undersea Research Center at UNCW (NA 96RU-0260), by the Biological Oceanography program at NSF (OCE-0550468), and by project CTM2007-66635 from the Spanish Government.

REFERENCES

- Blanquer, A. and M.J. Uriz. – 2007. Cryptic speciation in marine sponges evidenced by mitochondrial and nuclear genes: A phylogenetic approach. *Mol. Phylogenet. Evol.*, 45: 392-397.
- Boury-Esnault, N., A.M. Solé-Cava and J.P. Thorpe. – 1992. Genetic and cytological divergence between colour morphs of the Mediterranean sponge *Oscarella lobularis* Schmidt (Porifera, Demospongiae, Oscarellidae). *J. Nat. Hist.*, 26: 271-284.
- Desqueyroux-Faúndez, R. – 1999. Convenient genera or phylogenetic genera? Evidence from Callyspongiidae and Niphatidae (Haplosclerida). *Memoir. Queensl. Mus.*, 44: 131-146.
- Desqueyroux-Faúndez, R. and R.C. Valentine. – 2002. Family Callyspongiidae. In: J.N.A. Hooper and R.W.M. van Soest (eds.) *Systema Porifera: A guide to the classification of sponges*, pp. 835-851. New York, NY.
- Duran, S. and K. Rützler. – 2006. Ecological speciation in a Caribbean marine sponge. *Mol. Phylogenet. Evol.*, 40: 292-297.
- Erwin, P.M. and R.W. Thacker. – 2007a. Phylogenetic analyses of marine sponges within the order Verongida: a comparison of morphological and molecular data. *Invertebr. Biol.*, 126: 220-234.
- Erwin, P.M. and R.W. Thacker. – 2007b. Incidence and identity of photosynthetic symbionts in Caribbean coral reef sponge assemblages. *J. Mar. Biol. Ass. U.K.* 87: 1683-1692.
- Felsenstein, J. – 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, 39: 783-791.
- Folmer, O., W. Hoeh, M. Black, R. Lutz and R. Vrijenhoek. – 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotech.*, 3: 294-299.
- Heim, I., M. Nickel and F. Brümmer. – 2007. Molecular markers for species discrimination in poriferans: a case study on species of the genus *Aplysina*. In: M.R. Custódio, G. Lôbo-Hajdu, E. Hajdu and M. Muricy (eds.), *Porifera research: Biodiversity, innovation and sustainability*, pp. 361-371. Rio de Janeiro, Brazil.
- Holland, B.S. – 2000. Genetics of marine bioinvasions. *Hydrobiologia*, 420: 63-71.
- Kelly-Borges, M. and S.A. Pomponi. – 1994. Phylogeny and classification of lithistid sponges (Porifera: Demospongiae): a preliminary assessment using ribosomal DNA sequence comparisons. *Mol. Mar. Biol. Biotechnol.*, 3: 87-103.
- Klautau, M., C.A.M. Russo, C. Lazoski, N. Boury-Esnault, J.P. Thorpe and A.M. Solé-Cava. – 1999. Does cosmopolitanism result from overconservative systematics? A case study using the marine sponge *Chondrilla nucula*. *Evolution*, 53: 1414-1422.
- Knowlton, N. – 2000. Molecular genetic analyses of species boundaries in the sea. *Hydrobiologia*, 420: 73-90.
- López-Legentil S. and J.R. Pawlik. – 2009. Genetic structure of the Caribbean giant barrel sponge *Xestospongia muta* using the I3-M11 partition of COI. *Coral Reefs*, 28: 157-165.
- López-Legentil, S. and X. Turon. – 2005. How do morphotypes and chemotypes relate to genotypes? The colonial ascidian *Cystodytes* (Ascidiacea, Polycitoridae). *Zool. Scr.*, 34: 3-14.
- López-Legentil, S., R. Dieckmann, N. Bontemps-Subielos, X. Turon and B. Banaigs. – 2005. Chemical variation of alkaloids in color morphs of *Cystodytes* (Ascidiacea). *Biochem. Systemat. Ecol.*, 33: 1107-1119.
- Maldonado, M. and M.J. Uriz. – 1996. Skeletal morphology of two controversial Poecilosclerid genera (Porifera, Demospongiae): *Discorhabdella* and *Crambe*. *Helgoland Mar. Res.*, 50: 369-390.
- Miller, K., B. Alvarez, C. Battershill, P. Northcote and H. Parthasarathy. – 2001. Genetic, morphological, and chemical divergence in the sponge genus *Latrunculia* (Porifera: Demospongiae) from New Zealand. *Mar. Biol.*, 139: 235-250.
- Palumbi, S., A. Martin, S. Romano, W.O. MacMillan, L. Stice and G. Grabowski. – 1991. *The Simple Fool's Guide to PCR, Ver. 2.0*, Department of Zoology, Kewalo Marine Laboratory, University of Hawaii, Honolulu, HI.
- Pawlik, J.R., B. Chanas, R.J. Toonen and W. Fenical. – 1995. Defenses of Caribbean sponges against predatory reef fish: I. Chemical deterrence. *Mar. Ecol. Prog. Ser.*, 127: 183-194.
- Pawlik, J.R., T.P. Henkel, S.E. McMurray, S. López-Legentil, T-L. Loh and S. Rohde. – 2008. Patterns of sponge recruitment and growth on a shipwreck corroborate chemical defense resource trade-off. *Mar. Ecol. Prog. Ser.*, 368: 137-143.
- Peterson, K.J. and J.S. Addis. – 2000. *Clypeatula cooperensis* gen. n., sp. n., a new freshwater sponge (Porifera, Spongillidae) from the Rocky Mountains of Montana, USA. *Zool. Scr.*, 29: 265-274.
- Posada, D. and K.A. Crandall. – 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics*, 14: 817-818.
- Rogers, S.D. and V.J. Paul. – 1991. Chemical defenses of three *Glossodoris* nudibranchs and their dietary *Hyrtios* sponges. *Mar. Ecol. Prog. Ser.*, 77: 221-232.
- Ronquist, F. and J.P. Huelsenbeck. – 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19: 1572-1574.
- Rozas, J., J.C. Sanchez-DelBarrio, X. Messeguer and R. Rozas. – 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, 19: 2496-2497.
- Schneider, S., D. Roessli and L. Excoffier. – 2000. *Arlequin ver. 2000. A software for population genetics data analysis*. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Geneva.
- Solé-Cava, A.M. and J.P. Thorpe. – 1986. Genetic differentiation between morphotypes of the marine sponge *Suberites ficus* (Demospongiae: Hadromerida). *Mar. Biol.*, 93: 247-253.
- Tamura, K. and M. Nei. – 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.*, 10: 512-526.
- Tamura, K., J. Dudley, M. Nei and S. Kumar. – 2007. MEGA 4: Molecular Evolutionary Genetic Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.*, 24: 1596-1599.
- Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins. – 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, 25: 4876-4882.
- Van Soest, R.W.M. – 1980. Marine sponges from Curaçao and other Caribbean localities. Part II. Haplosclerida. *Stud. Fauna Curaçao Caribb. Isl.*, 62: 1-173.
- Voogd, N.J. – 2004. *Callyspongia (Euplaccella) biru* spec. nov. (Porifera: Demospongia: Haplosclerida) from Indonesia. *Zool. Meded.*, 78: 477-483.
- Wang, X. and D.V. Lavrov. – 2007. Mitochondrial genome of the Homoscleromorph *Oscarella carmela* (Porifera, Demospongiae) reveals unexpected complexity in the common ancestor of sponges and other animals. *Mol. Biol. Evol.*, 24: 363-373.
- Wiedenmayer, F. – 1977. A monograph of the shallow-water sponges of the Western Bahamas. *Experientia*, (Suppl.) 28: 1-287.
- Wörheide, G. – 2006. Low variation in partial cytochrome oxidase subunit I (COI) mitochondrial sequences in the coralline demospongia *Astrosclera willeyana* across the Indo-Pacific. *Mar. Biol.*, 148: 907-912.
- Zea, S. – 1987. *Espanjas del Caribe Colombiano*, pp. 99-110. Catálogo Científico, Colombia.
- Zwickl, D.J. – 2006. *Genetic algorithm approaches for the phylogenetic analysis of large biological sequences datasets under the maximum likelihood criterion*. Ph.D. thesis, Univ. Texas at Austin.

Scient. ed.: M.J. Uriz.

Received January 8, 2009. Accepted October 30, 2009.

Published online May 10, 2010.