Characterization of Genetic Markers for In Vitro Cell Line Identification of the Marine Sponge *Axinella corrugata*

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The marine sponge *Axinella corrugata* is being developed as a model organism for in vitro marine invertebrate research. Molecular genetics methods such as DNA fingerprinting [amplified fragment length polymorphism (AFLP) and single-stranded conformation polymorphism (SSCP)] and single-locus DNA sequence analyses were applied to this model to meet the primary objective of identifying positive *A. corrugata*–specific molecular markers that will aid in verifying cell identity in vitro and distinguish sponge cells from potential microbial contaminants. The extent of intra- and interspecific variation in these markers from geographically distinct samples of *A. corrugata* and closely related sponge taxa was also assessed. Two novel nuclear loci along with intervening transcribed spacer (ITS) regions of nuclear rRNA were characterized, although the latter appeared to better meet primary marker criteria, such as taxonomic specificity and high frequency of detection (via polymerase chain reaction [PCR]) from different individuals ($n > 40$) and cell cultures. Phylogenetic and phylogeographic analyses of ITS DNA sequences helped clarify taxonomies and also suggested species boundaries between and among western Atlantic and eastern Atlantic/Indian Ocean *A. corrugata* and Axinellidae samples. Patterns of genetic variation have important implications for the systematics, evolution, and chemical ecology of *A. corrugata* and related axinellids and are discussed.

Establishment of marine invertebrate cell cultures and the characterization of their genomes are at an early stage of development. To meet supply demands for development of marine natural products, efforts to establish in vitro production using model sponges have been initiated (Ilan et al. 1996; Osinga et al. 1998; Pomponi 1999; Pomponi et al. 1997, 1998; Rinkevich 1999; Willoughby and Pomponi 2000). For example, the shallow water sponge *Axinella corrugata* (class Demospongiae, order Axinellida, family Axinellidae, synonymous with *Teichaxinella morchella*) (Alvarez et al. 1998) produces the antitumor compound stevensine (odiline) (Wright et al. 1991) and is being developed as a model marine sponge for cell culture (Pomponi et al. 1997). The family Axinellidae encompasses many cosmopolitan species that range from polar to tropical regions, with about 50 nominal genera (Alvarez et al. 1998; Hooper et al. 1992; Hooper and Levi 1993; van Soest 1994). Despite this, the systematics of the more than 5000 species remain poorly resolved at most phyletic levels (Hooper 1999; Hooper et al. 1992; van Soest 1994), and studies of intra- and interspecific genetic variation in marine sponges remain few and far between (Chombard et al. 1997; Hooper et al. 1999; Sole-Cava 1994).

Sponge cell culture is beset by several major hurdles, such as slow cell proliferation, cell type verification, and microbial contamination (Pomponi and Willoughby 1994, 2000; Rinkevich 1999). Since the last two problems remain significant in even the most common cell lines (Gilbert et al. 1990; Green et al. 1972; Macleod et al. 1999; Nelson-Rees et al. 1981), the objectives of this study were to (1) identify positive species-specific molecular markers for *A. corrugata,* (2) assess each genetic marker's efficacy for distinguishing sponge cells from potential microbial contaminants in vitro, and (3) determine the extent of intra- and interspecific variation in these markers from geographically distinct samples of *A. corrugata.* Many marine sponge species host a wide microbial consortium (Burja et al. 1999; Lopez et al. 1999b; Wilkinson 1987), and distinguishing sponge host cells from microbial associates would serve as an adequate test for marker utility. Because our collection of

samples of Axinellidae covers wide geographical areas including the Caribbean, western Atlantic, eastern Atlantic, and Indian Ocean basins, we also viewed this study as an opportunity to investigate the phylogeography and potential population structure within and among western Atlantic *A. corrugata* and eastern Atlantic/Indian Ocean Axinellidae and among closely related congeners and axinellid family members. Although "fixed" genetic loci specific to *A. corrugata* are desired for the practical purposes of cell identification in vitro, determining levels of intraspecific variation could concomitantly provide useful data on the population structure, phylogeography, and molecular evolution of axinellid sponge species.

Molecular genetics offers a wide array of techniques for genetic marker development for distinguishing biological specimens (Ross et al. 1999; Sunnucks 2000). In general, the development of molecular markers can be divided into at least two broad categories: (1) genomic "fingerprinting" in the form of DNA banding patterns that are highly specific to the individual and sample-wide swaths of a genome (Gilbert et al. 1990; Lopez et al. 1999a; Welsh and McClelland 1990), and (2) single-locus characterization by DNA sequencing (Avise 1994; Hoelzel and Green 1992; Karl and Avise 1993). As one example of the first category, Lopez and Knowlton (1997) used amplified fragment length polymorphism (AFLP) analysis to distinguish coral sibling species in the reef-building *Montastraea* species complex. Alternative DNA fingerprinting techniques, such as random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSRs), have been previously described (Avise 1994; Coffroth 1997; Sunnucks 2000).

As the second approach, single-locus markers hold greater advantages over multilocus fingerprinting, such as the identification of discrete alleles and gene genealogies (Sunnucks 2000). For example, several studies have proven the utility of mitochondrial genes and their differences with nuclear markers (Burton and Lee 1994; Avise 1994). Unfortunately data on sponge mtDNA sequences and concomitant universal PCR primers remain relatively scarce (Hooper 1999; Watkins and Beckenbach 1999).

Fortunately rDNA internal transcribed spacer (ITS) regions have proven extremely useful for molecular evolution and systematics studies (Baldwin et al. 1995; Beauchamp and Powers 1996; Hershkovitz and Lewis 1996; Hunter et al. 1997; van Hoek et al. 1998; Wörheide et al., in press). Because both ITS-1 and ITS-2 sequences are not part of functional 5.8S, 18S small (SSU), and 28S large subunit (LSU) ribosomal RNA molecules, the ITS regions accumulate mutations more rapidly than the flanking rRNA sequences and are thus often useful as genetic markers at the species and subspecies (population) levels. Evidence of functional constraints that are correlated with a species' translational apparatus has also been found (van der Sande et al. 1992), and supports the utility of ITS sequences as species-specific markers.

Along with the traditional ITS locus, two novel genetic loci, TL13B and TL13C, can help to positively identify cultured *A. corrugata* cells. The novel TL13 loci were derived from cloning AFLP bands, which parallels strategies used to isolate polymorphic single-copy nuclear (SCN) loci (Karl and Avise 1993). The observed low intraspecific variation levels in these genetic markers supports *A. corrugata* species identifications when analyzed by either sequence analysis or single-strand conformation polymorphism (SSCP) electrophoresis.

Methods

Sponge Samples

Samples were collected from 1985 to 1997 from the western Atlantic and Caribbean (Figure 1) and were identified using morphological systematics characters (Alvarez et al. 1998) as *A. corrugata* (abbreviated as "Ac") or other species within the Axinellidae. The identity of *A. corrugata* samples was further verified by the presence/absence of stevensine, which is a discrete *A. corrugata*-derived secondary metabolite, using thin layer chromatography (see below and Wright et al. 1991). The source sample for novel nuclear markers was *A. corrugata* (sample Ac13) (Harbor Branch Oceanographic Museum catalog number 003:00969). Sponge samples morphologically identified as *Axinella* congeners or as closely related axinellid family members (e.g., *Ptilocaulis* sp.) were also included for molecular analyses and are hereafter labeled as "Ax" samples. The nonaxinellid species *Hymeniacidon* (family Halichondriidae) and *Anthosigmella varians* (order Hadromerida, family Spirastrellidae) were used as outgroups.

Cell Culture

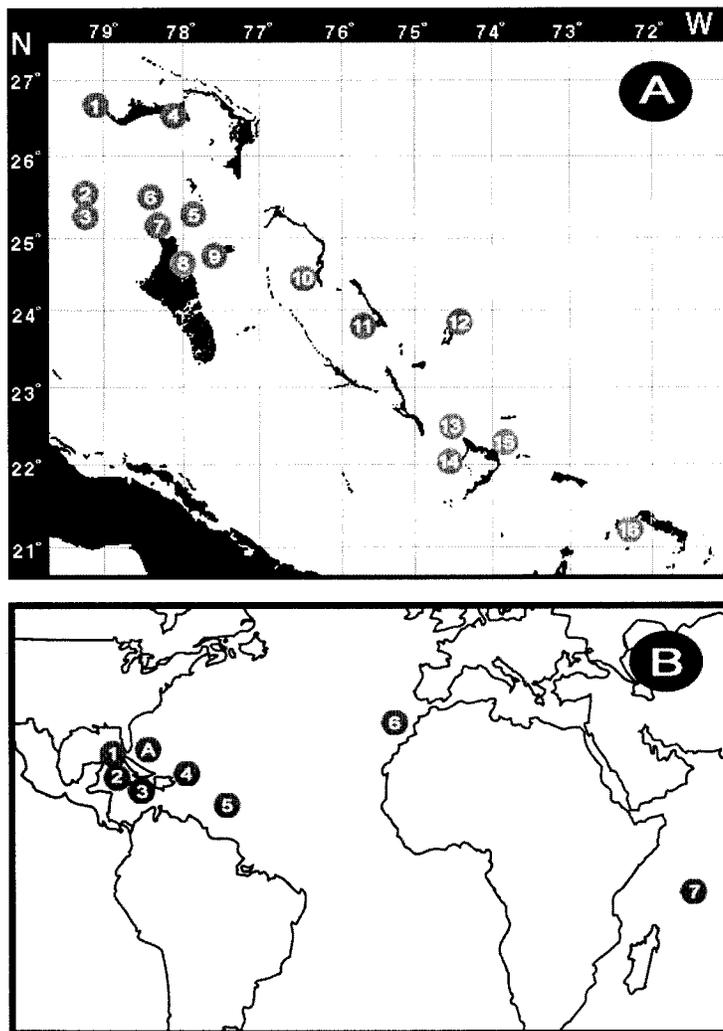Cryopreserved *A. corrugata* cells were thawed, and cultured for at least 7 days as described in Pomponi et al. (1997), with the following exceptions and additions: Cells were cultured at 10^6 viable cells/ml, 5 ml per flask, at 20°C, with medium containing the mitogen, phylohemagglutinin (1.5% by volume). This medium was changed every 3–4 days. After 2 weeks the cells were harvested and frozen for subsequent DNA analyses. To control fungal contamination, culture medium was supplemented with Fungizone (Life Technologies, Grand Island, NY) (1% by volume).

Thin Layer Chromatography

Small pieces of the sponge (approximately 0.5 cm³) were placed into 1 ml of methanol, macerated with a spatula, and steeped for approximately 4 h. The supernatant was transferred to a clean vial and the solvent removed by evaporation under a stream of nitrogen. The residue was reconstituted in 100 μl of methanol to provide a crude extract for chromatography. Four microliters of each extract were spotted onto the origin of DC-Plastikfolien Kieselgel 60 F_{254} plates cut to 10 cm × 10 cm (EM Separations, Gibbstown, NJ) The plates were eluted with a solvent mixture containing *n*-propanol-ethyl acetate-water (7:2:1 v/v/v). After the chromatography was complete, dried plates were sprayed with a solution of 2% ninhydrin in acetone (w/v), followed by heating. Stevensine can be detected as one of the major compounds present in the extract and appears as a bright salmon-red spot with a retention factor of 0.6.

DNA Extraction

DNA was isolated from 0.6–1.5 g of sponge mesohyl using a modified guanidium isothiocyanate method [based on Pitcher et al. (1989)]. Samples were ground to a fine powder in liquid nitrogen and incubated for about 1 h at 37°C in 5–10 ml of GES [60% (w/v) guanidium isothiocyanate, 20 mM EDTA, 0.5% sarcosyl]. Ammonium acetate was added to a final concentration of 2.5 M, and phenol:chloroform:isoamyl alcohol (25:24:1) extractions were performed until no material was visible at the aqueous-organic interface, followed by a final chloroform:isoamyl alcohol (24:1) extraction. The DNA was precipitated with 0.54 volumes isopropanol, washed with 70% ethanol, and resuspended in 300 μl dH_2O or TE (10 mM Tris, 1 mM EDTA) pH 8.0. For AFLP assays, *A. corrugata* DNA samples were further purified by either Geneclean (Bio 101, La Jolla, CA) or secondary phenol:chloroform extractions.

subunit 18S rRNA gene was amplified with highly conserved universal primers 18SA—5′ CCT GGT TGA TCC TGC CAG 3′ (Medlin et al. 1988)—and 18S–579rc—5′ TGCTG GCACC AGACTT GCCCT C 3′—using standard PCR conditions (Table 1).

Cloning

PCR products were cloned by spin-column purifying the amplified DNA from the reaction components (QIAquickTM PCR Purification Kit, Qiagen, Valencia, CA), ligating each product into a PCRII TA cloning vector (Invitrogen, Carlsbad, CA), transforming the recombinant plasmids into TOP10F′ competent cells, and plating the cells on Lennox L agar containing 50 μg/ml ampicillin for selection and spread with 40 μl 20 mg/ml Bluo-gal (Life Technologies) for colorimetric screening of clones. Then success of cloning was determined by culturing colonies from these plates overnight at 37°C in Lennox L broth containing 50 μg/ml ampicillin and performing plasmid minipreps (Promega Wizard PlusTM) and/or chelex preparations [10 μl culture added to 125 μl 5% chelex resin (Bio-Rad), heated 10 min at 98°C, vortexed, then centrifuged to pellet resin]. These samples were used for PCR and the products electrophoresed as described above to verify the presence of the insert ligated into the vector.

AFLP and SSCP Electrophoresis

As a relatively rapid and inexpensive method for assessing potential intraspecific genetic variation and single-copy status prior to more extensive DNA sequencing, candidate marker fragments that were amplifiable as small (<400 bp) PCR products were analyzed using "cold" SSCP electrophoresis (Girman 1996; Orita et al. 1989). PCR primers were designed to amplify portions of the candidate locus (Table 1). Purified PCR products (10–100 ng) were denatured by heating to 95°C for 4 min and then electrophoresed through precast 20% TBE gels with a Thermoflow/MiniCell polyacrylamide gel apparatus (NOVEX, San Diego, CA). Typically bands were separated at 300 W with a buffer temperature of 17°C for 8 h. Polyacrylamide gels were stained with ethidium bromide for 30 min to visualize the bands.

Extensive descriptions of both SSCP and AFLP protocols have been described in detail elsewhere (Lopez et al. 1999a; Lopez and Knowlton 1997; Vos et al. 1995). For AFLP, primers with the following arbitrary 3′ base extensions were tested: AF1

Figure 1. *Axinella corrugata* and other sponge samples were obtained from the following geographical locations with the number of samples shown in parentheses: A—(Bahamas archipelago): 1—Grand Bahama Island (west end)/Wood Cay (3) (Ac29, Ac30, Ac32); 2—Bimini (1) (Ac25); 3—Riding Rocks (1) (Ac31); 4—Sweetings Cay (1) (Ac10); 5—Chub Cay (6) (Ac4, Ac7, Ac16, Ac22, Ac35, Ac37); 6—Great Bahama Bank (NW channel) (3) (Ac5, Ac18, Ac23); 7—Joulters Cay (1) (Ac2); 8—Andros (Tongue of the Ocean) (1) (Ac34); 9—New Providence (2) (Ac15, Ac36); 10—Eleuthera (1) (Ac33); 11—Cat Island (1) (DD 10); 12—San Salvador (2) (Ac13, Ac14); 13—Crooked Island (1) (Ac19); 14—Long Cay (0); 15—Acklins Island (4) (Ac1, Ac3, Ac11, Ac20); 16—Turks and Caicos (2) (Ac9, Ac28). B—Caribbean, eastern Atlantic, and Indian Ocean: 1—Florida Keys, Marquesas, and SW Gulf of Mexico (16) (Ac12, Ac21, Ac24, Ac38, Ac39, Ac40, Ac41, Ac42, Ax1, Ax5, Keys-1, Keys-2, Keys-3, Keys-4, Keys-6, Keys-8; 2—Honduras (1) (Ac26); 3—Jamaica (3) (Ac6, Ac8, Ac17); 4—Puerto Rico (1) (Ac27); 5—Grenada (1) (Ax14); 6—Canary Islands, Tenerife (4) (Ax2, Ax3, Ax6, Ax9); 7—Seychelles (La Digue, Praslin) (3) (Ax7, Ax16, Ax18).

PCR Conditions

PCR was performed on genomic DNA templates in 30 μl volumes containing the following reactants: 0.1 mM each dNTP, 0.5 units *Taq* polymerase (Sigma or Promega), 10–100 ng DNA template, 25 pM each primer, 10 mM Tris pH 8.3, 50 mM KCL, 1.5 mM MgCl$_2$, and 0.001% gelatin. The typical reaction profile was 30 cycles at 94°C for 40 s, 53°C for 1 min, and 72°C for 1 min preceded by 1 min at 94°C and followed by 30 min at 72°C. Five microliters of each reaction were electrophoresed through a 1% agarose gel in 0.5× TBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA). All PCRs were repeated at least once to verify the results.

Sponge ITS and the 5.8S rRNA gene regions were most efficiently amplified with primers ITS RA2 and ITS 2.2 (Table 1) at an annealing temperature of 53°C (Adlard and Lester 1995; Wörheide 1998; Wörheide et al., in press). Another ITS primer in the ITS 2 region was designed and called 670rc—5′ CCTGC AGAAC ACTGC TGTCA 3′ (Figure 4)—to amplify recalcitrant *A. corrugata* samples.

To support the taxonomic identification and source of DNA templates used in PCRs, a 600–800 bp segment of the small

Table 1. Sequence profiles for proposed *Axinella corrugata* genetic markers

Genetic marker	Base composition				Predicted PCR product (size in bp)[a]	Closest database relative (no. matching residues/% identity)[b]	Primers (written 5′ → 3′)	Frequency of positive PCR amplification no. positive/total no. tested (%)		
	A	C	G	T				*A. corrugata*	*Axinella* sp.	Outgroup sponges
TL13A	20	25.3	26	28	316 (430)	Silkworm transposon (57/43)	375f—CTG CAC CCA GCC CAT ATG AGG ATG CATC; ESG-rc – CAT GCT GAT CCT GA	35/42 (83)	6/13 (46)	0/20 (0)
TL13B	26	24.6	22	27	311 (409)	No hit w BLASTX	47-f—TGG ATG TTA CTA TTT GGA CCT CG; 357rc—AGT TAT GCG CTA TAT GCA TGA GG	35/42 (83)	5/13 (38)	0/20 (0)
TL13C	25.7	21	27.8	25.4	294 (385)	Actin binding protein (22/32)	f—AATTT ATAGC GTGAG TTGAA CCG; rc—GGTAT CTGAC AGACC TCCAA CAG	37/42 (88)	6/13 (46)	1/20 (5)
ITS[a]	22.2	23.3	26.6	27.8	784	*Axinella* 18S or 23S rRNA (532/90–97)	RA2-f—GTCCCTGCC CTTTGTACA CA; 2.2rc – CCTGGTTAGTTTCTT TTCCTCCGC	42/42 (100)	9/13 (69)	12/20 (60)
Control 18S rRNA[b]	26.5	20.3	25	26.5	532	*Axinella polypoides* 18S rRNA (532/ 95)	18SA—CCT GGT TGA TCC TGC CAG (Medlin et al., 1988); 18S-579rc TGCTG GCACC AGACTT GCCCT C	42/42 (100)	12/13 (92)	16/20 (80)

[a] Sizes of amplified PCR products from representative individuals are shown for each locus. The actual size of original clones are shown in parentheses. Smaller subfragments of the original clones were amplified for optimal SSCP analyses.

[b] Searches were performed with BlastX. Therefore matching residues for the ITS and rRNA indicate DNA residues.

[c] The tabulated scores for the ITS locus reflect a compilation of two different sets of ITS-specific primers (RA2 and 2.2 or RA2 and 670rc). Highest success was obtained with the RA2 and 2.2 combination shown above. ITS amplification was considered positive if any of the primer combinations produced a product in the predicted size range for that primer pair. The nucleotide statistics are based on the representative sequences of Ac25 submitted to GenBank (AF300463).

[d] Base composition based on SSU rRNA gene of Ac35 in GenBank (AF300464).

(GGAG), AF32 (TGG), AF33 (AGCC), AF34 (CAAG).

DNA Sequences and Phylogenetic Analysis

Novel sponge DNA sequences were generated by cycle-sequencing reactions using Applied Biosystems Inc. (ABI, Foster City, CA) "Big Dye" sequencing kits. Completed reactions were sent to DNA sequencing laboratories at either Texas A&M University (Department of Veterinary Pathobiology) or the University of Florida (Interdisciplinary Center for Biotechnology Research). Sequence chromatograph data were analyzed and edited with Seqed (ABI). Multiple sequence alignments were constructed with PILEUP [Genetics Computer Group (GCG) 1994]. Alignments were optimized by testing various gap weights and also by aligning each ingroup separately prior to composite alignments. Alignments used for phylogenetic analysis are available from the authors upon request.

Sequences were queried to nonredundant databases with the BLAST family of programs (Altschul et al. 1990, 1997)—BLASTN (nucleotide query against a nucleotide database) and BLASTX (six frame conceptual translation of a nucleotide query against a protein sequence database)—and also analyzed by various programs in the GCG software package (GCG 1993). Optimal primers for PCR were designed using PRIMER 3 software available on the Internet (Rozen and Skaletsky 1996–1997).

Phylogenetic analyses with maximum parsimony, maximum likelihood, and distance methods were performed using PAUP 4.0b3* (Swofford 1999) on a Macintosh G3 PowerPC. Typical reconstructions used random stepwise addition with at least five replications, tree bisection reconnection and branch swapping, and 500–1000 replicates in bootstrap analyses. To help determine the topological order of clades, both midpoint and outgroup rooting and Bremer support indices were used (Bremer 1994). Distance matrices were based on uncorrected *P* values or implemented Kimura's 2*N* parameter correction scheme (Hillis et al. 1996). The concordance of derived topologies or clade reconstructions using different methods and parameters was taken to be reflective of true phylogenies (Pecon-Slattery and O'Brien 1998).

The following outgroup ITS sequences from sponge, ctenophoran, cnidarian, and fungal sequences were obtained from GenBank and used in phylogenetic analyses: sponge, *Halichondria panicea* (AF062607); sponge, *Hymeniacidon heliophila* (U65485); cnidarian, *Heteractis magnifica* (AF050206); ctenophore, *Bolinopsis* (U65480); fungus, oat root basidiomycetes (AJ246161); and fungus, *Penicillium minioluteum* (L14505). *Anthosigmella varians* (Avv) sequences are derived from samples kindly provided and identified by Dr. M. Hill (Fairfield University, CT).

The following GenBank accession numbers have been assigned for representative *A. corrugata* sequences: TL13A (AF300460), TL13B (AF300461), TL13C (AF300462), Ac25 ITS (AF300463), and Ac35 partial 18S rRNA gene (AF300464). Additional ITS sequences have the following accession numbers: AY055455–AY055461.

Results and Discussion

Genomic Fingerprinting and Detection of Microbial Contaminants

To test the efficacy of a relatively rapid DNA fingerprinting method for generating species-specific markers, AFLP-PCR was applied to *A. corrugata* genomic DNA samples (Figure 2A). Unlike previous studies

A.

29 31 32 33 19 20 22

400 bp->

B.

C2 26 C4 27 C5 28
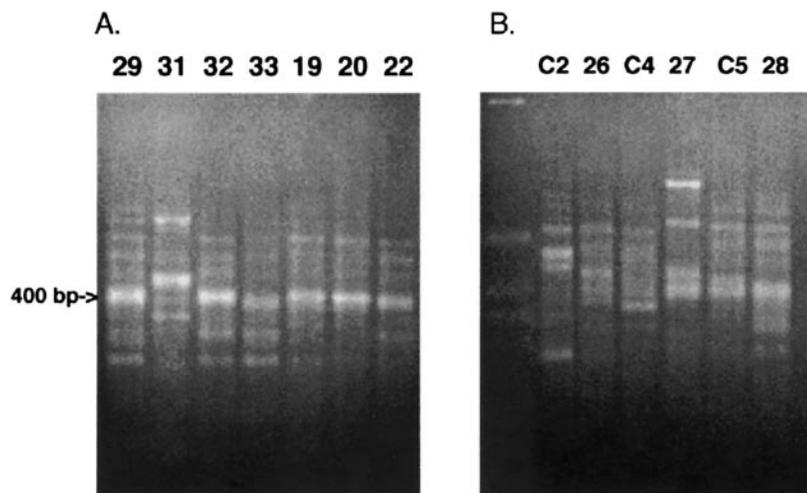
Figure 2. Representative agarose gel of *Axinella corrugata* products after AFLP PCR amplification with extension primer GAG. Numbers correspond to a specific *A. corrugata* sample. Panel A shows a random sample of *A. corrugata* AFLP profiles, while panel B shows *A. corrugata* samples (Ac26, Ac27, Ac28) that are paired with their corresponding cell culture samples (C2, C4, and C5, respectively). Although the size marker is not shown, the arrow points to the most consistent fragments in the range of 300–400 bp, which were later cloned and sequenced.

using the modified AFLP technique (Lopez et al. 1999a), sponge AFLP patterns appeared predominantly as low molecular weight (<700 bp) bands with high background smearing. This may be attributable to some of the harsh physical conditions of the DNA purification protocol. The AFLP results may have also been compromised by poor restriction digestion of sponge genomic DNA, a necessary step for AFLP template preparation. Nevertheless, after testing several different AFLP extension primers, a single primer (AF1) gave relatively consistent banding patterns (Figure 2A). Conserved fragments in the 300–400 bp size range appeared in most *A. corrugata* individuals, with only a few exceptions. For example Ac31 produced a highly deviant AFLP pattern, which is not likely due to phylogeography, but could be related to depth. Ac31 was collected from a deeper site than any other sample analyzed by AFLP. Overall, none of the AFLP band variation correlated with the respective geographical sources of the samples.

Although it is difficult to prove that these patterns stem solely from sponge genomic DNA, the same AFLP assay and primer set were applied to paired samples derived from *A. corrugata* cell cultures and matching source sponge somatic mesohyl. Three pairs of cultured and somatic sponge samples (C2 = Ac26, C4 = Ac27, C5 = Ac28) were analyzed. Comparison of somatic and cell culture patterns (Figure 2B) indicated that only the C5/Ac28 pair gave corresponding AFLP patterns between somatic and cultured samples, while the first two pairs significantly dif-

fered in banding patterns. These results supported microscopic analyses that the C2 and C4 cultured samples had become overgrown with microbes (see other cell culture results below).

DNA fingerprinting (with minisatellite probes) has been used successfully for verification of human cell lines in the past (Gilbert et al. 1990). However, the variable number of tandem repeats (VNTR) has been surpassed by more rapid and accessible fingerprinting methods such as AFLP and RAPD (Green et al. 1972; Kawai and Mitsuhashi 1997). Although our application of AFLP-PCR was limited in this study, this method could be attempted again once a sponge cell line is established.

Cloning and Sequence Analyses of Candidate Genetic Markers

Because of the probability that some of the AFLP bands in the fingerprint patterns of somatic *Axinella* samples may be derived from microbial symbionts commonly associated with marine sponges (Lopez et al. 1999b; Wilkinson 1987), it was decided that total reliance on AFLP patterns for sponge cell verification in vitro was not possible. Furthermore, in an effort to demonstrate that some of the more conserved AFLP marker bands (e.g., 300–400 bp region) originated from the sponge and not a microbial symbiont or contaminant, cloning of individual AFLP bands was initiated. This strategy resembles previously successful strategies for cloning SCN loci as a source of polymorphic nuclear genetic markers for natural history studies (Karl and Avise 1993).

Analysis of clones in the 300 bp region revealed the presence of multiple comigrating DNA PCR products in this size range, which is not uncommon in some DNA fingerprints (Grosberg et al. 1996). Three of these fragments, cloned as TL13A, TL13B, and TL13C, were sequenced and analyzed in greater detail as candidate single-locus markers (Table 1). BLAST searches gave no significant nucleotide or protein matches for clone TL13B, and only a distant relationship with a human actin binding protein across 22 amino acid residues for TL13C (32% identity). TL13A resembled retrotransposon reverse transcriptase amino sequences (43% identity) across about 57 residues after BLASTX searches. No significant open reading frames were detected greater than 150 bp or 300 bp for TL13B and TL13C, respectively.

Pairwise comparisons of either TL13B and TL13C sequences among different *A. corrugata* individuals using GCG's GAP program indicated low intraspecific sequence divergences on the order of less than 1%, which precluded the need for further sequencing of multiple sequences (also see SSCP results). Further, the base composition of all three novel loci did not deviate substantially from an average base composition profile of sponge genes (primarily *Geodia cydonium*) derived from current databases (Müller et al. 1998). Base composition analysis (Table 1) showed that the percent GC content ranged from about 0.46 to 0.51 and also supported elimination of possible protistan sources of contamination of sponge genomic DNA, since many protistan genomes are AT rich (Adje et al. 1998; Sogin and Silberman 1998). The possibility for a bacterial or fungal genomic source for the TL13B and TL13C loci is very difficult to eliminate entirely because the efficiency of obtaining pure microbial symbiont cultures for comparative testing is considered to be less than 1% (Button et al. 1993; Lopez et al. 1999b). Nevertheless, the high frequency of 18S rRNA PCR products amplified from the same *A. corrugata* DNA templates (Table 1) and identified as marine sponge in origin (e.g., *Hymeniacidon*) strongly suggested that sponge genomic DNA was the predominant DNA template present in all PCRs shown here.

To determine the reliability of novel marker loci, specific oligonucleotide primers were designed from the respective cloned sequences. Development of specific primers for consistent PCR, as well as verifying a single-copy status are vital cri-
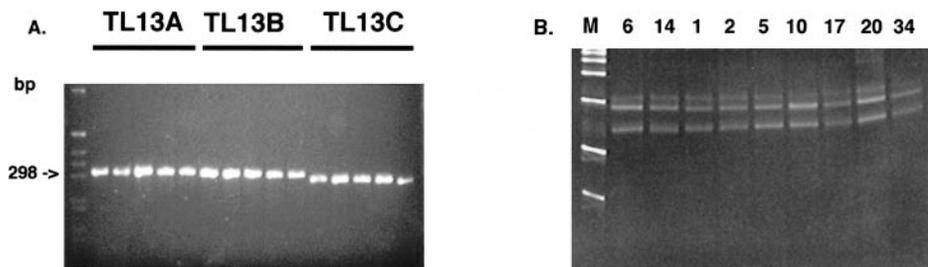
Figure 3. Agarose gel of representative candidate marker loci after standard PCR amplifications (A). Lanes 2–6 show TL13A PCR products from *Axinella corrugata* samples Ac12, Ac13, Ac22, Ac24, and Ac25, respectively. Lanes 7–11 show TL13B products derived from *A. corrugata* samples Ac3, Ac12, Ac13, Ac24, and Ac25, respectively. Lanes 12–16 show TL13C PCR products derived from *A. corrugata* samples Ac8, Ac12, Ac14, Ac25, and Ac28, respectively. (B) TL13B products after SSCP electrophoresis. Ac identification numbers are indicated above each lane. These products represent single-stranded molecules with an apparent heterozygous condition producing at least three strands with resolvable conformation differences. Parental genotypes and the exact ploidy of this species are currently unknown. The fourth strand most likely is comigrating with one of these. Lane 1 contains the 1 kb molecular weight marker fragments.

teria for their utility as reliable genetic markers. Figure 3A shows that predicted PCR products of TL13A, B, and C candidate marker loci were amplified from different *A. corrugata* samples and clearly resolved on agarose gels. Table 1 summarizes the results of all PCR amplifications for each candidate marker locus. The ITS locus (discussed in more detail below), using both ITS primer pairs, gave the highest frequency of positive amplification for *A. corrugata* templates (100%), followed by TL13C (37/42 = 88%), TL13A (35/42 = 83%), and TL13B (35/42 = 83%). In contrast (albeit with lower sample sizes), a much lower frequency (0–60%) of PCR success was observed using the same locus primers with congeneric *Axinella* (Ax) species, non-*Axinella* axinellids (*Ptilocaulis*), or anonymous/unidentified sponge samples as templates (Table 1), supporting higher *A. corrugata* specificity for candidate TL13B and C marker loci.

Consistent PCR amplification of novel genetic markers TL13B and TL13C was also tested on primary cell cultures of *A. corrugata*. First, TL13B and TL13C PCR products in the expected size range were amplified from three *A. corrugata* primary cell culture samples. These products resembled fragments obtained from the somatic *A. corrugata* samples shown in Figure 3A. Cell culture fragment identities were confirmed by either DNA sequencing or dot-blot hybridization with a digoxigenin–duTP–labeled marker locus gene probe. Second, since cell culture samples had fewer cells and lower yields of DNA than somatic mesohyl samples, minimum threshold amounts of 70 pg DNA template for the successful detection of TL13B and TL13C by PCR was determined by dilution experiments.

The ability to PCR amplify these two markers from many geographically distinct samples of *A. corrugata,* as well as from several separate cell cultures from this species, indicates that TL13B and TL13C will be useful for distinguishing *A. corrugata* cells from other sponge or contaminant cells in culture. Moreover, even if these markers stem from a microbial associate, the high frequency of amplification from *A. corrugata* individuals suggests a specific symbiosis that is not found in other sponges.

Analysis of Intraspecific Variation Among and Within Genetic Marker Loci

The SSCP technique has the capacity to resolve single base pair mutations in single-stranded DNA arising from different equilibrium state molecular conformations resolvable as altered mobilities on nondenaturing polyacrylamide gels (Orita et al. 1989). Due to the high resolution of the technique, SSCP was used to (1) rapidly evaluate levels of intraspecific variation and (2) estimate the copy number of novel marker loci. For the second application, more than four polymorphic single-stranded fragments would exceed the expected two alleles and suggest multiple copies of the respective locus. Although precise chromosome numbers are unknown for most sponges, diploidy was assumed for *A. corrugata,* along with low or no genetic homogenization of multiple copies of the novel candidate marker loci.

In a representative SSCP gel, TL13B PCR products revealed no intraspecific variation among nine *A. corrugata* samples (Figure 3B). SSCP experiments with several ITS and TL13C products also indicated few or no resolvable differences in banding patterns (data not shown). By contrast, TL13A PCR fragments appeared to be

more variable than previously observed on agarose gels, with complicated banding patterns that were possibly composed of multiple fragments or loci (data not shown). Multiple copies of TL13A were further suggested when direct sequencing of TL13A PCR products exhibited superimposed or unresolvable chromatographs. Since this phenomenon may be attributable to the retrotransposon-like identity of TL13A sequences (Table 1), and since concomitant multiplicity of TL13A loci would hinder interpretations of later assays, TL13A was eliminated from further consideration as a consistent genetic marker.

Evaluation of intraspecific variation at candidate marker loci helped determine their reliability for cell identification of in vitro cultures derived from contaminated, geographically isolated, or anonymous sponge samples. Overall, marker loci TL13B and TL13C displayed low genetic variation within *A. corrugata,* which enhanced their utility, despite less than 100% PCR success. SSCP was applied as a relatively rapid screen of genetic variation of candidate loci and indicated that the technique could be used for verifying the degree of fixation of specific *A. corrugata* genetic markers and also infer the single-copy status. The frequency of amplification may be improved by testing new TL13B and TL13C PCR primers and reaction conditions. Although enough sequence identity in these loci exists to allow amplification from distant axinellid species (38–46% in Table 1), these two novel loci may be used in tandem with other markers (e.g., ITS) for elimination or confirmation purposes to enhance their utility.

ITS DNA Sequence and Phylogenetic Analyses

As the more common gene marker, ITS loci met the expectations of reliable amplification by PCR from at least 42 different *A. corrugata* samples (Table 1). Sequence lengths for each region were roughly 300 bp for ITS-1, 150 bp for 5.8S rRNA, and 200 bp for ITS-2. A sponge origin for all the *A. corrugata* ITS sequences was confirmed after BLAST searches revealed an *A. damicornis* SSU sequence identity in the region of the ITS RA2 primer. The full length of the sequenced region (including SSU and LSU) spanned about 800 bp, with a mean GC content of about 49%. Individual ITS fragments were chosen for sequencing based on the hypothesis that geographically separated samples would exhibit the

```
Ac7          GAGGTGTTGTC--TGACAGCAGT--GTT---CTGCAGGCATCCCTTGAAA
Ac9          ...........--.......--...---...........
Ac6          ...........--.......--...---...........
Ac25         ...........--.......--...---...........
Ac21         ...........--.......--...---...........
Ac1          ...........--.......--...---...........
Ac38         ...........--.......--...---...........
Ax7          ..........--C.......--...CTG...........
Ax18         ..........--C......G..--..CTG...........
Ax9          ..........--.TGTT..-------.TTG....G........
Ax16         ..........--C.......--...CTG...........
Ax18         ..........--C....C.G..--...CTG.............C.TTC
keys1        ..........--..GTG.....TGC..TTG..A..........
keys2        ..........--..GTG.....TGC..TTG..A..........
CL           .GC..C.GAGAGACTG.CTTC..CC.G.TCGAGCGTTCG...G..C.TTC
Halichondria CGTT.TGA.G.GT..T.T..G.GCCC.CGCG.CA.G.........C...G
Hymeniacidon AGCTC.C..GTCGC.TAC.TT..GA.GAGTTG.CTCC.AGCGG..C.TCG
Bolinopsis   CCTT..C..ATGAA.CG.A.G..CCC..GAAG.AA..CG.....GA.TTG
Heteractis   .C..CCC.CCGCAA.CA....AGCA.C.G-CG.TGC...C..GTCCCTTG
oatrootbasid .T.TA.GCT.GGA..TGGAGGC.TT.CAGGT.CA.TCTTT.GTGG.CTGC
asco1        CCCCCTCAAC.GGC.GG.CGT.CCCCGAAATGAAGTT.GCGGTG.C..GT
```

Figure 4. Portion of ITS-2 sequence alignment chosen to distinguish *Axinella corrugata* (Ac) from closely related Axinellidae (Ax) sponges. This region occurs within ITS-2, about 100 bp downstream of the 5S rRNA gene. ITS boundaries were identified by the presence of highly conserved 5S rRNA motifs such as 5′ CATCGATGAAGAACGCA 3′, which is 100% conserved between sponge and outgroup fungal sequences. The entirety of new *Axinella* ITS sequences are available from GenBank with the following accession numbers: AY055455–AY055461. Outgroup sequences are represented or abbreviated as follows: *Bolinopsis* (ctenophore), *Heteractis* (cnidarian), CL—anonymous calcarea sponge, oat root basidiomycete (fungus), asco1 (fungus).

greatest sequence divergence and possible evidence of intraspecific population structure among *A. corrugata* individuals. Many more axinellid sponge samples from the Harbor Branch collection could have been added to the phylogenetic analyses, but a comprehensive systematics reevaluation of this family was beyond the scope of the objectives.

Alignment of multiple *A. corrugata* ITS sequences indicated low intraspecific variation. Although sample numbers were low, several single-base polymorphisms may represent private alleles such as the presence of a T at position 292 of the Ac25 ITS sequence in the San Salvador and Jamaican *A. corrugata* individual samples. Some polymorphisms stemmed from
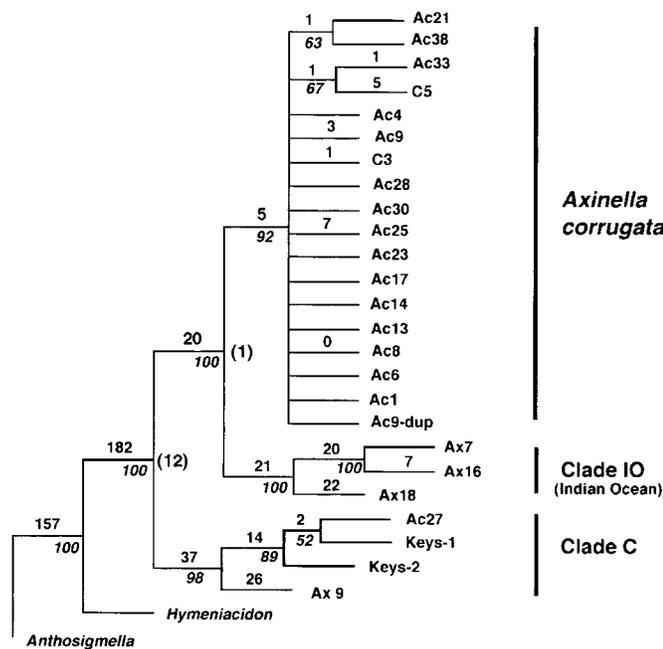
Figure 5. Maximum parsimony reconstruction based on complete ITS-1 and ITS-2 sponge sequences (800 bp). This phylogram was derived by a heuristic search with the tree bisection reconnection (TBR) branch-swapping options. A fourth cell culture sample (C3) derived from individual Ac25, along with C5 and a duplicate Ac9 sample are also analyzed. A strict consensus of 9893 trees recapitulated the presence of three major axinellid sponge clades and the same topology. In other reconstructions, transversions were weighted from 2 to 50 times transitions, but did not significantly alter tree topology. Two nonaxinellid sponges are included as outgroups. Assigned branch lengths and bootstrap percentages after 500 iterations are shown above and below the branches, respectively. The number of steps required before a specific clade collapses (Bremer indices) are shown in parentheses. The total tree length was 756 steps, with a consistency index of 0.9299 and retention index of 0.9047.

"double peak" ambiguities (likely heterozygotes) in sequence chromatograms. Conversely, much higher sequence divergences were evident between *A. corrugata* and outgroup (nonaxinellid sponge, cnidaria, and fungal) ITS sequences. For example, the mean pairwise uncorrected P difference values were (1) less than 1.0% among *A. corrugata* individuals; (2) 6.4% between *A. corrugata* and putative congeneric *Axinella* spp. (from the Indian Ocean); and (3) 10–12% between *A. corrugata* and *Ptilocaulis* or closely related axinellids. Similar distance values were obtained using Kimura two-parameter model corrections. Distances derived from sponge and outgroup comparisons were typically beyond error limits due to the lack of reliable sequence alignments at levels of more than 70% sequence divergence. A segment of the ITS-2 region was chosen to distinguish *A. corrugata* from other congeneric sponges because of the presence of multiple insertion/deletion mutations and thus for the design of *A. corrugata*-specific PCR primers (Figure 4).

Phylogenetic analyses of complete sponge ITS sequences using maximum parsimony and maximum likelihood supports genetic distance measurements. First, strong evidence of a phylogenetic signal among ITS sequences was indicated by a g statistic of −3.00 after evaluating 1000 trees with the Random Trees test. Among confirmed *A. corrugata* samples, only 116 parsimony informative variable sites were observed, in contrast to 292 informative sites after adding non-*A. corrugata* (Ax) sponge ITS sequences to the comparison. The maximum parsimony tree (Figure 5) has a topology of three major clades [hereafter labeled clades Ac (*A. corrugata*), IO (Indian Ocean), and C (Axinellidae mixture)] and represents a consensus reconstruction of 16 *A. corrugata* somatic samples, 2 *A. corrugata* cell culture samples, 6 axinellid family members, and 2 sponge outgroup samples. Overall, the same three-clade topology was reconstructed with smaller ITS sequence subsets and running exhaustive and branch-and-bound parsimony options. Likewise, this branching order was recapitulated in strict consensus trees and also with different rooting options such as midpoint and unrooted trees.

Although *H. heliophila* and *A. varians* were found to be the most suitable outgroups for the analyses of *A. corrugata* ITS sequences, the large divergences (>60% mean uncorrected P distances) between ingroup and candidate outgroups (e.g.,

fungi, cnidaria) leave open the possibility of large systemic errors. Also, although the true root of the tree is difficult to ascertain for these reasons, a separate phylogenetic reconstruction using only 171 bp of the conserved 5S region of the same taxa yielded the same topology of Figure 5 showing *Hymeniacidon* and the most basal *Anthosigmella*. Consequently the topology shown in Figure 5 more reliably reflects the clustering of specific axinellid samples rather a final phylogeographic conclusion.

Within clade Ac, it was interesting to observe the grouping of Ac21 with Ac38 (albeit with only one synaptomorphy), since these samples were both from the Florida Keys (see Figure 1 legend). This shared node was not observed in neighbor-joining trees (data not shown).

All reconstructions indicated that the *A. corrugata* clade (Ac) is most closely allied to clade IO (Indian Ocean), since the two clades collapsed after only a single Bremer index step. Clade IO was comprised of Ax7, Ax16, and Ax18 individuals, which all came from the Seychelles region and were identified by morphological characters as Axinellidae, but not *A. corrugata* species. Ax7 and Ax16 are the same species, an *Axinella* sp. very similar to *A. corrugata*; Ax18 is similar to Ax7 and Ax16, but not exactly the same. The ITS phylogenetic reconstruction supports the morphological similarities and differences both within the clade and between the *A. corrugata* and IO clades. The presence of stevensine has been a fairly robust chemotaxonomic marker for *A. corrugata* identification, as it has been found in 36 of 38 of our morphologically identified *A. corrugata* samples, but the compound is missing from all clade IO samples. However, all novel TL13 genetic markers were amplified from these three clade IO samples, further supporting the close similarity of clade IO to *A. corrugata*.

The third cluster, clade C, was comprised of geographically mixed samples (from the Florida Keys, Puerto Rico, and the eastern Atlantic) belonging to Axinellidae. For example, sample Ax9 was identified as an *Axinella* congener, while morphologically and chemically, Keys-1 and Keys-2 individuals resembled the axinellid *Ptilocaulis* sp. (Alvarez et al. 1998). The relatively high intracladal percent distances (mean > 5%; Table 2) within clade C indicated that its members probably constitute different axinellid species. Analysis of more samples that may belong to this clade from both Atlantic provinces are re-

Table 2. Mean percent distances (uncorrected *P*) of ITS DNA sequences

	AC	IO	C
Clade AC	(0.46 + 0.6)		
Clade IO	6.39 + 0.8	(3.7 + 2.0)	
Clade C	11.84 + 1.8	14.50 + 1.1	(5.82 + 1.8)

Pairwise distances were derived by PAUP for all taxa shown in Figure 5. Intra-clade distances are shown in parentheses.

quired for definitive conclusions on their biogeographical and taxonomic identity. All except one of the *A. corrugata* samples (Ac27) that were originally identified using morphological characters clustered as a single monophyletic clade with high bootstrap support (Figure 5). Because the ITS phylogenetic reconstruction strongly grouped Ac27 within clade C, this sample was reexamined using morphological characters and was found to be more similar to the other samples in clade C than to *A. corrugata*. The outlying position of clade C to either clade Ac or IO is supported by the large internode branch lengths (more than 20 steps) and Bremer indices. Overall, although morphological analyses suggest that clade C individuals most closely resemble *Ptilocaulis walpersi* (Alvarez et al. 1998), the more extensive sampling and analysis within this clade which is needed for their precise taxonomic identification is beyond the scope of this study.

This study confirms that the ITS region contains sufficient genetic variation to yield species-specific sequence signatures for distinguishing somatic or primary culture cells of *A. corrugata* from congeneric samples or potential microbial contaminants, respectively. For example, the large ITS sequence distances observed just within phylum Porifera allude to even greater sequence differences with microbial symbionts which are even more distant. Seen in another light, these distances also hinder efforts to find ITS sequences from appropriate outgroup taxa for tree rooting that are not saturated. Conversely, the low intraspecific variation (less than 1.0%) among *A. corrugata* ITS sequences meets gene marker criteria and is consistent with the low divergence values observed with ITS characterizations of other taxa (Hunter et al. 1997; Sugita et al. 1999). Wörheide et al. (in press) have suggested that homogenization among ITS sequences may be rapid in Porifera, as evidenced by the low intraindividual variability in some species.

The present data have interesting impli-

cations for sponge phylogeography and evolution. As Mace et al. (1996) have indicated, "Change in genetic biodiversity among localities can reflect steepness of ecological gradients and degree of evolutionary isolation and localities. . .." However, relatively little information on the most important mechanisms affecting the structure and evolution of marine sponge populations exists in comparison to other marine invertebrate species (Hooper 1999; Mayr 1954; Vermeij 1978; Wörheide et al., in press). Few studies on the population genetics of marine sponges exist, though Sole-Cava and Boury-Esnault (Boury-Esnault et al. 1999) report a wide range of genetic identities among *Axinella* congeners. Moreover, although some members of the order Axinellida are oviparous with free swimming larvae (Alvarez et al. 1998; Bergquist 1970, 1978), the real extent of *A. corrugata* larva dispersal is not known. Nonetheless, *A. corrugata* ITS sequence data were consistent with previous phylogeographic studies that show relatively low genetic differentiation between high-dispersal marine invertebrate populations (Helberg 1994; Palumbi 1994; Wörheide et al., in press). It is also notable that virtually no genetic differentiation was observed among Bahamian *A. corrugata* samples (spanning an area of 13,940 km²). Genetic uniformity among Bahamian samples may stem from asexual propagation (although this has not been reported for *A. corrugata*) or larvae tracking northwestern currents through the Bahamas (Kinder et al. 1985; Roberts 1997). The present study is a case where molecular data suggesting relatively high dispersal and "connectivity" (at least among western Atlantic populations) precedes knowledge of the dispersal characteristics of the organism in question.

In contrast to the above, some incipient *A. corrugata* population subdivision may be occurring between the Bahamas and the Florida Keys, as indicated by a distinct Florida Keys ITS subclade (Ac21 and Ac38) (Figure 5). The Florida Straits are expected to represent a formidable geographic barrier to slow-moving, planktotrophic larvae, since currents may reach up to 30 million ft³/s or 25–100 cm/s (Mayr 1954; Messing et al. 1990; Niiler and Richardson 1973; Reed 2001). Also the ITS DNA sequence data revealed clear genetic boundaries (either phylogeographic or systematic) between Axinellidae populations in different oceanic areas (i.e., the western and eastern Atlantic and Indian Oceans).

Lastly, the ITS sequence data may also

be useful for clarifying some of the many taxonomic questions still remaining within the genus *Axinella* (Alvarez et al. 1998; Hooper et al. 1992). For example, ITS sequences are congruent with analyses of morphological and chemical characters that underlie the reconstruction of each of the three major clades in Figure 5. The precise identification of clade IO samples—Ax7, Ax16, and Ax18—has not yet been resolved, and these may be included with the several axinellid taxa still in question (Hooper and Levi 1993; Vosmaer 1912). The actual number of different axinellid species (Ax) were not determined in this study, but their presence is alluded to in the amount of intracladal variation shown in Table 2 and Figure 5 relative to *A. corrugata*. Moreover, the presence of two novel markers (TL13B and C) and the relatively low ITS sequence divergence and Bremer index between clade Ac and IO, in spite of the great geographical distance between western Atlantic and Indian Ocean provinces, justifies the congeneric status and implies either a slow evolutionary rate in the included taxa or the possibility of significant gene flow in the past.

Conclusion

Cell type verification and microbial contamination of marine invertebrate cell cultures remains a persistent problem (Macleod et al. 1999; Pomponi and Willoughby 1994; Rinkevich 1999). The genetic markers in this study represent a "first pass" development of positive molecular probes that can be utilized for species-specific verification of *A. corrugata* sponge cells. They are also highly consistent with previously determined chemotaxonomic and morphological data. Moreover, it was demonstrated that the presence of microorganisms in either somatic or cell culture samples does not prohibit the detection of sponge genetic markers and band patterns, which suggests that such molecular probes would be useful even if there are obligate (endo-) symbionts (Burja et al. 1999; Friedrich et al. 1999; Fuerst et al. 1999; Lopez et al. 1999b; Wilkinson 1987).

The present data indicate that ITS loci appear more reliable with respect to PCR amplifiability and phylogenetic utility when compared to the novel TL13B/C loci. The lack of closer outgroups to Axinellidae made reliable rooting of the ITS tree difficult despite the strong support for three major clades. Also, it was unfortunate that we could not expand the scope of this study to include a comparison of

TL13 marker phylogeny with ITS, since the ITS phylogenies clearly demarcated species boundaries.

Developing reliable species markers remains an ongoing task for many groups of organisms, and especially for marine invertebrate fauna that are targeted for cell culture development (Rinkevich 1999; van Oppen et al., in press). This study highlights the importance of a comparative and phylogenetic approach in evaluating different methods as well as disparate loci within the same genome, and utilizing all available data in order to find concordance. Molecular genetics can provide the tools and a fertile resource of taxonomic characters, as manifested by gene sequences or banding patterns, which carry the specificity to distinguish cell cultures, individuals, and higher levels of organization.

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