RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS CAN REVEAL INTRASPECIFIC EVOLUTIONARY PATTERNS IN PORIFERA

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Random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) techniques were used to generate polymorphic markers to investigate evolutionary patterns in Porifera. Three primers were selected based on their amplification profiles using genomic DNA from 18 sponge species (17 Demospongiae and 1 Calcarea), collected from various localities along the Brazilian coast. The total number of amplified scorable bands per primer varied from 9 (primers OPS-17 and UBC-322) to 32 (primer OPG-19). The level of genetic polymorphism was measured in a population (N=9) of *Hymeniacidon heliophila* from Itaipu Beach (Rio de Janeiro State). The percentage of polymorphic fragments for primer UBC-322 was 31% (mean intrapopulation genetic distance=0.32). The interpopulational genetic diversity was calculated using two individuals from each of five different populations of *H. heliophila*. The percentage of polymorphic fragments varied from 50-68% (mean genetic distance between populations=0.53). A monomorphic band isolated from an individual of the Itaipu population of *H. heliophila* was labelled and used to probe dot blots containing genomic DNA from 18 species of sponges, fruit fly, rat and 15 individuals from the 5 populations of *H. heliophila*. Only *H. heliophila* DNA was hybridised with this marker. We also generated RAPD band patterns using genomic DNA from 6 different species of the genus *Mycale* and primers OPG-19 and OPS-17, with an average percentage of polymorphic bands of 91 and 98%, respectively. The genetic distance between species of *Mycale* varied from 0.45-0.93 (mean genetic distance=0.76) for primer OPG-19 and 0.64-0.93 (mean genetic distance=0.80) for OPS-17. The RAPD technique was shown to be a useful tool to generate molecular markers and to measure polymorphism and genetic distances in Porifera. ❒ *Porifera, molecular markers, RAPD, genetic diversity, SW Atlantic, Hymeniacidon heliophila, Mycale.*

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Sponges (phylum Porifera) show considerable morphological variability. At the biochemical level this variability is supported by heterogeneous allozyme banding patterns reflecting high genetic heterozygosity in sponges (Solé-Cava & Thorpe, 1991, 1994). These characteristics together with the limited data on aspects of reproduction, life history, ecology and cell biology make the study of sponge genetics and systematics complicated tasks. In fact, these problems are general to many marine organisms where complexes of sibling species are known to be common (Knowlton, 1993).

Very few molecular genetics studies have been initiated on sponges, the majority of them using allozyme electrophoresis or ribosomal RNA sequence comparisons (Kelly-Borges et al., 1991; Kelly-Borges & Pomponi, 1994; Solé-Cava & Thorpe, 1994; Collins, 1998). The application of molecular techniques to study sponge genetics and the evolution of the phylum is a difficult proposition due to the lack of knowledge on the complexity of the sponge genome.

On the other hand, fingerprinting techniques have been widely used in studying ecology and taxonomy of several marine phyla, and constitute a good integration bridge between environmental and molecular sciences (Burton, 1996). These techniques provide possibilities to reach more stable classifications by quickly assessing vast series of polymorphic molecular characters of potential phylogenetic significance. Characterisation of sponge genomes by fingerprinting techniques allow better estimation of their intra- and interspecific diversity. This estimation is essential for effective management of biological resources and sound species delimitations, particulary in sibling species complexes.

The study of polymorphisms in the eukaryotic genome is a way to discover the evolutionary relationships between populations or species. Among the many molecular methods currently available for genetic diversity studies, the random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR), also known as arbitrarily primed (AP) PCR (Williams et al., 1990; Welsh & McClelland, 1990), appears particularly suitable for population genetics. In RAPD analysis there is no need for a priori knowledge of DNA sequences of samples, cost and efforts are reasonable, and many individuals and loci can be efficiently assayed.

In RAPD analysis a single nonspecific primer is used to randomly amplify DNA segments in the genome. The DNA sequence between inverted hybridisation sites is amplified, and, because the distance between primer sites can vary among individuals, different length fragments (or fingerprints) are generated. As the primers are small (normally 10-mers) and not specific to any given gene, many different fragments are produced in an amplification. Fragments present in one individual (or species), but not in others, are defined as polymorphic markers. The fingerprint generated is consistent for the same primer, DNA, and PCR conditions used. This fingerprint is useful in the assessment of affinities along the lower levels of biological and classificatory hierarchies, from individuals and populations to species within a genus (Hillis et al., 1996).

The primary goal of this study was to generate molecular markers by RAPD analysis to demonstrate their usefulness in the investigation of genetic variation within, and among populations of sponges, and in the assessment of species relationships. This technique has already been

FIG. 1. Map of the Brazilian coast, showing collection sites for populations of *Hymeniacidon heliophila* (inset). 1) Cabelo Gordo Beach, São Sebastião, São Paulo State (23°48'S, 45°26'W); 2) Praia Vermelha Beach; 3) Urca Beach; Rio de Janeiro City, Rio de Janeiro State (23°0'S, 43°12'W); 4) Boa Viagem Beach; 5) Itaipu Beach, Niterói, Rio de Janeiro State (22°52'S, 43°0'W). The distances between beaches are: Itaipu-Boa Viagem=20km; Boa Viagem-Urca= 10km; Urca-Praia Vermelha=3km; Praia Vermelha-Cabelo Gordo=300km.

used successfully for population genetics in, for example, leguminous trees (Chalmers et al., 1992), prosobranch snails (Jacobsen et al., 1996) and weevil insects (Taberner et al., 1997). It has also been effective in phylogenetic studies of plants of the genus *Brassica* (Demeke et al., 1992), papaya cultivars (Stiles et al., 1993) and mangrove species (Lakshmi et al., 1997). These studies have shown that RAPD is a rapid, accurate and sensitive method to detect genetic variation, and to assist in taxonomic investigation at levels from populations to species.

In this report we used RAPD analysis to: 1) assess the genetic diversity within and between populations of the sponge *Hymeniacidon heliophila* Parker, 1910; 2) generate a potential species-specific molecular marker for *H. heliophila*; and 3) generate molecular markers which could be used to investigate interspecific relationships within the genus *Mycale* Gray, 1867.

MATERIALS AND METHODS

SPONGE COLLECTIONS. Samples of *Hymeniacidon heliophila* were collected using SCUBA at Praia Vermelha Beach, Urca Beach

TABLE 1. Sponge species, classification, collection sites, and voucher numbers. Abbreviations: Ba = Bahia State, PE = Pernambuco State, RJ = Rio de Janeiro State, SP = São Paulo State. MNRJ and UFRJPOR = Porifera Collections of the Museu Nacional, Universidade Federal do Rio de Janeiro.

Species	Classification	Collection site	Voucher number
Aiolochroia crassa (Hyatt, 1875)	Verongida, Demospongiae	Fernando de Noronha, PE	UFRJPOR 4766
Amphimedon viridis Duchassaing & Michelotti, 1864	Haplosclerida, Demospongiae	Praia do Sono, RJ	UFRJPOR 4747
Aplysina fulva (Pallas, 1766)	Verongida, Demospongiae	Abrolhos, BA	UFRJPOR 4699
Arenosclera sp.	Haplosclerida, Demospongiae	Búzios, RJ	UFRJPOR 4628
Callyspongia sp.	Haplosclerida, Demospongiae	Fernando de Noronha, PE	UFRJPOR 4783
Cinachyrella alloclada (Uliczka, 1929)	Spirophorida, Demospongiae	Parati, RJ	UFRJPOR 4755
Ectyoplasia ferox (Duchassaing & Michelotti, 1864)	Poecilosclerida, Demospongiae	Fernando de Noronha, PE	UFRJPOR 4772
Gastrophanella sp.	Lithistida, Demospongiae	Fernando de Noronha, PE	UFRJPOR 4773
Hymeniacidon heliophila Parker, 1910	Halichondrida, Demospongiae	Itaipu, RJ	UFRJPOR 4759
Hymeniacidon heliophila Parker, 1910	Halichondrida, Demospongiae	Boa Viagem, RJ	MNRJ 809
Hymeniacidon heliophila Parker, 1910	Halichondrida, Demospongiae	Urca, RJ	UFRJPOR 4605
Hymeniacidon heliophila Parker, 1910	Halichondrida, Demospongiae	Praia Vermelha, RJ	UFRJPOR 4246
Hymeniacidon heliophila Parker, 1910	Halichondrida, Demospongiae	São Sebastião, SP	MNRJ 1307
Leucilla sp.	Leucosoleniida, Calcarea	Praia Vermelha, RJ	UFRJPOR 4838
Mycale (Aegogropila) aff. americana Van Soest, 1984	Poecilosclerida, Demospongiae	São Sebastião, SP	MNRJ 1584
Mycale (Aegogropila) escarlatei Hajdu, Zea, Kielman & Peixinho, 1995	Poecilosclerida, Demospongiae	São Sebastião, SP	UFRJPOR 4451
Mycale (Arenochalina) laxissima (Duchassaing & Michelotti, 1864)	Poecilosclerida, Demospongiae	São Sebastião, SP	MNRJ 1027
Mycale (Carmia) microsigmatosa Arndt, 1927	Poecilosclerida, Demospongiae	São Sebastião, SP	MNRJ 1331
Mycale (Mycale) arenaria Hajdu & Desqueyroux-Faundez, 1994	Poecilosclerida, Demospongiae	Búzios, RJ	UFRJPOR 2438
Mycale (Zygomycale) angulosa (Duchassaing & Michelotti, 1864)	Poecilosclerida, Demospongiae	Parati, RJ	UFRJPOR 4743
Mycale (Zygomycale) angulosa (Duchassaing & Michelotti, 1864)	Poecilosclerida, Demospongiae	São Sebastião, SP	MNRJ 429
Plakortis sp.	Homosclerophorida, Demospongiae	Fernando de Noronha, PE	UFRJPOR 4774
Pseudaxinella reticulata (Ridley & Dendy, 1886)	Halichondrida, Demospongiae	São Sebastião, SP	MNRJ 289
Topsentia ophiraphidites (de Laubenfels, 1934)	Halichondrida, Demospongiae	Fernando de Noronha, PE	UFRJPOR 4779

(Rio de Janeiro City, Rio de Janeiro State), Boa Viagem Beach, Itaipu Beach (Niterói, Rio de Janeiro State) and Cabelo Gordo Beach (São Sebastião, São Paulo State) (Fig. 1). Samples of *Mycale* (*Mycale*) *arenaria* were collected at João Fernandinho Beach (Búzios, Rio de Janeiro State), while *M.* (*Aegogropila*) *aff. americana*, *M.* (*A.*) *escarlatei*, *M.* (*Arenochalina*) *laxissima*, *M.* (*Carmia*) *microsigmatosa* and *M.* (*Zygomycale*) *angulosa* were collected at Cabelo Gordo Beach and surroundings (São Sebastião, São Paulo State). The other twelve species used were collected in several sites along the Brazilian coast (Table 1).

Voucher specimens were deposited in the Porifera Collections of the Museu Nacional of the Universidade Federal do Rio de Janeiro (MNRJ and UFRJPOR) (Table 1). Specimens were frozen in dry ice immediately after removal from sea water and kept frozen at -20°C or -70°C prior to DNA extraction.

EXTRACTION OF DNA. After careful dissection to remove macroscopic symbionts on an ice-cooled Petri dish, specimens were ground

with a glass rod in a solution of 4M guanidine hidrochloride, 0.1M Tris-HCl pH 8.0, 0.5% sarcosil and 1% ß-mercaptoethanol. The suspension was incubated at 50°C for 1hr and centrifuged at 3000g/20mins. The supernatant was extracted with an equal volume of phenol: chloroform:isoamyl alcohol (25:24:1) and nucleic acids were precipitated with 1 volume of isopropanol. The pellet was washed in 70% ethanol and air dried. The dried pellet was dissolved in 0.01M Tris-HCl pH 8.0, 0.005M EDTA, 0.5% SDS, 50µg/ml proteinase K and 20µg/ml RNAse A and incubated at 50°C for 2hrs. Another extraction with phenol:chloroform: isoamyl alcohol (25:24:1) was followed by a single extraction with chloroform/0.3M sodium acetate pH 5.2 and the DNA was precipitated with two volumes of ethanol. After eentrifugation the DNA pellet was washed in 70% ethanol, air dried and dissolved in sterile water. The DNA concentration was estimated in 1% agarose gels and, followed by dilution in water to a final concentration of 1ng/µl.

RAPD- PCR PROCEDURE. RAPD analysis of genomic DNA was undertaken using a set of three 10-mer random oligonucleotide primers selected on the basis of the high reproducibility of the patterns and the signal intensity of the bands from a total of seventeen decamer primers screened (Table 2). Each PCR amplification reaction mixture of 25µl eontained 2ng of genomic DNA, 2.5µl of 10 x buffer (0.5M KCl, 0.1M Tris-HCl pH 8.4, 1mg/ml gelatin), 1.5µl of 50mM $MgCl_2$, 0.5µl of 5mM dNTPs (Pharmacia), 20ng of primer and 1 unit of Taq DNA polymerase (USB). The reaction mixture was overlaid with one drop of mineral oil, and amplification was carried out in a DNA thermal cycler (Perkin Elmer 480). An initial denaturation step of 3mins at 94°C was followed by 35 cycles of 30secs at 94°C, 30secs at 37°C and 90secs at 72°C, with an additional final step of 10mins at 72°C. The amplified bands were separated by electrophoresis on 6% vertical nondenaturing polyacrylamide gels (PAGE) and visualised after silver staining following the procedure of Sanguinetti et al. (1994). The size of amplified fragments was estimated by comparison with a standard DNA marker: λ (lambda) DNA, double digested with *Eco*Rl-*Hind*III.

RAPD DATA INTERPRETATION AND ANALYSIS. Relationships between RAPD profiles obtained from DNA amplifications of sponge individuals with each primer were explored by eomparing the presence or absence of shared bands. We scored bands using sharpness and differentiation from the background, and although this is an empirical technique, it produced a consistent outcome which was reproduced by at least two people in blind tests. This process of fragment selection was used throughout all experiments reported in this study. From these assays we recorded the selected markers as either present (1) or absent (0) in each DNA sample. Data recording and calculations were performed using the RAPDistance package, version 1.03 (Armstrong et al., 1994), which allowed us to construet a matrix of pairwise distances. Data from these comparisons were used to calculate similarities between pairs of samples using the Jaecard eoeffieient (Jaeeard, 1901, 1908). This algorithm provides similarity values in the range 0-1, and the software converts these to genetic distances as (1-s).

TABLE 2. Name/number, sequence and G+C content of the primers used.

Name/ No. of primer	Sequences 5`-3`	G+C content
OPS-17/ no. 6	TGG GGA CCA C	70%
OPG-19/ no. 13	GTC AGG GCA A	60&
UBC-322/ no.15	GCC GCT ACT A	60%

The first requirement, and a potential problem of the RAPD method, is its reproducibility. Small changes in reaetion conditions are known to cause variation in the amplification pattern (Khandka et al., 1997, and references therein). These difficulties may be overeome if care is taken to ensure consistent PCR reaetion eonditions during amplification. To optimise these conditions and demonstrate RAPD reproducibility we eondueted multiple (four or more) PCR amplifications with the same primer and individual samples (Lobo-Hajdu, unpublished data). In the conditions standardised in our laboratory the PCR-RAPD reactions were reproducible.

Many studies have shown Mendelian inheritance of RAPD markers (Lewis & Snow, 1992; Levitan & Grosberg, 1993), with a few exceptions (e.g. Riedy et al., 1992). Although RAPD analysis of DNA does provide a random sample of the genome, the banding patterns cannot be interpreted allelically because bands are dominant markers and preclude the discrimination of heterozygotes and homozygotes (Welsh & McClelland, 1990; Williams et al., 1990). Consequently, following Williams et al. (1990), we assumed that DNA profiles were the

result of single alleles when computing RAPD data in this study.

DOT BLOTS. Aproximately 500ng of genomic DNA from fruit fly, rat, 18 species of sponges and 15 individuals of *H. heliophila* were denatured with NaOH and bound to a Gene Screen plus nylon membrane (Dupont) using a Bio Dot microfiltration apparatus (Bio Rad), following the manufacturer's recommendations. The membrane was dried in an oven at 80°C for 1hr and pre-hybridised for 1hr at 65°C in 10ml of buffer containing 50mM Tris-HCl pH 7.4, 1M NaCl, 10% PEG-8000, 1% SDS and 100µg/ml of heat denatured salmon sperm DNA. A monomorphic band obtained with primer UBC-322 in the interpopulation assay was excised after separation on a 1.6% agarose gel. The fragment contained in the agarose plug was isolated using a Geneclean Kit (BIO 101) following the manufacturer's recommendations, and then reamplified under standard conditions with the same primer originally employed to generate the band (UBC-322). The reamplified product was labelled with α–^{32}P dCTP using a T7 Quick Prime Kit (Pharmacia Biotechnologies) and added to the pre-hybridisation solution. Hybridisation was carried out for another 12hrs. The membrane was then washed with 0.2 × SSC at 65°C and exposed overnight to an X-Ray film (Kodak) in a cassete at -70°C.

RESULTS

Fourteen of 17 primers used to amplify DNA of 12 specimens of *Hymeniacidon heliophila* from Praia Vermelha Beach, produced clear and distinct banding patterns in a standard RAPD reaction. Three primers were selected to compare banding patterns within and among different sponge species and to test if these primers would work in general for sponges. Each primer generated a different number of fragments for the same species of sponge. The total number of bands per primer ranged from 9-32 (average =22). The majority of fragments scored were found in the molecular weight range between 300-2500 base pairs (bp) (Table 3). Commonly, bands with molecular weight above 2000bp are very compressed and difficult to score.

DNA amplification profiles in 9 individuals of *H. heliophila* from Itaipu Beach, produced using primer UBC-322 (Fig. 2), yielded both monomorphic and polymorphic bands (arrows in Fig. 2). The diversity of RAPD markers generated for this sample of a natural sponge population was estimated by the percentage of polymorphic fragments which ranged between 15-42% (average =31%; Table 4). Estimated genetic distance between individuals ranged from 0.17-0.45 (average =0.32; Table 5).

TABLE 3. Sponge species and average number of bands, polymorphic plus monomorphic, obtained with each primer used (range of molecular weight in base pairs).

Sponge sample	OPS-17	OPG-19	UBC-322
Amphimedon viridis	20 (400-2100)	23 (400-2200)	22 (100-2100)
Aplysina fulva	22 (200-2100)	32 (200-2500)	13 (100-2000)
Arenosclera sp.	23 (400-2500)	30 (200-2500)	27 (650-2500)
Callyspongia sp.	22 (450-2000)	21 (150-1900)	09 (700-1900)
Ectyoplasia ferox	20 (500-3000)	13 (500-1600)	23 (400-1900)
Gastrophanella sp.	28 (400-2500)	25 (600-3000)	28 (600-2500)
Leucilla sp.	17 (700-2300)	26 (600-3000)	19 (600-2500)
Mycale (Aegogropila) aff. *americana*	11 (600-3000)	27 (400-2500)	20 (300-2100)
Mycale (Aegogropila) escarlatei	23 (300-2500)	29 (400-2500)	21 (600-2500)
Mycale (Arenochalina) laxissima	24 (300-2500)	32 (300-2500)	24 (400-2500)
Mycale (Carmia) microsigmatosa	23 (400-2500)	30 (450-2500)	10 (700-2500)
Mycale (Mycale) arenaria	18 (300-2100)	31 (200-2500)	24 (400-2000)
Mycale (Zygomycale) angulosa	21 (400-2500)	28 (300-2500)	25 (300-2500)
Plakortis sp.	09 (400-1700)	22 (400-3000)	17 (600-1600)

The percentage of polymorphic fragments obtained from RAPD amplification profiles of 2 individuals from each of the 5 different populations of *H. heliophila* (Fig. 3) varied from 50-68% (mean of 5 populations=61%; Table 6). Genetic distances between individuals ranged from 0.22-0.73 (average=0.53; Table 7). Genetic distances between individuals of the same population are within the range of intrapopulation variation (0.17-0.45; Table 5): PV1-PV2=0.22; U1-U2=0.32; BV1-BV2=0.32; IT1-IT2=0.27 and SS1-SS2=0.42 (Table 7).

A monomorphic band of approximately 700bp (Fig. 3, middle left arrow) isolated from one individual of *H. heliophila* from Itaipu Beach was purified, reamplified, labelled and used as a probe in dot blots. These blots contained genomic DNA from 18 sponge species, fruit fly, rat and from 15 individuals of the 5 populations of *H. heliophila* (Fig. 4). The labelled monomorphic band hybridised only with *H. heliophila* DNA.

TABLE 4. Number and percentage of polymorphic fragments amplified with primer UBC-322 for each individual of *Hymeniacidon heliophila* shown in Fig. 2. Fragments scored in the range between 200-2000bp.

Specimens of *H. heliophila*	No. polymorphic fragments	Total no. fragments	% polymorphic fragments
Specimen H1	6	17	35
Specimen H2	3	14	21
Specimen H3	8	19	42
Specimen H4	5	16	31
Specimen H5	5	16	31
Specimen H6	8	19	42
Specimen H7	6	17	35
Specimen H8	2	13	15
Specimen H9	4	15	27
Mean	5	16	31

Genomic DNA from 6 species of *Mycale* was amplified with primers OPG-19 and OPS-17 (Fig. 5A, B). Genetic distances between *Mycale* species ranged from 0.45-0.93 (average=0.76; Table 8A) when using primer OPG-19 and from 0.64-0.93 (average=0.80; Table 8B) when using primer OPS-17. Genetic distances between the two *Mycale* (*Zygomycale*) *angulosa* from two different populations (Parati and São Sebastião) was equal to 0.65 with both primers used. Interestingly, this genetic distanee was within the range obtained for interpopulation variation in *Hymeniacidon* (0.22-0.73; Table 7), suggesting that the range for interpopulation variation on *Mycale* could be similar.

DISCUSSION

In this study we tested the utility of the RAPD method to estimate genetic variation in sponges and to generate molecular markers that could be used in population genetics, species delimitation and intrageneric phylogenetic studies. These markers were used to quantify the genetic diversity at the intrapopulation, interpopulation and interspecific levels in experiments undertaken on populations of one species, *Hymeniacidon heliophila*, and in six species within *Mycale*.

Major advantages in using RAPD techniques include their simplicity, reduced running time, and low cost. Moreover, the technique does not make use of radioactive probes and does not require large amounts of DNA or foreknowledge of the regions to be amplified. Another simple and popular way to analyse genetic variation, is to make use of allozyme electrophoresis (Shaw & Prasad, 1970; Avise, 1994; Hillis et al., 1996). This technique revealed different degrees of polymorphism than did the RAPD analysis, which can be explained by the nature of genetic markers generated by both methods. Allozymes are products of structural genes, subjected to relatively strict selective processes, and variation could also be due to post-translational modifications. In contrast, the RAPD amplification technique scans anonymous DNA sequences, whether or not they are coding regions, unique or repetitive, which are randomly distributed in the genome. RAPD markers generate a wide picture of the genome and are not strictly subjected to natural selection.

One of the advantages of RAPD analysis is that RAPD patterns can be easily generated from DNA from any living organism. However, this can also be a major disadvantage, especially when the target organism harbours symbionts. Sponges are notorious for the presence of a number of different organisms living in them, such as bacteria, cyanobacteria, protozoa, fungi, algae and several invertebrates (Berquist, 1978; Wilkinson, 1978; Cheshire & Wilkinson, 1991; Preston et al., 1996; Vacelet & Donadey, 1997).

A potential problem with the data and interpretations of RAPD analysis was the amplification of symbiotic DNA together with sponge DNA which could result in the presence of non-homologous, yet co-migrating bands. We

TABLE 5. Genetic distances (1-s, where s = Jaccard's similarity values in the range 0-1) between 9 individuals of *Hymeniacidon heliophila* from Itaipu Beach (see Fig. 2), calculated from the presence and absence of the amplification products of primer UBC-322. Complete identity is 0 and complete difference is 1. Mean genetic distance among the 9 individuals H1-H9 was 0.32.

	H1	H2	H3	H4	H5	H6	H7	H8
H2	0.28							
H3	0.36	0.35						
H4	0.35	0.24	0.41					
H5	0.35	0.24	0.25	0.22				
H6	0.29	0.26	0.35	0.33	0.41			
H7	0.38	0.28	0.29	0.17	0.17	0.36		
H8	0.33	0.20	0.40	0.29	0.29	0.40	0.24	
H9	0.40	0.29	0.30	0.45	0.28	0.38	0.40	0.35

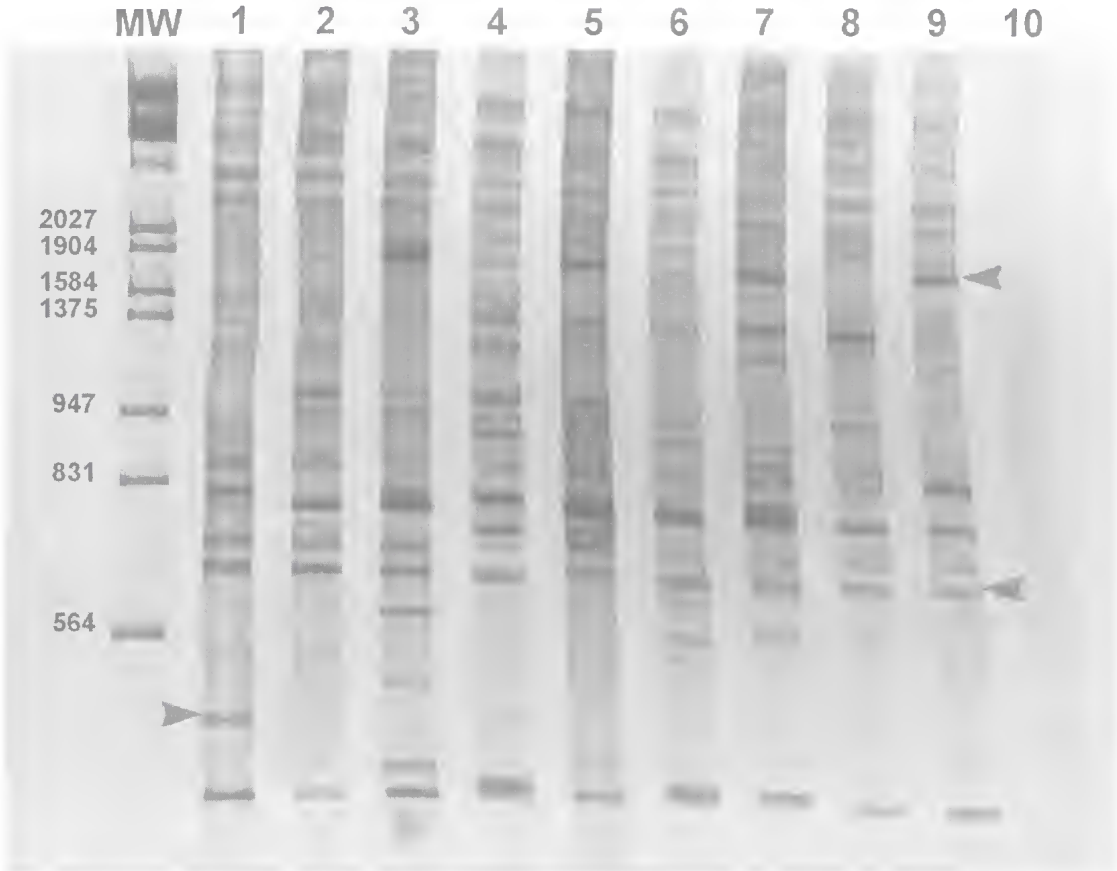

FIG. 2. DNA amplification profiles obtained by RAPD analysis of *Hymeniacidon heliophila* specimens using primer UBC-322. Key: MW, molecular-weight size markers (λ DNA digested with *Eco*RI and *Hind*III) in base pairs. Lanes 1-9, nine individuals from the Itaipu Beach population, lane 10, PCR control reaction without DNA. Arrows show polymorphic (upper right and lower left), and monomorphic (middle right) bands.

TABLE 6. Number and percentage of polymorphic fragments amplified with primer UBC-322 for each individual of *Hymeniacidon heliophila* shown in Fig. 3. Fragments scored in the range 200-1200bp. Abbreviations: PV, Praia Vermelha Beach; U, Urca Beach; BV, Boa Viagem Beach; IT, Itaipu Beach; SS, Cabelo Gordo Beach, São Sebastião.

Specimens of *H. heliophila* / beach site	No. polymorphic fragments	Total no. fragments	% polymorphic fragments
specimen 1/PV	11	17	65%
specimen 2/PV	9	15	60%
specimen 3/U	12	18	67%
specimen 4/U	13	19	68%
specimen 5/BV	12	18	67%
specimen 6/BV	8	14	57%
specimen 7/IT	6	12	50%
specimen 8/IT	8	14	57%
specimen 9/SS	8	14	57%
specimen 10/SS	10	16	63%
Mean	10	16	61%

account for this by taking into consideration that one of the first original works describing the technique (Williams et al., 1993), showed that in a competitive amplification assay using up to 460-fold molar excess of a cyanobacterial genomic DNA mixed with DNA from a soybean genome, all of the detectable amplified products were from soybean. This result suggests that the outcome of an amplification reaction is determined in part by competition for priming sites in the genome, and that a genome of high complexity (soybean) should have more target sites with a better complement to a RAPD primer, as compared to a genome of low complexity

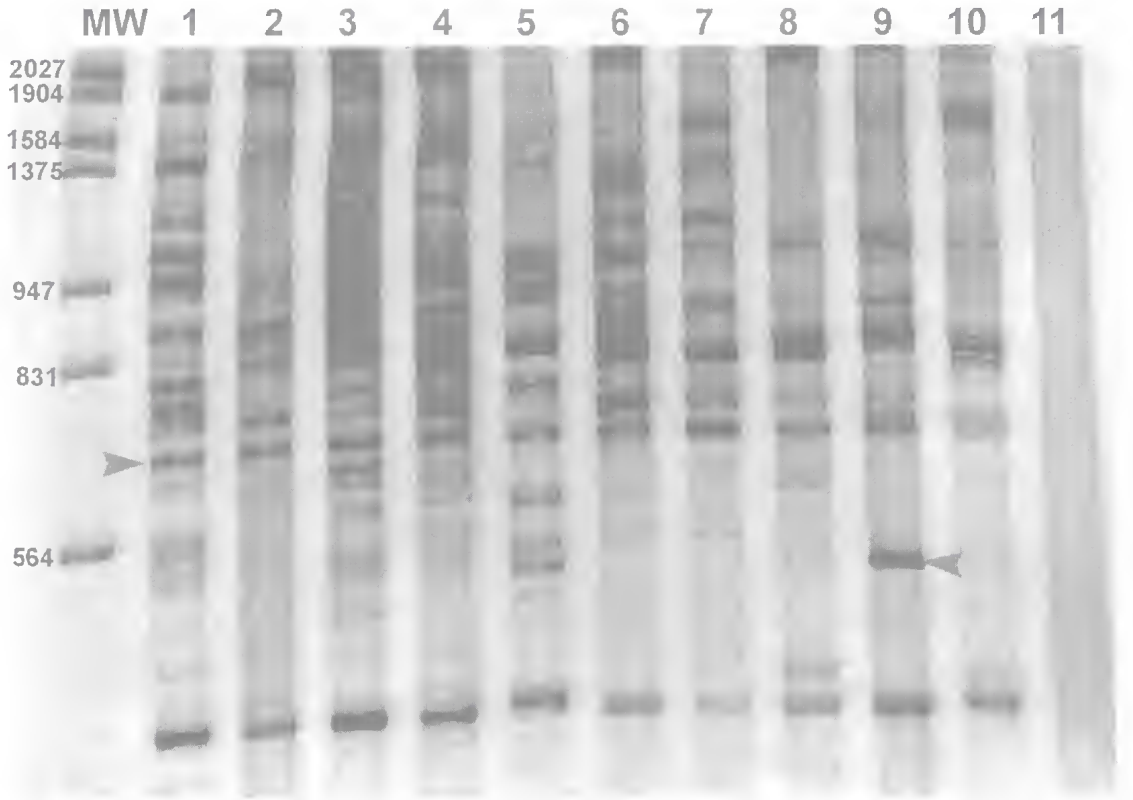

FIG. 3. DNA amplification profiles of individuals from different populations of *H. heliophila* obtained by RAPD analysis with primer UBC-322. Key: MW, molecular-weight size markers in base pairs. Lanes 1-2, Praia Vermelha Beach; lanes 3-4, Urca Beach; lanes 5-6, Boa Viagem Beach; lanes 7-8, Itaipu Beach; lanes 9-10, Cabelo Gordo Beach; lane 11, PCR control reaction without DNA. The arrows show polymorphic (lower right) and monomorphic (middle left) (this band was used on dot-blot) bands.

(cyanobacteria). Symbionts are a commom problem for the majority of the molecular techniques including allozyme analysis and DNA sequencing. Although, RAPD gels can be blotted and markers can be checked with sponge derived probes, we are currently approaching the 'symbiont problem' through the purification of sponge cells, the use of dissociated sponge cells in RAPD amplification reactions, and the comparison of the resulting RAPD pattern with the one obtained for the whole sponge.

Hymeniacidon heliophila showed high levels of genetic variation within and between populations. Allozyme electrophoresis studies

TABLE 7. Genetic distances, based on Jaccard's similarity index, among 10 individuals of *Hymeniacidon heliophila* from 5 populations calculated from the presence or absence of the amplification products of primer UBC-322. Complete identity is 0 and complete difference is 1. Mean genetic distance among the 10 individuals from the 5 beaches was 0.53. Abbreviations: PV, Praia Vermelha Beach; U, Urca Beach; BV, Boa Viagem Beach; IT, Itaipu Beach; SS, Cabelo Gordo Beach, São Sebastião.

	PV1	PV2	U1	U2	BV1	BV2	IT1	IT2	SS1
PV2	0.22								
U1	0.60	0.57							
U2	0.56	0.52	0.32						
BV1	0.65	0.73	0.62	0.58					
BV2	0.62	0.62	0.55	0.50	0.62				
IT1	0.62	0.65	0.50	0.52	0.57	0.38			
IT2	0.65	0.62	0.48	0.50	0.55	0.44	0.27		
SS1	0.71	0.68	0.48	0.50	0.48	0.44	0.38	0.35	
SS2	0.57	0.65	0.58	0.48	0.45	0.64	0.60	0.57	0.42

TABLE 8. Genetic distances, based on Jaccard's similarity index, among 6 species of *Mycale* calculated from the presence and absence of the amplification products of primers OPG-19 (A) and OPS-17 (B). Mean genetic distance among the 6 species (considering just *M. angulosa* SS) was 0.76 (A) and 0.80 (B). Abbreviations: PT, Parati, Rio de Janeiro State; SS, Cabelo Gordo Beach, São Sebastião.

(A) OPG-19	*M. arenaria*	*M.* aff. *americana*	*M. escarlatei*	*M. laxissima*	*M. microsigmatosa*	*M. angulosa* PT
M. aff. *americana*	0.85					
M. escarlatei	0.74	0.62				
M. laxissima	0.81	0.63	0.77			
M. microsigmatosa	0.80	0.87	0.93	0.92		
M. angulosa PT	0.75	0.67	0.71	0.78	0.76	
M. angulosa SS	0.79	0.45	0.70	0.67	0.86	0.65
(B) OPS-17						
M. aff. americana	0.90					
M. escarlatei	0.78	0.83				
M. laxissima	0.81	0.86	0.88			
M. microsigmatosa	0.78	0.78	0.64	0.69		
M. angulosa PT	0.90	0.90	0.93	0.68	0.93	
M. angulosa SS	0.89	0.85	0.84	0.71	0.71	0.65

have already shown that sponges are among the most genetically variable organisms (Solé-Cava & Thorpe, 1994, and references therein). For example, Solé-Cava & Thorpe (1994) showed that the average proportion of polymorphic loci for 21 sponge species was 0.50 (range 0.11-0.86). Our results, with an average genetic distance of 0.32 for the intrapopulation variation (Itaipu samples) and of 0.53 for the interpopulation samples, show that RAPD analysis also points to the existence of high levels of genetic variation, at least for this sponge species.

The dot-blot experiment was used to determine whether the isolated RAPD marker was specific to *Hymeniacidon*, or also present in other species. Our results indicate that this marker is specific to populations of *Hymeniacidon* within the regions studied, or perhaps to the genus *Hymeniacidon*. To determine more accurately the taxonomic value of this marker, we are presently expanding our sponge samples to other species of *Hymeniacidon* and other genera of the Halichondriidae. Cloning and sequencing of this marker is also in progress.

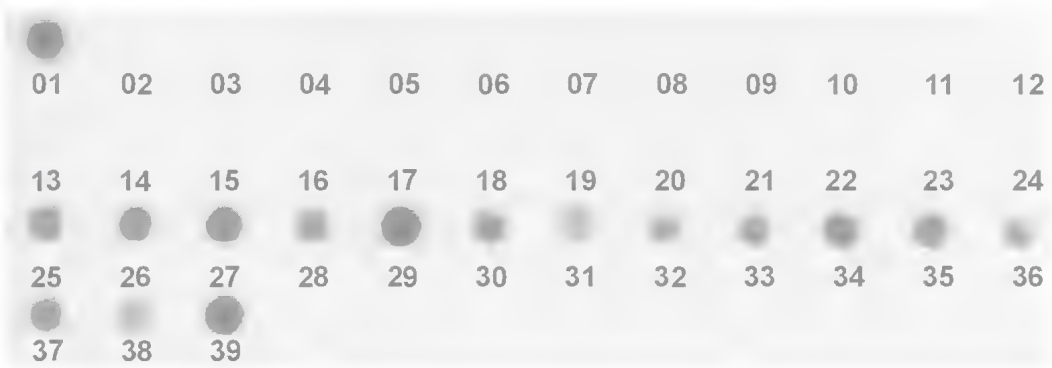

FIG. 4. Dot-Blot hybridised to a RAPD monomorphic marker (shown in Fig. 3) from *Hymeniacidon heliophila*. Key: Wells: 01, *H. heliophila*; 02, *Callyspongia sp.*; 03, *Cinachyrella alloclada*; 04, *Ectyoplasia ferox*; 05, *Gastrophanella* sp.; 06, *Aiolochroia crassa*; 07, *Mycale* (*Mycale*) *arenaria*; 08, *Mycale* (*Aegogropila*) *aff. americana*; 09, *Mycale* (*Aegogropila*) *escarlatei*; 10, *Mycale* (*Arenochalina*) *laxissima*; 11, *Mycale* (*Carmia*) *microsigmatosa*; 12, *Mycale* (*Zygonycale*) *angulosa*; 13, *Plakortis* sp.; 14, *Pseudaxinella reticulata*; 15, *Topsentia ophiraphidites*; 16, *Aplysina fulva*; 17, *Amphimedon viridis*; 18, *Leucilla* sp.; 19, fruit fly; 20, rat; 25-39, *H. heliophila* from five populations (25-27, Praia Vermelha; 28-30, Urca; 31-33, Boa Viagem; 34-36, Itaipu; 37-39, Cabelo Gordo).

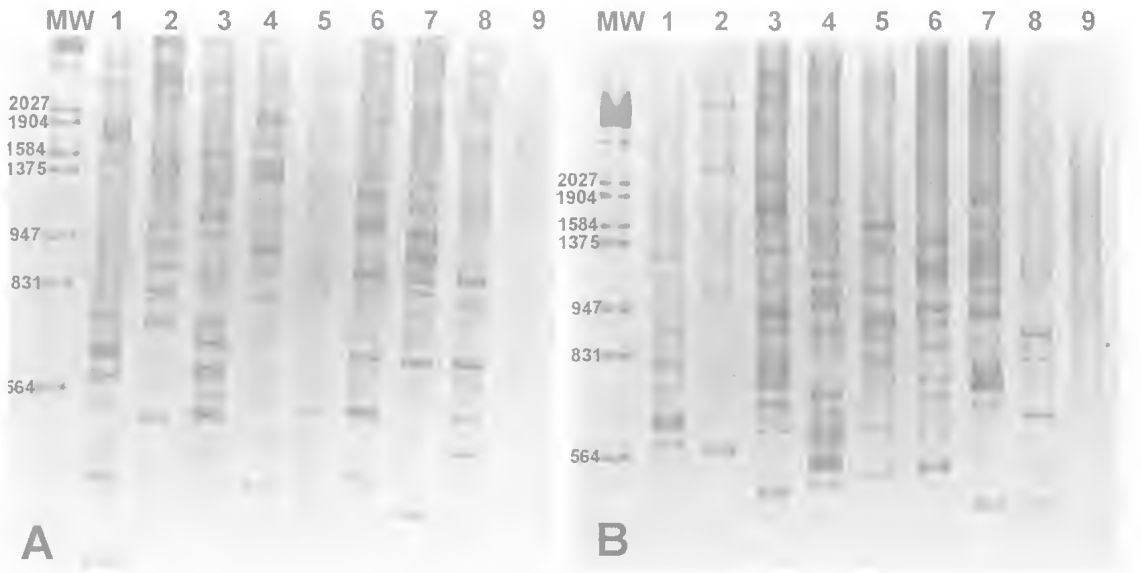

FIG. 5. DNA RAPD amplifications of *Mycale* species. A, Amplification with primer OPG-19. B, Amplification with primer OPS-17. Key: MW, molecular-weight size markers in base pairs. Lanes: 1, *Mycale* (*Mycale*) *arenaria*; 2, *M.* (*Aegogropila*) aff. *americana*; 3, *M.* (*A.*) *escarlatei*; 4, *M.* (*Arenochalina*) *laxissima*; 5, *M.* (*Carmia*) *microsigmatosa*; 6, *M.* (*Zygomycale*) *angulosa* (Parati, Rio de Janeiro State); 7, *M.* (*Z.*) *angulosa* (Cabelo Gordo Beach, São Sebastião, São Paulo State); 8, *Arenosclera* sp. (João Fernandinho Beach, Búzios, Rio de Janeiro State).

Markers generated by RAPD analysis have been used extensively to distinguish species (Bardakci & Skibinski, 1994; Coffroth & Mulawka, 1995; Appa Rao et al., 1996; Partis & Wells; 1996, De Bustos et al., 1998). We applied this method to a set of species of *Mycale* to check its utility in exploring phylogenetic affinities. *Mycale* is well represented at the SE Brazilian coast and phylogenetic affinities within this genus were recently generated (Hajdu & Desqueyroux-Faúndez, 1994; Hajdu & Rützler, 1998; Hajdu, 1999, this volume). Due to the high levels of intra- and interpopulational genetic diversity obtained for *Hymeniacidon*, it will be necessary to survey several (5-10 at least) specimens of each *Mycale* species before deriving conclusions at the interspecific level. This work is in progress, even though the levels of interspecific variation for the six *Mycale* studied here were consistently high for the two primers used. We recognise that our results are still preliminary and stress that our experiments have been conducted to explore the potential utility of RAPD-PCR techniques to assess phylogenetic relationships of congeneric species. We are currently directing our efforts towards increasing the number of primers, specimens and species considered.

RAPD-PCR divergence rates cannot be used as a universal clock with an invariant rate in all animals (Borowsky, 1998). However, a strong relationship seems to exist between degrees of RAPD pattern divergence and time since separation of isolated taxa (Borowsky, 1998). Data from this study show that the mean genetic distance increases from the intrapopulation (0.32) through the interpopulation (0.53) to the interspecific level (0.76 and 0.80). In fact these results support our assumption that there was no amplification of symbiotic DNA, as the presence of symbionts would not correlate well with the degree of relatedness of individuals; in other words, would not be homogeneous at all levels. Our main conclusion is that RAPD patterns show a good correlation between genetic distances and taxonomic level.

Our results demonstrate that molecular markers generated by the PCR-RAPD technique provide an effective tool to assess the existing genetic polymorphism within and between populations and species of sponges. As a future outcome of the present study, specific markers for each sponge population or species can be isolated and sequenced, and population/species-specific primers can be generated. This would allow rapid screening of a larger number

of individuals and thus ease the genetic identification of field samples, more detailed study of the genetic structure of sponge populations, and construction of phylogenies for congeneric species.

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