Invertebrate Biology 126(3): 220–234.

DOI: 10.1111/j.1744-7410.2007.00092.x

Phylogenetic analyses of marine sponges within the order Verongida: a comparison of morphological and molecular data

Patrick M. Erwin and Robert W. Thacker[a]

University of Alabama at Birmingham, Birmingham, Alabama 35294, USA

Abstract. Because the taxonomy of marine sponges is based primarily on morphological characters that can display a high degree of phenotypic plasticity, current classifications may not always reflect evolutionary relationships. To assess phylogenetic relationships among sponges in the order Verongida, we examined 11 verongid species, representing six genera and four families. We compared the utility of morphological and molecular data in verongid sponge systematics by comparing a phylogeny constructed from a morphological character matrix with a phylogeny based on nuclear ribosomal DNA sequences. The morphological phylogeny was not well resolved below the ordinal level, likely hindered by the paucity of characters available for analysis, and the potential plasticity of these characters. The molecular phylogeny was well resolved and robust from the ordinal to the species level. We also examined the morphology of spongin fibers to assess their reliability in verongid sponge taxonomy. Fiber diameter and pith content were highly variable within and among species. Despite this variability, spongin fiber comparisons were useful at lower taxonomic levels (i.e., among congeneric species); however, these characters are potentially homoplasic at higher taxonomic levels (i.e., between families). Our molecular data provide good support for the current classification of verongid sponges, but suggest a re-examination and potential reclassification of the genera *Aiolochroia* and *Pseudoceratina*. The placements of these genera highlight two current issues in morphology-based sponge taxonomy: intermediate character states and undetermined character polarity.

Additional key words: 28S ribosomal subunit, ITS-2, spongin, *Aplysina, Verongula*

Marine sponges are recognized as a basal phylum of metazoans (Hooper & van Soest 2002). The poriferan body plan is simple, consisting of a series of canals and chambers through which water is circulated. These canals and chambers are lined by a single layer of cells termed the choanoderm. Another single layer of cells, the pinacoderm, separates the external environment from the internal sponge environment. Between the pinacoderm and choanoderm is the mesohyl, forming the main body of the sponge and consisting of connective tissue, spongin fibers, and inorganic matter (e.g., spicules and sand particles, Bergquist 1978). Adult sponges do not exhibit organization beyond the cellular level because these ''tissue'' layers lack basal laminae (Degnan et al. 2005). Owing to this simplistic body plan and cellular organization, many sponge phenotypes are plastic and vary with respect to environmental, rather than genetic, factors (Niegel & Schmahl 1984; Palumbi 1984). The low number of informative characters and high degree of phenotypic plasticity found in sponge anatomy has hindered traditional taxonomic and systematic efforts (Hooper & van Soest 2002). Although the limitations of a purely morphological sponge classification system have been recognized for nearly half a century (Levi 1957), current sponge classifications are based primarily on morphological characters, often using only one or two diagnostic characters to define a taxon (Hooper & van Soest 2002).

Molecular data have been used more recently to resolve the relationships among sponge taxa and can have several advantages over morphological data (Borchiellini et al. 2000). Nuclear ribosomal DNA (rDNA) has been most commonly used in poriferan phylogenetics, with the more conserved 18S rDNA region used in the resolution of higher taxonomic levels (e.g., paraphyly of sponges, Borchiellini et al. 2001), the variable 28S rDNA region used for lower

[a] Author for correspondence.
E-mail: thacker@uab.edu

taxonomic levels (e.g., inter-ordinal relationships, Erpenbeck et al. 2004), and the highly variable internal transcribed spacer (ITS) regions used at the lowest taxonomic levels (e.g., intra-specific variation, Wörheide et al. 2002). In addition to rDNA, other regions of the poriferan genome are being explored for their phylogenetic utility, including mitochondrial DNA (Erpenbeck et al. 2002; Duran et al. 2004b; Nichols 2005), microsatellite loci (Duran et al. 2002, 2004a), and nuclear protein-coding genes (Erpenbeck et al. 2005).

In previous studies, the amount of congruence observed between molecular and morphological data sets has varied among different types of molecular data and among sponge taxa. An analysis of several species of *Chondrilla* from Mediterranean and Atlantic populations using allozyme electrophoresis and spicule dimensions found evidence for five distinct species based on molecular evidence, with no consistent variation in spicule dimensions among the five species (Klautau et al. 1999). A similar study of species of *Chondrilla* from Australia, using rDNA sequence data and spicule dimensions, found evidence for three distinct species based on both molecular and morphological evidence (Usher et al. 2004). Studies of other sponge taxa using rDNA sequence data and morphological data suggest some congruence between traditional classifications and rDNA phylogenies (Chombard et al. 1998; Alvarez et al. 2000). These studies also demonstrate potential difficulties in the assessment of the homology of morphological characters (Alvarez et al. 2000) and suggest alternate views of morphological character homology and homoplasy based on DNA sequence data (Chombard et al. 1998). Predicting which morphological characters accurately reflect evolutionary relatedness (i.e., those exhibiting variation based on genetic factors) and which characters do not has proven difficult in sponge taxonomy. The use of molecular data in conjunction with traditional morphological analyses may provide a means of elucidating which morphological characters are homologous and diagnostic and which are homoplasic and uninformative (Alvarez et al. 2000; Hart et al. 2004).

Based on morphological and biochemical analyses, verongid sponges form a cohesive and distinct order of marine sponges. Verongid sponges lack a mineral skeleton and instead have a heavily collagenous mesohyl supported by spongin fibers that exhibit a granulated ''pith'' interior and a laminated ''bark'' exterior (Bergquist 1978). These spongin fiber skeletons may either be anastomizing (reticulated) or dendritic (branching). Verongid sponges also produce a suite of secondary metabolites, termed bromotyrosine derivatives, found only within the order Verongida (Bergquist & Cook 2002). Although clearly delineated from other sponge orders, the intra-ordinal relationships among verongid sponges are less studied and more difficult to resolve.

The order Verongida is comprised of four families, distinguished almost exclusively by the structure and composition of their spongin fibers (Bergquist & Cook 2002). Aplysinidae is the largest verongid family, consisting of 26 species from three genera (*Aplysina, Verongula,* and *Aiolochroia*), and is defined by an anastomizing fiber skeleton with both pith and bark elements. Ianthellidae is the second largest verongid family, consisting of 12 species from three genera (*Ianthella, Anomoianthella,* and *Hexadella*), and is distinguished from other verongid families by the presence of eurypylous choanocyte chambers. Aplysinellidae consists of nine species from three genera (*Aplysinella, Porphyria,* and *Suberea*) and is defined by a dendritic fiber skeleton with both pith and bark elements. Pseudoceratinidae consists of four species from a single genus (*Pseudoceratina*) and is defined by a dendritic fiber skeleton with only pith elements. Sponges within the family Aplysinidae are primarily distributed in the Caribbean and Mediterranean regions, whereas the remaining three families are widespread in the Indo-Pacific region (Bergquist & Cook 2002).

To resolve the intra-ordinal relationships among verongid sponges, we used both morphological and molecular phylogenetic analyses of samples representing all four families, with a focus on Caribbean species. To assess the taxonomic utility of spongin fiber characteristics, we analyzed variability in total fiber diameter and pith content within and among species, and compared these results with the molecular phylogeny. We used three specimens of *Chondrilla nucula* Schmidt 1862 (order Chondrosida, family Chondrillidae) and three specimens of *Smenospongia aurea* (Hyatt 1878) (order Dictyoceratida, family Thorectidae) as outgroup comparisons for our analyses. Recent molecular phylogenies of sponge orders suggest that Chondrosida is the most closely related order to Verongida (Nichols 2005), whereas traditional morphological systematics place Verongida closest to the other ''keratose'' (spicule-free) orders: Dictyoceratida and Dendroceratida (van Soest 1978).

Methods

Sample collection

We examined the verongid sponge species *Aiolochroia crassa* (Hyatt 1878), *Aplysina aerophoba* Nardo 1834, *Aplysina cauliformis* (Carter 1882),

Aplysina fistularis (Pallas 1766), *Aplysina fulva* (Pallas 1766), *Aplysinella rhax* (De Laubenfels 1954), *Ianthella basta* (Pallas 1766), *Pseudoceratina arabica* (Keller 1889), *Verongula gigantea* (Hyatt 1875), *Verongula reiswigi* (Alcolado 1984), and *Verongula rigida* (Esper 1794). Additionally, we examined *Smenospongia aurea* (order Dictyoceratida) and *Chondrilla nucula* (order Chondrosida) as outgroup species. Sponge samples were collected by SCUBA at the Caribbean Marine Research Center, Lee Stocking Island, Bahamas, during the summer of 2001 and fall of 2003; at the University of Guam Marine Laboratory during the summer of 2000 and 2002; at the Coral Reef Research Foundation, Koror, Palau, during the summer of 2003; at La Tixera, Spain, during the summer of 2002; and at Gray's Reef National Marine Sanctuary, during the summer of 2004 (Table 1). Each sponge was photographed *in situ,* and descriptive notes were taken during the collection and processing of all sponge samples. Samples were divided in half and preserved separately in RNAlater (Ambion, Austin, TX) for molecular analyses, and 70% ethanol, for morphological analyses. Sponge identifications were verified by Dr. M. Cristina Diaz (Smithsonian Institution) using external morphology (shape, color, surface texture, and oscula) and skeletal characteristics (fiber diameter, pith content, mesh diameter, and mesh area).

Spongin fiber analysis

Sponge samples preserved in 70% ethanol were digested using a 50% bleach solution, then rinsed with deionized water, and preserved in 95% ethanol. Whole spongin skeletons were examined using light microscopy for branching patterns and fiber arrangement. For each individual, ten separate spongin fibers were removed from the skeleton and examined using light microscopy with $\times 200$ magnification with a compound microscope. For each fiber, the total diameter and pith diameter were measured. Percentage pith was calculated by dividing the pith diameter by the total fiber diameter. Before statistical analyses, data were ranked-transformed to meet the assumptions of normality and homogeneity of variances. Nested analyses of variance (individuals nested within species) were used to compare spongin fiber measurements among species. To assess the significance of inter-specific differences, *post hoc* pairwise comparisons of mean differences were conducted with a Bonferroni correction (Sokal & Rohlf 1995).

Morphological analysis

Thirteen traditional morphological characters (55 total character states) were used to construct a morphological character matrix. Field and specimen observations used as characters included growth form, surface texture, aerophobicity, inclusion of debris in the mesohyl, and oscular arrangement (Fig. 1A–F). Other characters used in the analysis were determined by light microscopy, including skeletal arrangement, spongin fiber specialization (i.e., primary and/or secondary fiber structure; Bergquist 1978), and the presence/absence of spongocytes within spongin fibers (Fig. 1J–L). Information on choanocyte chamber morphology (diplodal or eurypylous) and mesohyl composition (heavy or light collagen content) was taken from Hooper & van Soest (2002). Spongin fiber diameter, pith diameter, and percentage pith data from the spongin fiber analysis were coded into the matrix with statistically different values representing unique character states. A numerical coding scheme was created for each morphological character (Table 2). From the coding scheme, a morphological matrix was created for phylogenetic analysis (Table 3).

DNA extraction and PCR amplification

Genomic DNA from 44 sponge samples (representing 13 species; Table 1) preserved in RNAlater (Ambion) was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) and cleaned using the Wizard DNA Clean-Up System (Promega), following the manufacturer's protocol. The sponge-specific oligonucleotide primers SP58bF and SP28cR (Thacker & Starnes 2003) were used to amplify a segment of nuclear ribosomal DNA corresponding to the second ITS (ITS-2) region (214–268 bp) and the 5′ end of the 28S subunit (350–358 bp) for all verongid and outgroup samples. The total PCR reaction volume was 50 μL, including 25 pmol of each primer, 10 nmol of each dNTP, 1× MasterTaq PCR Buffer (Eppendorf), and 1× TaqMaster additive (Eppendorf, Westbury, NY). Thermocycler reaction conditions were an initial denaturing time of 2 min at 94°C, followed by the addition of 0.5 U MasterTaq DNA polymerase (Eppendorf), then 34 cycles of 0.5 min at 94°C, 0.5 min at 54°C, and 1.5 min 72°C, and a final extension time of 2 min at 72°C. PCR reactions were repeated in triplicate to increase amplicon yield; pooled PCR reactions were gel-purified and cleaned using the Wizard PCR Preps System (Promega) following the manufacturer's protocol. PCR products were cloned using the pGEM-T Easy Vector System

Table 1. Classification, collection location, and GenBank accession numbers for sponge samples used in this study. Numbers in parentheses identify multiple individuals of a single species collected from the same location.

Order	Family	Genus	Species	ID code	Location	GenBank no.
Verongida	Aplysinidae	*Aplysina*	*aerophoba*	LT-S(1)	La Tixera, Spain[f]	DQ411443
				LT-S(2)	La Tixera, Spain[f]	DQ411444
		Aplysina	*cauliformis*	WM-B	Washing Machine[b]	DQ411442
				BW-B	Bock Wall[b]	DQ411440
				SR-B	Shark Rock[b]	DQ411438
				WH-B	White Horse[b]	DQ411441
		Aplysina	*fistularis*	WH-B(1)	Washing Machine[b]	DQ411439
				WH-B(2)	Washing Machine[b]	DQ411436
				RG-B	Rainbow Gardens[b]	DQ411435
				BP-B	Big Point[b]	DQ411437
		Aplysina	*fulva*	NN-B	North Norman's[b]	DQ411433
				WM-B	Washing Machine[b]	DQ411432
				SR-B	Shark Rock[b]	DQ411434
				BP-B	Big Point[b]	DQ411431
		Aiolochroia	*crassa*	NN-B(1)	North Norman's[b]	DQ411420
				NN-B(2)	North Norman's[b]	DQ411423
				BW-B(1)	Bock Wall[b]	DQ411422
				BW-B(2)	Bock Wall[b]	DQ411421
				WH-B	White Horse[b]	DQ411424
		Verongula	*gigantea*	WH-B	White Horse[b]	DQ411449
				BP-B(1)	Big Point[b]	DQ411450
				BP-B(2)	Big Point[b]	DQ411451
		Verongula	*reiswigi*	WH-B(1)	White Horse[b]	DQ411457
				WH-B(2)	White Horse[b]	DQ411455
				WH-B(3)	White Horse[b]	DQ411456
		Verongula	*rigida*	WH-B(1)	White Horse[b]	DQ411454
				WH-B(2)	White Horse[b]	DQ411452
				RG-B	Rainbow Gardens[b]	DQ411453
	Pseudoceratinidae	*Pseudoceratina*	*arabica*	NL-P(1)	Ngeruktabl Lake[c]	DQ411445
				NL-P(2)	Ngeruktabl Lake[c]	DQ411446
				NB-P(1)	Neco Bay[c]	DQ411447
				NB-P(2)	Neco Bay[c]	DQ411448
	Ianthellidae	*Ianthella*	*basta*	SM-G(1)	Sponge Mound[d]	DQ411425
				SM-G(2)	Sponge Mound[d]	DQ411426
				SM-G(3)	Sponge Mound[d]	DQ411427
	Aplysinellidae	*Aplysinella*	*rhax*	NP-P	Ngerikuul Pass[c]	DQ411429
				KC-P	KB Channel[c]	DQ411428
				WC-P	Wonder Channel[c]	DQ411430
Chondrosida	Chondrillidae	*Chondrilla*	*nucula*	MS-GR	Monitoring Site I[e]	DQ411462
				RT-GR	R2 Tower[e]	DQ411461
				TI-BE	Trunk Island[a]	DQ411463
Dictyoceratida	Thorectidae	*Smenospongia*	*aurea*	RG-B	Rainbow Gardens[b]	DQ411460
				WM-B	Washing Machine[b]	DQ411459
				SR-B	Shark Rock[b]	DQ411458

[a]Bermuda.
[b]Lee Stocking Island, Bahamas.
[c]Palau.
[d]Guam.
[e]Gray's Reef National Marine Sanctuary.
[f]Spain.

(Promega); plasmids were harvested using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Three separate clones were sequenced for one sample from specimens of *A. cauliformis, A. fistularis,* and *A. fulva* to screen for multiple copies of the ITS-2 region. For subsequent analyses, a consensus

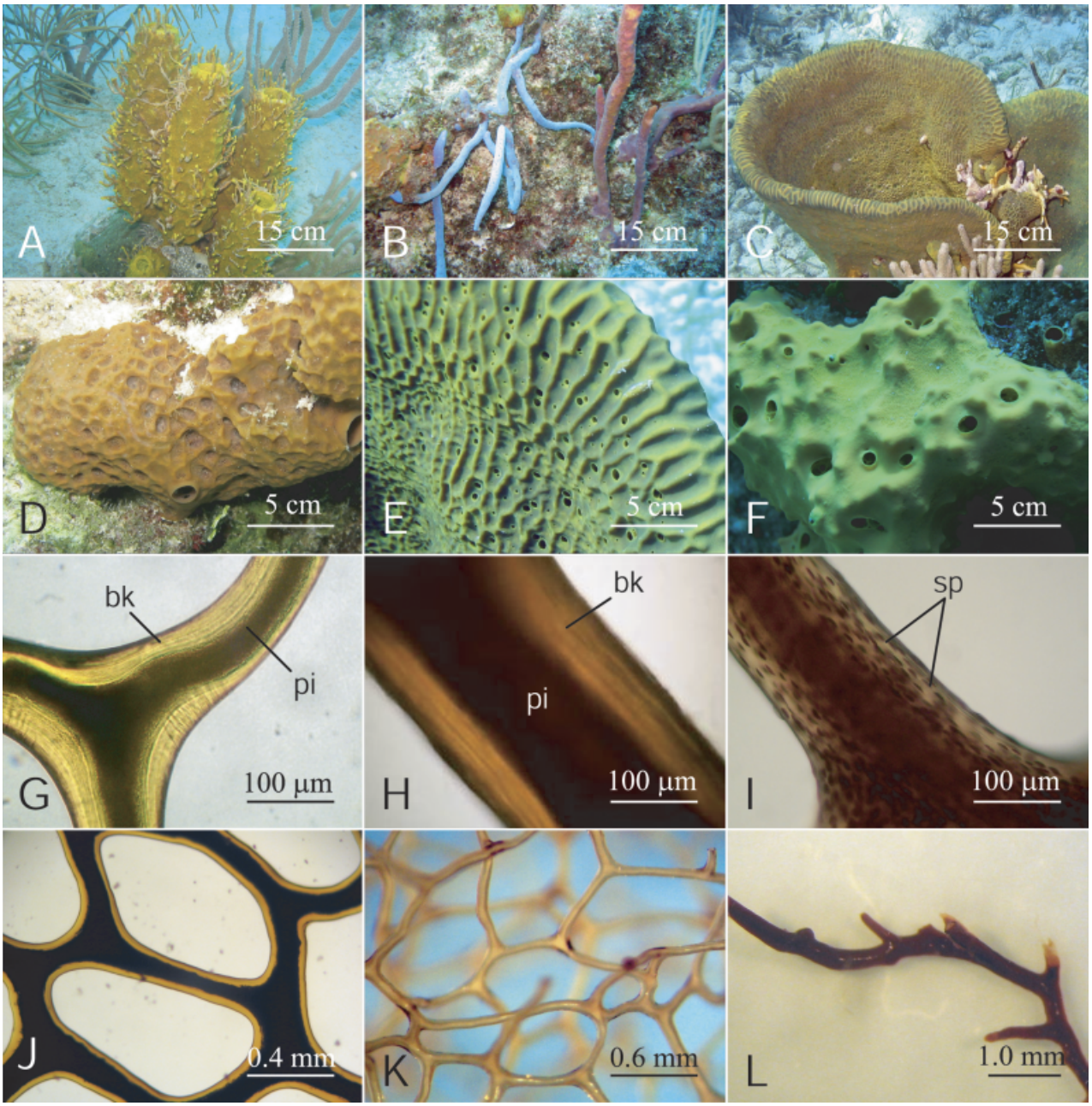

Fig. 1. Photographs and micrographs of representative sponge morphological characters. **A.** The verongid sponge *Aplysina fistularis* exhibited the tubular growth form. **B.** The verongid sponges *Aplysina cauliformis* (left) and *Aplysina fulva* (right) exhibiting the rope-like growth form. **C.** The verongid sponge *Verongula gigantea* displaying the turbinate growth form. **D.** The dictyoceratid sponge *Smenospongia aurea* displaying the ramose growth form and pitted surface texture. **E.** *Verongula gigantea* displaying the honeycomb surface texture. **F.** The verongid sponge *Aiolochoria crassa* displaying the conulose surface texture. **G**, **H.** Spongin fibers of *A. fistularis* and *A. crassa,* respectively, showing pith (pi) and bark (bk) elements. **I.** Spongin fibers of *Ianthella basta* showing spongocytes (sp). **J**, **K.** Reticulated fiber skeleton of *V. gigantea* and *A. fistularis,* respectively. **L.** Dendritic (i.e., branching) fiber skeleton of *A. crassa*.

sequence was created incorporating information from all clones, with polymorphic sites coded using IUPAC multiple base pair codes (e.g., clone 1 with "C"+clone 2 with "T" = consensus with "Y"). Forward and reverse sequencing reactions were performed for each sample at the University of Alabama at Birmingham Center for AIDS Research DNA Sequencing Core Facility.

Table 2. Morphological characters and character states used to create the morphology matrix and indices of congruence for each character when mapped onto the molecular phylogeny (CI, consistency index; RI, retention index). Spongin fiber diameter, pith diameter, and pith percentage data are from the spongin fiber analysis (see Fig. 2). —, unused character states.

Character	Character states							Congruence	
	0	1	2	3	4	5	6	CI	RI
1. Growth form	Mound	Tubular	Rope-like	Turbinate	Fan	Encrusting	Massive	0.75	0.33
2. Surface texture	Smooth	Finely conulose	Conulose	Ridged	Honeycomb	Pitted	—	0.67	0.50
3. Aerophobic	Yes	No	—	—	—	—	—	1.00	1.00
4. Fiber skeleton	Reticulated	Dendritic	Absent	—	—	—	—	0.67	0.50
5. Debris in mesohyl	Yes	No	—	—	—	—	—	1.00	0.00
6. Fiber specialization	Primary	Primary/ secondary	Absent	—	—	—	—	1.00	0.00
7. Oscular arrangement	Single	Row	Staggered	Apical	Random	—	—	0.50	1.00
8. Choanocyte chambers	Diplodal	Eurypylous	Aphodal	—	—	—	—	1.00	0.00
9. Spongocytes in fibers	Yes	No	—	—	—	—	—	1.00	0.00
10. Collagen in mesohyl	Heavy	Light	—	—	—	—	—	1.00	0.00
11. Fiber diameter	A	B	CDE	F	G	H	—	0.83	0.50
12. Pith diameter	A	B	C	D	E	F	G	1.00	1.00
13. Pith percentage	A	B	C	D	E	F	—	1.00	1.00

Sequence analysis and alignment

Forward and reverse sequences were compared using Sequencher (Gene Codes, Ann Arbor, MI) to ensure the accuracy of sequencing results, yielding a final consensus sequence. Consensus sequences were aligned in Clustal X (Thompson et al. 1997) using five different gap opening and gap extension costs (8/2, 8/4, 20/2, 24/4, and 24/6) to assess the effects of alignment parameters on tree topology (Hedin &

Table 3. Morphological character matrix used in phylogenetic analysis. Dashes (—) represent missing or unavailable character states.

Species	Character number												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Aiolochroia crassa ($n = 5$)	0	3	0	1	1	0	3	0	1	0	1	0	3
Aplysina aerophoba ($n = 2$)	0	0	0	0	1	0	3	0	1	0	5	4	2
Aplysina cauliformis ($n = 3$)	2	0	0	0	1	0	1	0	1	0	4	5	5
Aplysina fistularis ($n = 3$)	1	0	0	0	1	0	0	0	1	0	4	5	5
Aplysina fulva ($n = 3$)	2	0	0	0	1	0	2	0	1	0	3	5	5
Aplysinella rhax ($n = 3$)	6	2	0	1	1	0	3	0	1	0	0	—	—
Ianthella basta ($n = 3$)	4	1	0	0	1	0	4	1	0	0	2	3	4
Pseudoceratina arabica ($n = 4$)	2	3	0	1	0	0	4	0	1	0	2	6	0
Smenospongia aurea ($n = 3$)	0	4	1	0	1	1	3	0	1	1	—	—	—
Verongula gigantea ($n = 3$)	3	5	0	0	1	0	0	0	1	0	2	2	3
Verongula reiswigi ($n = 3$)	1	5	0	0	1	0	4	0	1	0	2	1	1
Verongula rigida ($n = 3$)	0	5	0	0	1	0	3	0	1	0	4	3	1
Chondrilla nucula ($n = 3$)	5	0	1	2	1	2	4	2	1	0	—	—	—

Table 4. Nested analyses of variance for total fiber diameter, pith diameter, and pith percentage. All three fiber characteristics displayed significant variation among and within sponge species. Analyses were performed on rank-transformed data with individuals nested within species.

Variable	Source	Sum of squares	df	Mean square	F-ratio	p
Fiber diameter	Species	7,941,465	10	794,147	712.396	<0.001
	Individual (within species)	212,228	27	7860	7.051	<0.001
	Error	381,246	342	1115		
Pith width	Species	3,179,937	9	353,326	472.584	<0.001
	Individual (within species)	140,330	25	5613	7.508	<0.001
	Error	235,509	315	748		
Pith percentage	Species	2,901,586	9	322,398	307.846	<0.001
	Individual (within species)	334,636	25	13,385	12.781	<0.001
	Error	329,891	315	1047		

Maddison 2001; Arnedo et al. 2004). Three specimens of *C. nucula* (order Chondrosida, family Chondrillidae) and three specimens of *S. aurea* (order Dictyoceratida, family Thorectidae) were used as outgroup comparisons for all alignments. An elision matrix was created in BioEdit (Hall 2000) by combining all alignments (Wheeler et al. 1995). Topological congruence was calculated as the number of nodes in common between the consensus tree of each alignment and the elision matrix. The gap opening/gap extension alignment most closely resembling the topology produced by the elision matrix (opening cost: 24, extension cost: 4) was chosen for subsequent phylogenetic analyses. This final alignment is available from the authors upon request.

Phylogenetic analysis

The morphology matrix and DNA sequence alignment were analyzed using the maximum parsimony (MP) criterion in PAUP*4.0 (Swofford 1998). In all data sets, gaps were treated as missing data, because treating gaps as fifth character states did not significantly affect the topology of the resulting phylogenies. For each data set, a heuristic search was performed with 100 random additions, a maximum of ten trees retained at each step, and an overall maximum of 1000 trees. The stepwise addition and tree bisection reconnection algorithms were implemented with default settings. The data were resampled using 100 bootstrap replicates. The DNA sequence alignment was also analyzed using the maximum likelihood (ML) criterion in PAUP*4.0. MODELTEST 3.06 (Posada & Crandall 1998) was used to select a model of DNA substitution. ML analysis was performed using a heuristic search; data were resampled using 100 bootstrap replicates. Neighbor-joining (NJ) analysis was performed using MEGA 3.1 (Kumar et al. 2004) with the Kimura two-parameter model of nucleotide substitution. Data were resampled using 1000 bootstrap replicates. To compare the congruence between the morphological and molecular phylogenies, a partition homogeneity test (PHT) was conducted in PAUP*4.0. Additionally, each morphological character was mapped onto the molecular phylogeny in MacClade 3.07 (Maddison & Maddison 1997). Congruence between each character and the molecular phylogeny was evaluated by examining the degree of homoplasy (consistency index; CI) and synapomorphy (retention index; RI).

Results

Spongin fiber analysis

Verongid sponges displayed a wide range of fiber diameters, pith diameters, and pith percentages (Table 4, Fig. 2). Among the Caribbean species of *Aplysina,* members of *Aplysina fulva* had a significantly greater fiber diameter than those of both *Aplysina fistularis* ($p<0.001$) and *Aplysina cauliformis* ($p<0.001$). The absolute pith diameter and percentage of total fiber diameter were not significantly different among the three species of *Aplysina*. The Mediterranean species *Aplysina aerophoba* was clearly differentiated from the Caribbean species of *Aplysina* and exhibited a significantly lower fiber diameter ($p<0.001$), a higher pith diameter ($p<0.001$), and a higher pith percentage ($p<0.001$) than the three Caribbean species of *Aplysina*. Specimens of *Aiolochroia crassa* had significantly larger fiber and pith diameters than all other verongid species, except *Aplysinella rhax* ($p<0.001$), but the same pith percentage as *Verongula gigantea* ($p=1.00$). Among the species of *Verongula,* specimens of *Verongula reiswigi* and *V. gigantea* had a larger fiber diameter than those

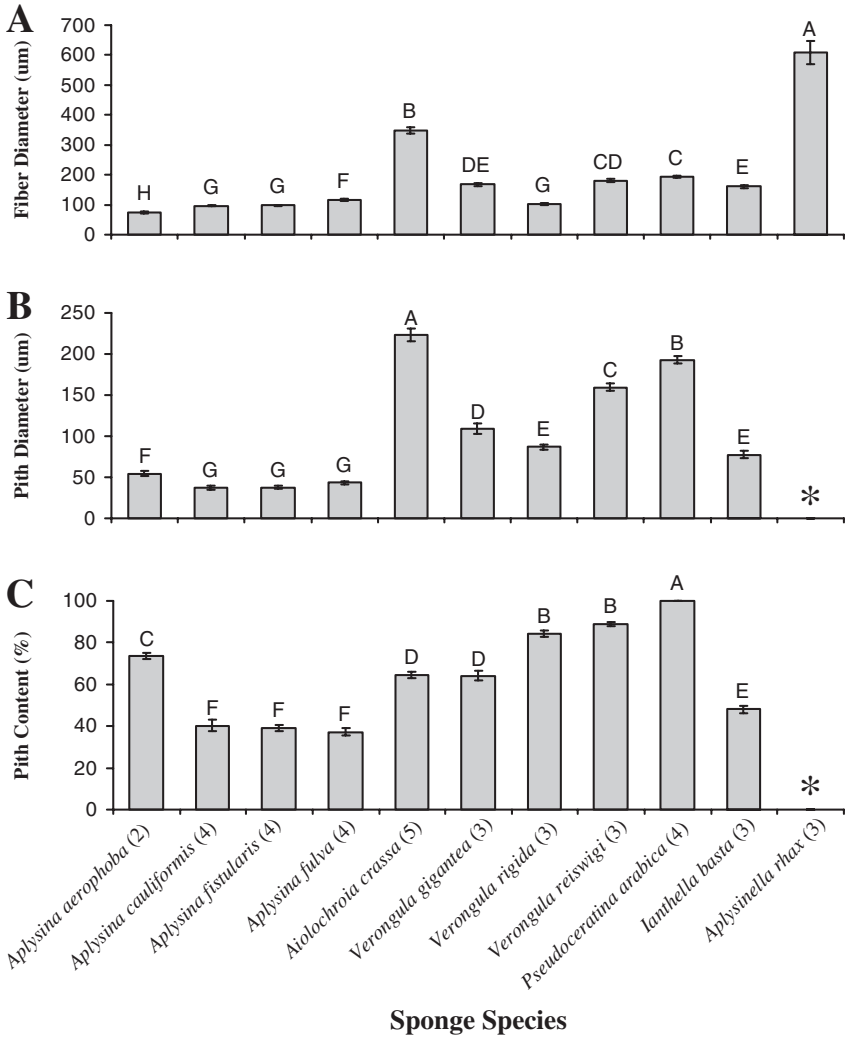

Fig. 2. Comparisons of mean spongin fiber diameter (**A**), pith diameter (**B**), and pith content as a percentage of total diameter (**C**) for 11 verongid sponge species. Numbers in parentheses indicate the number of individuals examined. For each individual, ten separate fibers were measured. Error bars represent ± 1 SE. For each comparison, different letters above bars represent significantly different means. Pith content was not discernable for *Aplysinella rhax* (*).

of *Verongula rigida* ($p < 0.001$), and all three species varied significantly in pith diameter ($p < 0.05$). Individuals of *V. reiswigi* and *V. rigida* displayed no significant difference in pith percentage ($p = 1.00$), both significantly larger than in *V. gigantea* ($p < 0.001$). In *Pseudoceratina arabica,* fiber diameter was similar to that in *V. reiswigi* ($p = 1.00$), but with the highest pith percentage of all verongid species ($p < 0.001$). Members of *Ianthella basta* had a fiber diameter similar to that of *V. gigantea* ($p = 1.00$) and a pith diameter similar to that in *V. rigida* ($p = 0.556$). In *A. rhax,* a significantly larger fiber diameter was exhibited than in all other verongid species ($p < 0.001$); however, sand grains covering the fiber surface prevented the measurement of pith content in this species.

Morphological phylogeny

Phylogenetic analysis of the morphological character matrix produced a single most parsimonious tree (tree length = 71, CI = 0.5634, homoplasy index [HI] = 0.4366, RI = 0.8063; Fig. 3). All 13 characters in the data set were parsimony informative. Verongid sponges and *Chondrilla nucula* formed a monophyletic clade (94% bootstrap support) with respect to *Smenospongia aurea*. Within the verongid+*C. nucula* clade, *A. fulva* and *A. cauliformis* formed a weakly supported monophyletic clade (50% bootstrap support), and all species, except *A. fistularis* and *V. rigida,* formed monophyletic species-level clades with moderate to strong bootstrap support (70–100%).

Molecular phylogeny

The overall alignment of the ITS-2/28S rDNA region contained 638 bp, with 355 variable characters, of which 347 were parsimony informative. The aligned ITS-2 region of rDNA (278 bp; 78.78% parsimony-informative characters) exhibited a higher percentage of parsimony-informative characters

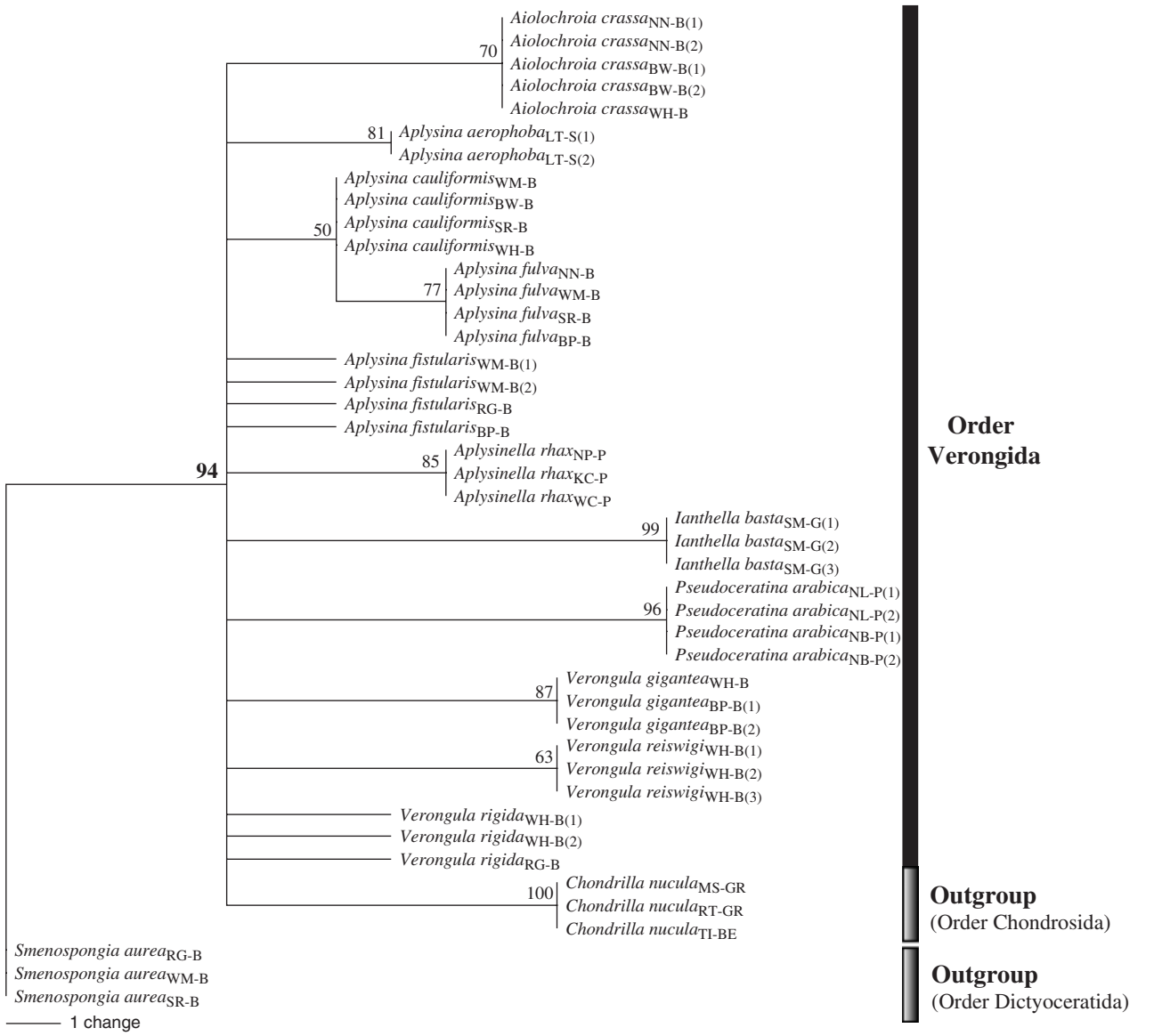

Fig. 3. Phylogeny resulting from maximum parsimony analysis of the morphology matrix. Numbers on nodes represent bootstrap percentages after 100 replicates. The scale bar depicts the number of character changes associated with horizontal branch distance.

compared with the aligned 5′ end of the 28S subunit (360 bp; 35.56% parsimony-informative characters). All verongid species exhibited 33.5–40.0% sequence divergence from outgroup taxa in the ITS-2 region and 15.0–19.4% sequence divergence from outgroup taxa in the partial 28S subunit.

Low levels of intragenomic variation were revealed by screening multiple clones per individual sample of the Caribbean species of *Aplysina*. In *A. cauliformis* and *A. fulva,* a similar number and percentage of polymorphic sites in the ITS-2 region and partial 28S subunit was exhibited; however, specimens of *A. fistularis* exhibited a higher number and percentage of polymorphic sites than those of *A. cauliformis* and *A. fulva* in the ITS-2 region, and no polymorphic sites in the partial 28S subunit (Table 5).

Phylogenetic analysis of the ITS-2/28S segment by MP, ML, and NJ analyses produced trees with nearly identical branching patterns (Fig. 4). MP analysis produced one most parsimonious tree (tree length = 637, CI excluding uninformative characters = 0.7917, HI excluding uninformative characters = 0.2083, RI = 0.9447). The best-fit ML model selected by hierarchical likelihood ratio tests was Tamura–Nei (Tamura & Nei 1993) with a γ distribution of substitution rates among sites (TrN+G).

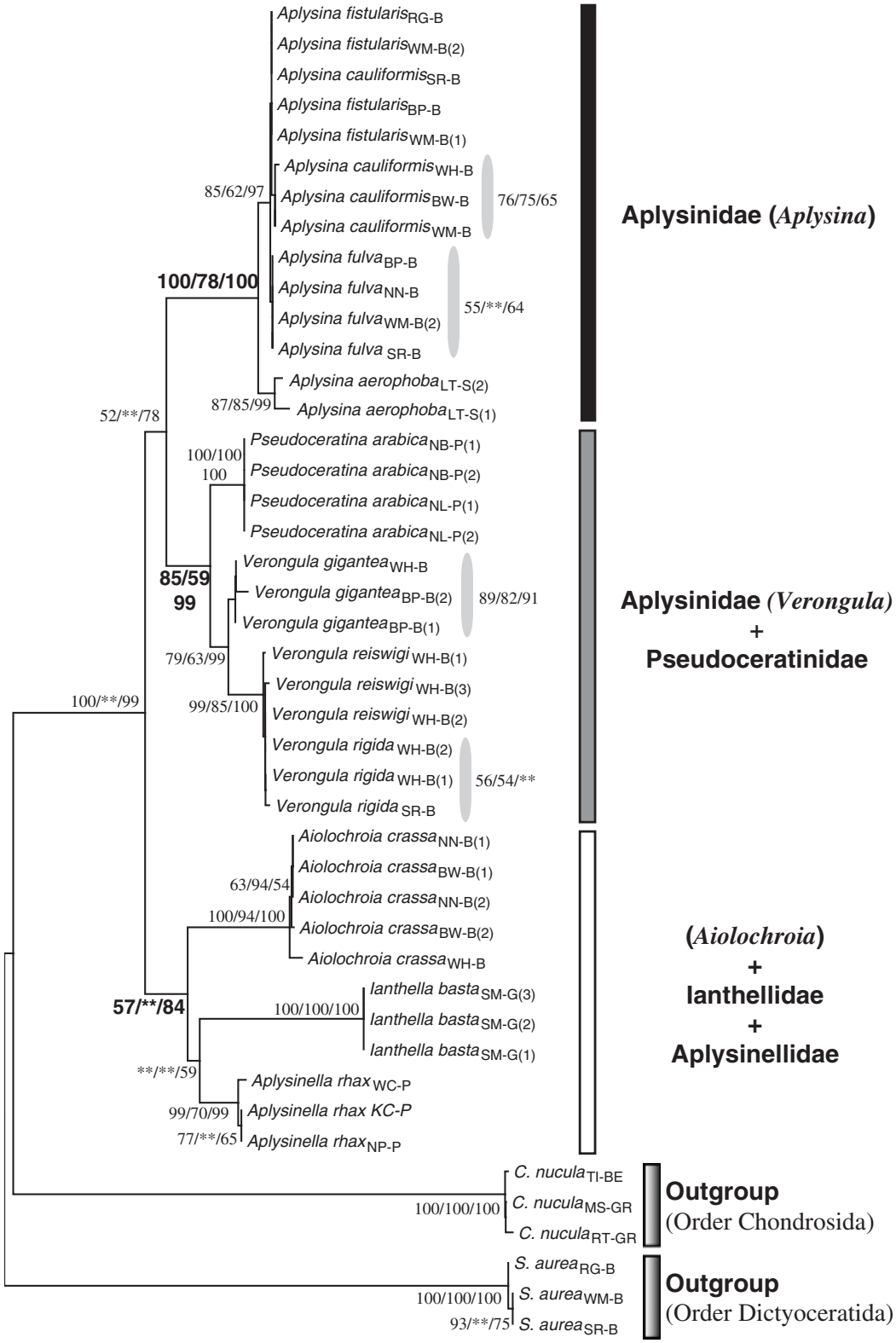

Fig. 4. Phylogeny resulting from neighbor-joining analysis of the ITS-2/28S rDNA segment. Numbers on nodes represent maximum parsimony/maximum likelihood/neighbor-joining bootstrap percentages after 100, 100, and 1000 replicates, respectively. Bootstrap values <50% are denoted with a double asterisk (**). The scale bar depicts the number of character changes associated with horizontal branch distance. Subscript text next to species names refers to the collection location; numbers in parentheses identify multiple individuals of the same species from the same site.

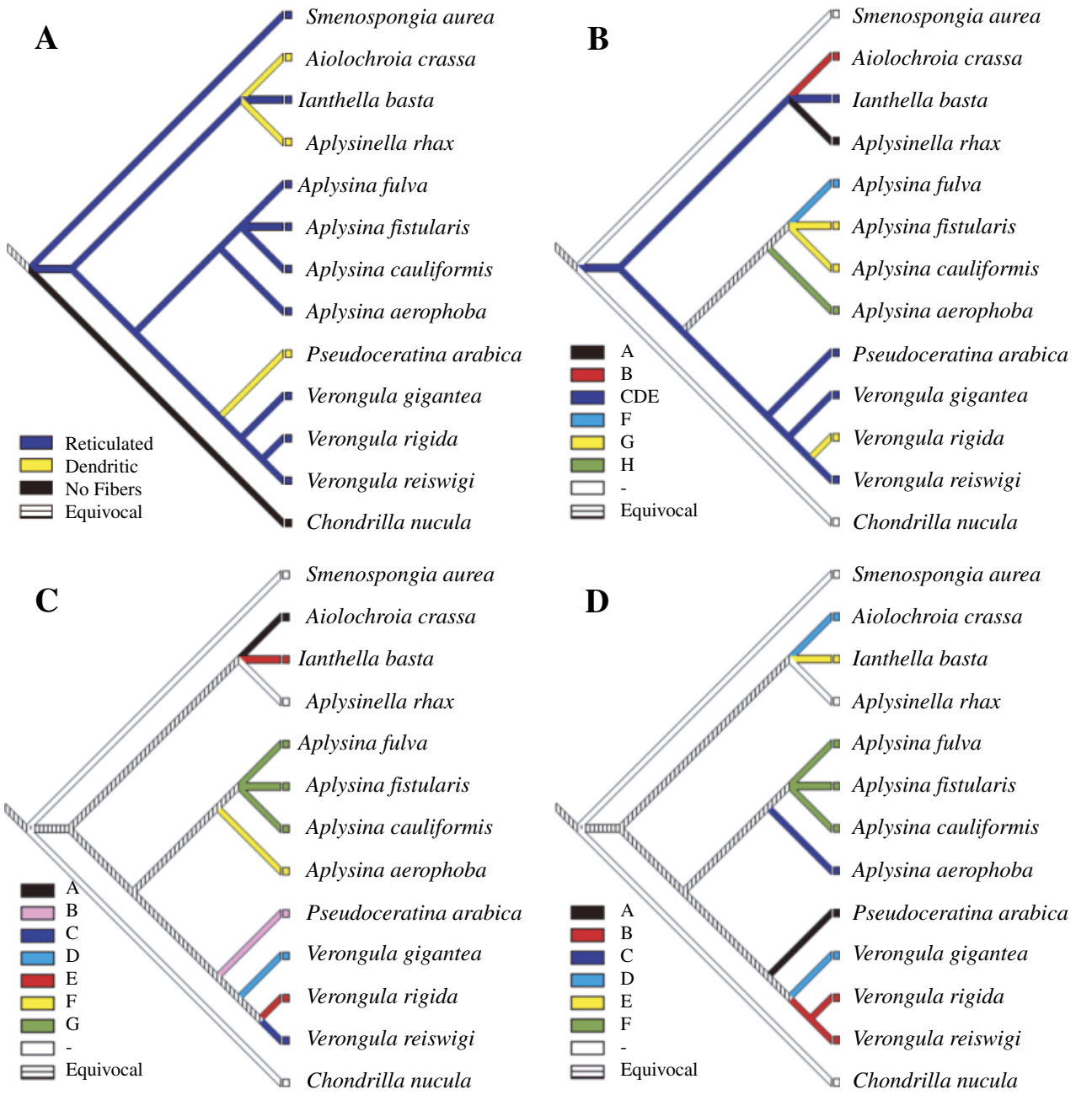

Fig. 5. Morphological characters mapped onto the consensus molecular phylogeny include: spongin fiber branching pattern (**A**), fiber diameter (**B**), pith diameter (**C**), and pith percentage (**D**). Labels for character states represent the significantly different groups illustrated in Fig. 2. The color of terminal nodes indicates the current morphological state of each character for each taxon and the color of supporting branches indicates the predicted ancestral states of each character. White coloration refers to missing or unavailable data (-), and black dotted coloration refers to equivalent ancestral character states (Eq).

ML and NJ analyses produced similarly well-resolved phylogenies. The major discrepancies between the three recovered phylogenies involved a monophyletic clade containing the genera *Aiolochroia, Ianthella,* and *Aplysinella* supported weakly by MP (57%) and moderately by NJ (84%), but not recovered in ML analysis.

Verongid sponges formed a very well-supported monophyletic clade in both MP and NJ analyses; however, ML analysis grouped the outgroup species

Table 5. Number of clones screened, number of different copies of the second internal transcribed spacer (ITS-2)/28S subunit found, and number (percentage) of variable bases among replicate clones of *Aplysina* spp.

Species	Number of clones screened	Number of copies	Number of variable bases (%)	
			ITS-2 region (229 bp)	Partial 28S subunit (425 bp)
Aplysina cauliformis	3	3	2 (0.87%)	2 (0.47%)
Aplysina fistularis	3	3	5 (2.18%)	0 (0.00%)
Aplysina fulva	3	2	2 (0.87%)	4 (0.94%)

C. nucula with the ingroup verongid species (100%). Within the verongid clade, the genera *Aplysina, Verongula,* and *Pseudoceratina* formed a clade with weak bootstrap support in MP (52%) and moderate bootstrap support in NJ (78%) analyses. Within this clade, the genera *Verongula* and *Pseudoceratina* formed a clade supported by MP, ML, and NJ analyses (85%, 59%, 99%, respectively, and hereafter). Within the genus *Verongula,* the species *V. gigantea* formed a well-supported clade (89%, 82%, 91%) separate from *V. reiswigi* and *V. rigida*. *Verongula rigida* formed a weakly supported clade (56%, 54%, $<50\%$), but *V. reiswigi* did not form a monophyletic clade. Within the genus *Aplysina,* the Mediterranean species *A. aerophoba* formed a well-supported clade (87%, 85%, 99%) basal to the three Caribbean species. Three of the four individuals of *A. cauliformis* formed a moderately supported clade in all analyses (76%, 75%, 65%), while *A. fulva* formed a weakly supported clade in the MP and NJ analyses (55%, 64%). *Aplysina fistularis* did not form a monophyletic clade under any criteria. Well-supported species-level monophyletic clades were recovered for *A. crassa* (100%, 94%, 100%), *I. basta* (100%, 100%, 100%), and *A. rhax* (99%, 70%, 99%).

Phylogenies reconstructed from morphological and molecular data exhibited statistically different topologies (PHT; $p<0.03$). The mapping of individual morphological characters onto the molecular phylogeny resulted in a wide range of CI and RI values (Table 2). Of the morphological characters examined, the spongin fiber data were the most informative, delineating closely related verongid species and providing the best fit to the molecular phylogeny (Fig. 5).

Discussion

Although the morphological character matrix produced a poorly resolved phylogeny of verongid sponges, rDNA sequence data produced a well-resolved and highly informative phylogeny. In the morphological phylogeny, verongid sponges were clearly differentiated from one outgroup species, but formed a clade with the other outgroup species and were thus paraphyletic. Eight of the 11 ingroup species formed monophyletic clades; however, the relationships among species, genera, and families were not well resolved. This high species-level resolution indicates the ability of morphological characters to distinguish species; however, the lack of resolution at the genus and family levels indicates the inability of this morphological data set to reconstruct phylogenetic history and relationships among these species. The low intra-ordinal resolution of the morphological phylogeny is not surprising, given the paucity of characters available for analysis and the potential plasticity and homoplasy of these characters. Although characters such as growth form and oscular arrangement are often used to distinguish between congeneric sponge species (van Soest 1978), homoplasic sponge morphotypes are common, as similar growth forms are found in distantly related sponge taxa (Hooper & van Soest 2002) and many sponge species exhibit different morphotypes with respect to environmental conditions (Niegel & Schmahl 1984; Palumbi 1984). Additionally, several morphological character states were found only in one species, providing no phylogenetic information beyond the cohesive nature of the species. Owing to these considerations, the ability of currently identified morphological characters to reconstruct the phylogenetic history of verongid sponges appears to be limited. In contrast, the phylogenies produced from rDNA sequence analyses were robust and well resolved from the ordinal to the species level.

We observed a high degree of variability in spongin fiber diameter, pith diameter, and pith percentage within and among species. The large amount of intraspecific variation implies that multiple measurements and careful analysis must accompany any spongin fiber examination. However, the degree of variation that we report is consistent with previous accounts of spongin fiber measurements (Wiedenmayer 1977; van Soest 1978; Zea 1987) that describe large ranges of fiber diameter and pith diameter within species. Despite this high degree of variation, the spongin fiber data distinguished several verongid species.

Some comparisons contradicted the molecular phylogeny; for example, the large sequence divergence between *Aiolochroia crassa* and *Verongula gigantea* was reflected in their differing total fiber diameters (Fig. 5B), but not in their similar pith percentages (Fig. 5D). Other spongin fiber data supported conclusions reached from molecular data. For example, members of the Mediterranean species *Aplysina aerophoba* had significantly smaller and more strongly pithed fibers than the three Caribbean species of *Aplysina,* results that agree with the rDNA phylogeny (Fig. 5A,B), which clearly delineates *A. aerophoba* from other species of *Aplysina* but offers less compelling evidence for distinctions among the Caribbean species of *Aplysina*. Similarly, the high percentage sequence similarity between *Verongula reiswigi* and *Verongula rigida* is reflected in their similar pith percentages (Fig. 5D). Overall, the results indicate that spongin fibers exhibit a high degree of intra-specific variability and appear to be most useful for distinguishing congeneric verongid species; comparisons of spongin fiber data between distantly related verongids can be homoplasic.

Wörheide et al. (2004) surveyed marine sponges for intragenomic polymorphisms (IGPs) within rDNA ITS regions. These authors reported that IGPs were infrequent; however, the incidence and extent of IGPs were unpredictable and varied considerably among taxa. Although Wörheide et al. (2004) demonstrated the phylogenetic utility of the ITS regions when IGP levels are moderate, they also cautioned against the use of ITS regions without screening for IGPs. Concordantly, we screened multiple clones from the three Caribbean species of *Aplysina* and found low frequencies of IGPs (Table 5). Although all three species exhibited multiple copies of ITS-2 and 28S, low levels of variation in *Aplysina cauliformis* and *Aplysina fulva* (<1%) may be a result of PCR or sequencing error, as polymorphisms were found in both DNA regions. Of the three species, *Aplysina fistularis* appears most likely to harbor variable copies of the ITS-2 region, exhibiting high variability among copies in the ITS-2 region (>2%) and no variation in the 28S region, possibly contributing to the paraphyly of this species in our phylogenetic analyses. Overall, the incidence of IGPs was low and did not significantly affect the branching pattern of our rDNA phylogeny.

Our molecular phylogenetic analysis of Verongida provided good support for the current classification of this order (Hooper & van Soest 2002). Sequence data supported Verongida as a separate and distinct sponge order in two of the three phylogenetic analyses, with only ML analysis not supporting the monophyly of Verongida by including *Chondrilla nucula* in the clade. Similarly, Nichols (2005) reported a close phylogenetic association between the orders Verongida and Chondrosida. All verongid genera were strongly supported by the molecular data. Phylogenetic analyses were largely congruent with species-level taxonomy, although there were low levels of genetic variation among the three Caribbean species of *Aplysina* (0.00–0.47%) and between two species of *Verongula* (0.00–0.47%), resulting in the paraphyly of *A. cauliformis* (only three of the four individuals form a monophyletic clade) and *A. fistularis,* and the paraphyly of *V. reiswigi* and *V. rigida* (Fig. 4). Similar low levels of genetic divergence have been reported previously for Caribbean species of *Aplysina* using ITS-2 and 18S rDNA sequence data (Schmitt et al. 2005). These low and inconsistent levels of genetic variation suggest that these species may represent recent speciation events and/or exhibit infrequent residual gene flow among species via hybridization. Further sampling of *Aplysina* and *Verongula* from geographically distant populations and exploration of new, more variable nuclear and mitochondrial markers will be needed to fully assess the potential for hybridization within these two genera.

The major discrepancy between the rDNA phylogenies and current verongid sponge classification involves the placement of the genus *Aiolochroia* and the legitimacy of the family Pseudoceratinidae. The species *A. crassa* was recently renamed (from *Pseudoceratina crassa*), given its own monospecific genus *Aiolochroia,* and placed *incertae sedis* in the family Aplysinidae with the genera *Aplysina* and *Verongula* (Bergquist & Cook 2002). The uncertain placement of the genus *Aiolochroia* is due to a fiber skeleton that shows both dendritic and reticulated portions. Although *A. crassa* was initially described as possessing a dendritic skeleton, recent observations found evidence of occasional reticulation among the fibers, which contain clear pith and bark elements. The removal of *A. crassa* from the genus *Pseudoceratina* is supported by the molecular evidence in this study; however, the placement of *A. crassa* within the family Aplysinidae is not supported because *A. crassa* exhibits high levels of sequence divergence from other members of Aplysinidae. Additionally, *A. crassa* forms a monophyletic clade with the genera *Ianthella* (family Ianthellidae) and *Aplysinella* (family Aplysinellidae) in two of the molecular phylogenetic analyses, providing preliminary evidence for a close association between *A. crassa* and these two families. A more thorough taxonomic sampling from all verongid families will be necessary to fully resolve the familial placement of the genus *Aiolochroia*.

The family Pseudoceratinidae consists entirely of the genus *Pseudoceratina* and is defined by skeletal fiber characteristics, which include a dendritic fiber structure and fibers composed entirely of pith, lacking laminar bark elements (Bergquist & Cook 2002). Although these characteristics are unique among verongid sponges, the molecular phylogenies presented here provide strong evidence for a close association between the genera *Pseudoceratina* and *Verongula*. The genus *Pseudoceratina* groups within the family Aplysinidae, suggesting that the unique fiber skeleton that defines *Pseudoceratina* may be more recently derived (Fig. 5A) than considered in previous investigations (Bergquist & Cook 2002).

Many sponge taxa exhibit a morphological simplicity that provides few diagnostic characters for systematic study and phylogenetic analysis (Nichols 2005). Although the importance of morphological systematics is not to be underestimated (Wiens 2004), the relative weight of specific morphological traits used to define taxa can be subjective and controversial, despite the existence of objective methods for weighting traits (Goloboff 1993). Molecular studies provide a means of testing the predictions of traditional morphological systematics, and often shed further insight into the interpretation of morphological characters (Alvarez et al. 2000; Borchiellini et al. 2004). This study demonstrates the ability of rDNA sequence data to elucidate intra-ordinal relationships within a sponge taxon exhibiting few diagnostic morphological traits. Although our results largely support the current classification of Verongida, they also highlight two issues associated with the morphology-based classification of sponges: the placement of taxa with intermediate characters (e.g., the placement of the genus *Aiolochroia*) and the determination of character polarity (e.g., the placement of the genus *Pseudoceratina*). Additional sequencing of verongid species not represented in this study and explorations of new regions of DNA will further enhance our understanding of verongid sponge phylogenetics.

Acknowledgments. Special thanks are due to M.C. Diaz for taxonomic verification of sponge species, M. Becerro for providing *Aplysina aerophoba* samples, and M. Slattery, D. Gochfeld, D. Gleason, and G. McFall for assisting with field collections. We thank the Caribbean Marine Research Center (Lee Stocking Island, Bahamas), the University of Guam Marine Lab, the Coral Reef Research Foundation (Koror, Palau), and NOAA's Gray's Reef National Marine Sanctuary for field support and assistance in obtaining collection permits. M. Salazar and the UAB CFAR DNA Sequencing Core Facility provided DNA sequencing support; this work has been facilitated by the infrastructure and resources provided by the NIH CFAR Core Grant P30 AI27767. This material is based on work supported by the National Science Foundation under Grant No. 0209329 awarded to RWT. This publication represents partial fulfillment of MS degree requirements by PME at the University of Alabama at Birmingham.

References

Alvarez B, Crisp MD, Driver F, Hooper JNA, & van Soest RWM 2000. Phylogenetic relationships of the family Axinellidae (Porifera: Demospongiae) using morphological and molecular data. Zool. Scr. 29: 169–198.

Arnedo MA, Coddington J, Agnarsson I, & Gillespie RG 2004. From a comb to a tree: phylogenetic relationships of the comb-footed spiders (Araneae, Theridiidae) inferred from nuclear and mitochondrial genes. Mol. Phyl. Evol. 31: 225–245.

Bergquist PR 1978. Sponges. Hutchinson and Company, London. 268 pp.

Bergquist PR & Cook SDC 2002. Order Verongida. In: Systema Porifera: A Guide to the Classification of Sponges, Vol. I. Hooper JNA & van Soest RWM, eds., pp. 1081–1096. Kluwer Academic/Plenum Publishers, New York.

Borchiellini C, Chombard C, Lafay B, & Boury-Esnault N 2000. Molecular systematics of sponges (Porifera). Hydrobiology 420: 15–27.

Borchiellini C, Manuel M, Alivon E, Boury-Esnault N, Vacelet J, & Le Parco Y 2001. Sponge paraphyly and the origin of Metazoa. J. Evol. Biol. 14: 171–179.

Borchiellini C, Chombard C, Manuel M, Alivon E, Vacelet J, & Boury-Esnault N 2004. Molecular phylogeny of Demospongiae: implications for classification and scenarios of character evolution. Mol. Phylogenet. Evol. 32: 823–837.

Chombard C, Boury-Esnault N, & Tillier S 1998. Reassessment of homology of morphological characters in tetractinellid sponges based on molecular data. Syst. Biol. 47: 351–366.

Degnan BM, Leys SP, & Larroux C 2005. Sponge development and antiquity of animal pattern formation. Integr. Comp. Biol. 45: 335–341.

Duran S, Pascual M, Estoup A, & Turon X 2002. Polymorphic microsatellite loci in the sponge *Crambe crambe* (Porifera: Poecilosclerida) and their variation in two distant populations. Mol. Ecol. Notes 2: 478–480.

——— 2004a. Strong population structure in the marine sponge *Crambe crambe* (Poecilosclerida) as revealed by microsatellite markers. Mol. Ecol. 13: 511–522.

Duran S, Pascual M, & Turon X 2004b. Low levels of genetic variation in mtDNA sequences over the Western Mediterranean and Atlantic range of the sponge *Crambe crambe*. Mar. Biol. 144: 31–35.

Erpenbeck D, Breeuwer JAJ, van der Velde HC, & van Soest RWM 2002. Unravelling host and symbiont phylogenies of halichondrid sponges (Demospongiae,

Porifera) using a mitochondrial marker. Mar. Biol. 141: 377–386.

Erpenbeck D, McCormack GP, Breeuwer JAJ, & van Soest RWM 2004. Order level differences in the structure of partial LSU across demosponges (Porifera): new insights into an old taxon. Mol. Phylogenet. Evol. 32: 388–395.

Erpenbeck D, Breeuwer JAJ, & van Soest RWM 2005. Identification, characterization and phylogenetic signal of an elongation factor-1 alpha fragment in demosponges (Metazoa, Porifera, Demospongiae). Zool. Scr. 34: 437–445.

Goloboff PA 1993. Estimating character weights during tree search. Cladistics 9: 83–91.

Hall TA 2000. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41: 95–98.

Hart MW, Johnson SL, Addison JA, & Byrne M 2004. Strong character incongruence and character choice in phylogeny of sea stars of the Asterinidae. Invert. Biol. 123: 343–356.

Hedin MC & Maddison WP 2001. A combined molecular approach to phylogeny of the jumping spider subfamily Dendryphantinae (Araneae: Salticidae). Mol. Phyl. Evol. 18: 386–401.

Hooper JNA & van Soest RWM 2002. Systema Porifera: A Guide to the Classification of Sponges, Vol. I. Kluwer Academic/Plenum Publishing, New York. 1101 pp.

Klautau M, Russo CAM, Lazoski C, Boury-Esnault N, Thorpe JP, & Sole-Cava M 1999. Does cosmopolitanism result from overconservative systematics? A case study using the marine sponge *Chondrilla nucula*. Evolution 53: 1414–1422.

Kumar S, Tamura K, & Nei M 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings Bioinform. 5: 150–163.

Levi C 1957. Ontogeny and systematics in sponges. Syst. Zool. 6: 174–183.

Maddison WP & Maddison DR 1997. MacClade: Analysis of Phylogeny and Character Evolution. Version 3.07. Sinuar Associates, Sunderland, MA.

Nichols SA 2005. An evaluation of support for order-level monophyly and interrelationships within the class Demospongiae using partial data from the large subunit rDNA and cytochrome oxidase subunit I. Mol. Phyl. Evol. 34: 81–96.

Niegel JE & Schmahl GP 1984. Phenotypic variation within histocompatibility-defined clones of marine sponges. Science 224: 413–415.

Palumbi SR 1984. Tactics of acclimation: morphological changes of sponges in an unpredictable environment. Science 225: 1478–1480.

Posada D & Crandall KA 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14: 817–818.

Schmitt S, Hentschel U, Zea S, Dandekar T, & Wolf M 2005. ITS-2 and 18S rRNA gene phylogeny of Aplysinidae (Verongida, Demospongiae). J. Mol. Evol. 60: 327–336.

van Soest RWM 1978. Marine sponges from Curacao and other Caribbean localities. Part I. Keratosa. Stud. Fauna Curacao 179: 1–91.

Sokal RR & Rohlf FJ 1995. Biometry, 3rd ed. W. H. Freeman, San Francisco.

Swofford DL 1998. PAUP*4.0, Version b10. Sinuaer, Sunderland, MA.

Tamura K & Nei M 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.

Thacker RW & Starnes S 2003. Host specificity of the symbiotic cyanobacterium *Oscillatoria spongeliae* in marine sponges, *Dysidea* spp. Mar. Biol. 142: 643–648.

Thompson JD, Gibson TJ, Plewniak F, Heanmougin F, & Higgins DG 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24: 4876–4882.

Usher KM, Sutton DC, Toze S, Kuo J, & Fromont J 2004. Biogeography and phylogeny of *Chondrilla* species (Demospongiae) in Australia. Mar. Ecol. Prog. Ser. 270: 117–127.

Wheeler WC, Gatesy J, & DeSalle R 1995. Elision: a method for accommodating multiple molecular sequence alignments with alignment-ambiguous sites. Mol. Phylogenet. Evol. 4: 1–9.

Wiedenmayer F 1977. Shallow-Water Sponges of the Western Bahamas. Birkhauser Verlag, Basel. 287 pp.

Wiens JJ 2004. The role of morphological data in phylogenetic reconstruction. Syst. Biol. 53: 653–661.

Wörheide G, Hooper JNA, & Degnan BM 2002. Phylogeography of Western Pacific *Leucetta 'chagoensis'* (Porifera: Calcarea) from ribosomal DNA sequences: implications for population history and conservation of the Great Barrier Reef World Heritage Area (Australia). Mol. Ecol. 11: 1753–1768.

Wörheide G, Nichols SA, & Goldberg J 2004. Intragenomic variation of the rDNA internal transcribed spacers in sponges (Phylum Porifera): implications for phylogenetic studies. Mol. Phylogenet. Evol. 33: 816–830.

Zea S 1987. Esponjas del Caribe Colombiano. Catalogo Cientifico, Bogota, Columbia. 286 pp.